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FACULTÉ DES SCIENCES ET TECHNIQUES DE FÈS

N° d'ordre : 89/2023

UAlg
UNIVERSIDADE DO ALGARVE

Centre des Etudes Doctorales : Sciences et Techniques et Sciences Médicales

Formation Doctorale : Sciences et Génie de la Matière de la Terre et de la Vie / Sciences Biologiques

Discipline : Biologie

Spécialité : Biochimie - Biotechnologie

Thèse pour l'obtention de DOCTORAT

Dans le cadre d'une cotutelle entre

L'Université Sidi Mohamed Ben Abdellah - Maroc

&

Universidade do Algarve - Portugal

Préparée par: Oumaima BOUTOUB

Sujet de Thèse :

Etude de La Composition Chimique et des activités Biologiques d'*Euphorbia resinifera* et d'*Euphorbia officinarum* et de Leur Miel

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2023

Study of chemical composition and biological activities of *Euphorbia resinifera* and *Euphorbia officinarum* and their honeys

Oumaima Boutoub

Thesis submitted to the Faculty of Science and Technology of the University Sidi Mohamed Ben Abdellah under a joint supervision with the University of Algarve in partial Fulfillment of the requirement for the degree of Doctor in Biology

2023

To my Father,

For your unwavering support, guidance, and love throughout my academic journey. Your belief in my abilities has been my constant motivation. This thesis stands as a tribute to your sacrifices and encouragement. Your strength and belief in me have been my greatest motivators. Thank you for being my rock and my inspiration.

To my Mother,

For your boundless love, your sacrifices. This thesis is a token of my deep appreciation for all that you have done to help me reach this milestone.

To my Sisters and Brother,

In appreciation of the unwavering support, love, and understanding you have provided.

To my Grandmother,

For your wisdom, love, and belief in my potential. This thesis is a tribute to the values you instilled in me and the strength I have gained from your love.

Long Life for You

To All my Family and the Dear Souls of my Grandparents…

Acknowlegdments

To the Department of Biology, Faculty of Science and Technology, University of Algarve and To the Deparetment of Biology, Faculty of Science and Technology, University Sidi Mohamed Ben Abdellah for accepting me as a PhD student under a joint supervision Doctoral Programe of Biological Sciences and Science and Engineering of Earth Matter and Life.

To my supervisor, Professor **Maria da Graça Costa Miguel**, for accepting to supervise my work. In recognition of your intense kindness, guidance, endless patience, and invaluable mentorship throughout this research endeavor. Your expertise, encouragement, and constructive feedback have been instrumental in shaping this thesis. I am grateful for the opportunity to learn from you and for your belief in my potential. I extremely appreciate your human qualities of understanding, listening and being always there for us not only in the lab work but also in integration during my period in Portugal. Thank you from the bottom of my heart

Muito Obrigada

To my supervisor, Professor **EL Ghadraoui Lahsen**, for the chance you gived me to became a PhD student. Your valuable advices, support, suggestions, patience, friendship and especially trust allowed me to complete this thesis. It is with your strong support the thesis came to reality. To have worked under your supervision since my Master made me grow up and recognize that professional success is the result of the effort and dedication. I won't ever be able to thank you enough for all what you have done for me.

Thank you

To Professor **Lazraq Abderrahim** from Biology Departement**,** Faculty of Science and Technology, University Sidi Mohamed Ben Abdellah Fes for your help, assistance to accomplish this work, and for the valuable advices.

To Professor **Ana Cristina Figueiredo** from Biology Department, Faculty of Sciences, University of Lisbon for welcoming me so well during my research trainings in Lisbon, and for the valuable advices and the detailed revisions and corrections each time it was needed.

To Professor **Maria Leonor Faleiro** and **Maria Clara Costa** from faculty of science and technology, university of Algarve for your visionary leadership and unwavering dedication in opening your laboratory during my last 5 years and making this research possible.

I am extremely thankful

To Professor **Monica Borghi** from Biology Department, Faculty of Sciences, Utah State University in United States of America for the chance, the opportunity, the help in the laboratory

and the talks about MS characterizations of flowers samples. Thank you for the care that you gave me throughout my stay in the United States. It was truly an unforgettable experience.

To Doctor **Soukaïna El-Guendouz** for creating an excellent atmosphere in the laboratory and constant help. For your help, assistance during the lab work in the beginning of my experience with scientific research abroad, for the work that you taught me, the patience, support, motivation, continuous advices, valuable suggestions and, more than everything, your unconditional friendship.

To Mr. **Jawad El-Amrani** the beekeeper and **Hakim Zouhri**, for there help and providing the all Plants, Honey, propolis and Flower samples.

To Mr. **Fouad Ouryagli** my dear life sciences professor, it is because of you that I not only chose but love biology. Thank you from the heart.

To Professor **Smail Aazza** from Biology Departement, Faculty Multidisciplinary of Nador your help, assistance to accomplish this work, and for the valuable advices.

To all my lab mate and colleagues, past and present, for the wonderfull moments spent in the lab and for all the shared laughs, in particular to Sara, Soukayna, Luis, Isabel, Ana, Liseta, Joao.

To all my friends outside the lab world Angelo, Erminio, Michele, Donata, Ana and especially Oumaima Chajai (my best friend), for always being present in the good and bad moments for the lifetime friendship, for the support, and long talks.

To my family, Dear father and mother, thank you for the unconditional love, support, encouragement, wise advices, for help in my life and believing in me. My lovely sister Doha, thank you for being always present and by my side in all the moments. To my aunt and my lovely cousins (Tasnime, Yassine and Jana) your presence has played a significant role in shaping my journey. To my dear brother and my beautiful sister-in-law and their little sons Ali.

To all my university professors at the Faculty of Science and Technology, Sidi Mohamed Ben Abdellah Fes University, I wanted to take a moment to express my most sincere thanks to you for your valuable advice and support during my university studies.

Finally, To Jury Members **Pr. DERRAZ Khalid**, **Pr. MIKOU Karima**, **Pr. BELWALHA Saadia**, **Pr. BOUR Abdellatif**, **Pr. ANJOS Ofélia, Pr. AZEVEDO SERRALHEIRO Ana Isabel** and **Pr. BOUCHAMMA El Ouazna**, I sincerely thank you for serving as my committee members even at hardship. I also want to thank you for letting my defense be an enjoyable moment, and for your brilliant comments and suggestions.

Je vous Remercie Énormément

Abstract

Euphorbia officinarum and Euphorbia resinifera are two species found in Morocco. They have been used in traditional medicine. Reports indicate the use of *Euphorbia officinarum* for the treatment of wounds, skin infections and abscesses and the aerial parts of *Euphorbia resinifera* in combination with honey or extracts obtained by decoction have been used by patients in Morocco to treat cancer. The "Morocco Green Plan" has played an important in terms of sustainable development of the agricultural sector, helping to increase its benefit, through the promotion of specific products from different territories. One of these products is honey. Propolis (a natural substance produced by bees from plant resins, sap and other botanical sources) is beginning to attract the interest of Moroccan beekeepers thanks to its biological properties. This work does not focus on latex, but on the aerial parts of *E. officinarum* and E. *resinifera,* the inflorescence part called cyathium of *E. resinifera*, which do not secrete latex; monofloral honey of *E. officinarum* and *E. resinifera*; as well as propolis from *E. officinarum* and *E. resinifera*. The solvent for extracting the aerial parts of both species was water, because it is the liquid used by the population to treat their illnesses. The extraction temperature; extraction time; and plant/solvent ratio are the parameters chosen to obtain extracts with the highest quantities of phenols and with the best in vitro antioxidant properties, chelating activity and capacity to inhibit α-glucosidase activity using response surface methodology (RSM). The best extraction conditions detected were using 1 g/50 mL during 60 minutes at 30 ºC for *E. resinifera* and for *E. officinarum*; the best conditions were temperature of 50 ºC, using1 g/100 mL for 270 minutes.

Euphorbia resinifera cyathium releases a nectar that bees collect and transform into honey, currently labeled as a Protected Geographical Indication, due to its qualities. Additionally, flower cyathium honey and water extracts have been used in traditional medicine in Morocco. Several compounds belonging to different metabolite classes were identified in the present investigation. Concerning the primary metabolites: in hexoses, glucose followed by fructose predominates; in organic acids, citrate and α-ketoglutarate were the most abundant; amino acids were also found in abundance, notably asparagine (Asn); and vitamins such as niacin, vitamin B3, threonate (a metabolite of vitamin C), and the antioxidants ascorbate and dehydroascorbate. Regarding secondary metabolites: lutein was dominant in the pigments identified; flavonoids were dominated by the flavonol glycosides quercetin, kaempferol and isorhamnetin; polyamines were also identified. The scavenging capacity of DPPH and superoxide radicals by the floral extracts corresponded to two thirds and half of the capacity of pure compounds used respectively as positive control. Out of seven honey samples, two were of floral origin from *E. resinifera* and after pollen analysis, the other five were found to have high percentages of *E. officinarum* pollen grains. The potassium (K) was the most important mineral element in in all samples with an average content of 409 mg/kg. The chromatographic profiles evaluated by liquid chromatography

coupled to diode array detection and mass spectrometry (LC-DAD-MS) of Euphorbia honey extracts were quite different. In all samples, it was possible to detect gallic acid, 4-hydroxybenzoic acid and p-coumaric acid, although in different proportions. Naringenin was identified in all but one sample. Abscisic acid was detected in five of seven honey samples, being the major compound detected in in one *E. resinifera* honey. The antioxidant properties of *E. officinarum* and *E. resinifera* as well as their ability to inhibit acetylcholinesterase, lipoxygenase, tyrosinase, and xanthine oxidase enzymes were evaluated and compared. At the same time, the *in vitro* biological activities of the entire honey and the extracts obtained from the respective honey were also evaluated and compared. Generally, the methanolic extracts of the honey recorded better activities in all the biological activities, than the corresponding entire honey. The antioxidant properties and the capacity to inhibit some enzymatic activities of E. resinifera and *E. officinarum* aqueous extracts and honeys were assessed. The aqueous extracts were obtained by decoction and two extraction parameters were chosen: extraction time (1 h, 2 h, and 8 h); and PSR (plant solvent ratio) of 1 g/20 mL; 1 g/50 mL, and 1 g/100 mL. The physicochemical characteristics of the honey were also evaluated and were within the range of the quality standards, E. officinarum honey showed higher amounts of total phenols and better capacity for scavenging superoxide anion free radicals and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals than *E. resinifera* honey, but poorer capacity for inhibiting lipoxygenase, acetylcholinesterase, tyrosinase, and xanthine oxidase. Moreover, the aqueous extracts had systematically higher in vitro activities than the respective honey samples. Concerning propolis samples, the study aimed to characterize the pollen type, the volatile compounds, the phenolic and mineral profiles of three Euphorbia propolis samples collected in Morocco. In addition, it was intended to evaluate its antioxidant and antimicrobial activities, as well has the enzymatic inhibition potential. Pollen analysis revealed that *E. resinifera* pollen dominated in the P1 sample (58%), while E. officinarum pollen dominated in the P2 and P3 samples (44%). The volatile compounds were primarily composed of monoterpenes hydrocarbons, with α-pinene being the major component. Calcium (Ca) was the predominant mineral element in all propolis samples. Higher levels of phenols, flavonoids, and dihydroflavonoids were detected in the *E. officinarum* which corresponded to superior antioxidant activity. This was determined by its ability to scavenge DPPH and nitric oxide (NO) free radicals, superoxide anion radicals, and to prevent lipid peroxidation. P1 and P3 exhibited the best inhibitory effects on glucosidase activity, while the P2 sample demonstrated the highest efficacy in inhibiting acetylcholinesterase, lipoxygenase, tyrosinase, and xanthine oxidase activities. *Euphorbia* propolis displayed the ability to inhibit quorum sensing in the biosensor Chromobacterium violaceum CV026 and disrupted bacterial biofilm formation, including that of resistant bacterial pathogens.

Keywords: *Euphorbia,* aerial parts, cyathia, aqueous extracts, honey, propolis, *in vitro* biological activities.

Resumo

Euphorbia officinarum e *E. resinifera* são duas espécies que se podem encontrar em Marrocos. Têm sido usadas em medicina popular. Relatos indicam a utilização de *E. officinarum* para o tratamento de feridas, infecções cutâneas e abcessos ou, em associação com outras espécies locais (*Ziziphus lotus* and *Opuntia ficus-barbarica*) ou com mel, no tratamento de pielonefrite ou nefrite. As partes aéreas de *E. resinifera* em associação com mel ou extractos obtidos por decoccão têm sido usadas, por doentes de Marrocos, no tratamento do cancro, enquanto que o látex fresco é usado em picadas venenosas, mordeduras e dores de dentes.

O "Plan Vert Marroc" tem sido de grande importância no que ao desenvolvimento sustentável do sector agrícola diz respeito, contribuindo para aumentar o seu valor acrescido, mediante a promoção dos produtos específicos dos diversos territórios com a indicação de Indicações Geográficas, Designação de Origem ou Rótulos Agrícolas. Um desses produtos é o mel. Dois méis de origem monofloral de *Euphorbia* foram registados, designadamente: indicação geográfica "mel do deserto *Euphorbia*" e indicação de protecção geográfica "mel de *Euphorbia* de Tadla-Azilal". A espécie da planta em concreto não foi providenciada, contudo é possível encontrar três tipos de mel monofloral em Marrocos: *Euphorbia officinarum* subsp. *echinus*, *E. regis-jubae*, and *E. resinifera*. *E. resinifera*.

Própolis (uma substância natural produzida pelas abelhas a partir de resinas de plantas, seiva e outras fontes botânicas) começa a suscitar o interesse dos apicultores marroquinos graças às suas propriedades biológicas. A nível mundial, o própolis tem sido genericamente usado na medicina tradicional ou em suplementos alimentares. Desta forma, este produto apícola pode constituir uma fonte adicional de receitas para os apicultores marroquinos.

A revisão bibliográfica efectuada sobre *E. officinarum* e *E. resinifera* demonstrou que o foco da investigação científica tem sido o látex. Tem existido grande interesse por *E. officinarum* para a obtenção de compostos por hemissíntese a partir de compostos isolados do látex com o objetivo de melhorar as atividades biológicas. A partir do látex de *E. resinifera* é possível isolar a resiniferatoxina (diterpeno extremamente irritante) que tem sido usado nalguns ensaios clínicos como um potencial analgésico no alívio de dor oncológica e artrítica, sendo cerca de 1000 vezes mais potente que a capsaicina.

O enfoque deste trabalho incide não sobre o látex, mas sim sobre as partes aéreas de *E. officinarum* e *E. resinifera* sem o látex e sem as pseudo-inflorescências, designadas ciátio; ciátio de *E. resinifera*, que não secretam látex; mel monofloral de *E. officinarum* e *E. resinifera*; bem como o própolis de *E. officinarum* e *E. resinifera*.

O solvente de extracção para as partes aéreas de ambas as espécies foi água visto ser este o líquido usado pela população no tratamento de suas enfermidades. Os parâmetros escolhidos para obter extratos com as mais elevadas quantidades de fenóis e com as melhores propriedades *in vitro* [capacidade de sequestrar 2,2-difenil-1-picril-hidrazilo (DPPH, do inglês *2,2-diphenyl-1-picrylhydrazyl*), actividade quelante, e capacidade de inibir α-glucosidase atividade] foram: e temperatura de extracção (24, 37 e 50 ºC); tempo de extração (1 h, 2 h, e 8 h); e razão planta/solvente (RPS) 1 g/20 mL; 1 g/50 mL e 1 g/100 mL. O método de extracção consistiu na maceração. Para atingir tal objetivo recorreu-se à metodologia de superfície de resposta (MSR) utilizando um delineamento factorial de três níveis. As melhores condições de extração foram diferentes para *E. officinarum* e para *E. resinifera*. Para esta última, as melhores condições foram: temperatura de extracção entre 30 e 35°C; extracção usando RPS de 1 g/50 mL durante 60 minutos. Já para E. *officnarum* as melhores condições foram temperatura de 50 ºC, RPS de 1 g/100 mL durante 270 minutos.

Euphorbia resinifera é uma espécie nativa de Marrocos. O ciátio exsuda néctar que as abelhas recolhem e transformam em mel, este atualmente encontra-se rotulado com a designação Indicação de Protecção Geográfica, pelas suas qualidades. Para além disso, o mel e os extratos aquosos do ciátio têm sido usados na medicina tradicional em Marrocos. Diversos compostos pertencentes a diferentes classes de metabolitos foram identificados na presente investigação. No que concerne os metabolitos primários: nas hexoses a glucose seguida da frutose predominaram; nos ácidos orgânicos foram o citrato e o α-cetoglutarato os mais abundantes; os aminoácidos também se encontravam em abundância, particularmente asparagina (Asn); e vitaminas como niacina, vitamina B_3, treonato (um metabolito da vitamina C) e os antioxidantes ascorbato e desidroascorbato. Relativamente aos metabolitos secundários: a luteína foi dominante nos pigmentos identificados; os flavonóides foram dominados pelos glucósidos flavonólicos de quercetina, canferol e isorramnetina; a poliamina tri-*p*-cumaroil espermidina foi a mais abundante entre as poliaminas identificadas. A capacidade de captação dos radicais DPPH e superóxido por parte dos extratos florais corresponderam a dois terços e a metade da capacidade de captação do 2,6-di-*terc*-butil-*p*-cresol (BHT, do inglês *butylated hydroxytoluene*) e ácido ascórbico, compostos puros usados como controlo positivo, respectivamente.

De sete amostras de mel, duas eram de origem floral de *E. resinifera* e após análise ao polén, verificou-se que as restantes cinco tinham elevadas percentagens de grãos de pólen de *E. officinarum*. No que diz respeito aos parâmetros físico-químicos geralmente alvo de avaliação no mel (mistura, acidez, pH, níveis de hidroximetilfurfural (HMF), prolina, actividade diastase, cinzas e condutividade eléctrica), estes encontravam-se geralmente dentro das gamas de valores recomendados pelos regulamentos internacionais de qualidade. A glucose e a frutose foram os principais açúcares presentes nas amostras de mel, como seria de esperar. Os açúcares redutores

foram significativamente mais elevados nas amostras de *E. resinifera* (66.67% e 70.67%) comparativamente aos méis monoflorais de *E. officinarum*. O potássio (K) foi o elemento mineral mais importante com uma concentração média de 409 mg/kg.

Os perfis cromatográficos avaliados por *liquid chromatography coupled to diode array detection and mass spectrometry* (LC-DAD-MS) dos extratos de méis *Euphorbia* foram bastante diferentes. Em todas as amostras foi possível detectar ácido gálico, ácido 4 hidroxibenzóico, e ácido *p*-coumarico, apesar de em diferentes proporções. A naringenina foi identificada em todas as amostras à excepção de uma. O ácido abscísico foi detetado em cinco das sete amostras de mel, sendo o composto principal detetado num mel de *E. resinifera*. As propriedades antioxidantes de *E. officinarum* e *E. resinifera*, bem como a sua capacidade para inibir as atividades das enzimas acetilcolinesterase, lipoxigenase, tirosinase e de xantina oxidase foram alvo de avaliação e comparação. Ao mesmo tempo, as atividades biológicas *in vitro* dos méis e dos seus extratos também foram avaliadas e comparadas. Os méis *E. resinifera* demonstraram uma melhor capacidade para inibir a atividade de lipoxigenase do que o mel monofloral *E. officinarum*, ao passo que nas restantes atividades biológicas não foi possível observar diferenças entre os dois tipos de mel. Genericamente, as atividades biológicas dos extratos metanólicos dos méis eram melhores do que aquelas encontradas nos respetivos méis.

Foram avaliadas as propriedades antioxidante e a capacidade para inibir algumas atividades enzimáticas dos extratos aquosos e dos méis de *E. resinifera* e *E. officinarum*. Os extratos aquosos foram obtidos por decocção e dois parâmetros de extração foram avaliados: tempo de extracção (1 h, 2 h e 8 h); e RPS de 1 g/20 mL; 1 g/50 mL, e 1 g/100 mL. As características físico-químicas do mel também foram avaliadas e encontravam-se dentro da gama de valores dos critérios de qualidade, embora um mel de *E. officinarum* tivesse quantidades relativamente elevadas de ferro (Fe), cobre (Cu) e alumínio (Al). O mel de *E. officinarum* demonstrou ter quantidades mais elevadas de fenóis totais e melhor capacidade para captar os radicais livres superóxido e DPPH relativamente ao mel *E. resinifera*, mas uma capacidade mais reduzida para inibir as atividades de lipoxigenase, acetilcolinesterase, tirosinase e xantina oxidase. De forma genérica, os extratos aquosos de *E. officinarum* tinham concentrações mais elevadas de fenóis totais, independentemente do tempo de extração e da RPS: RPS 1 g/100 mL e tempo de extracção de 1 h ou 2 h providenciaram extractos com concentrações mais elevadas de fenóis totais (11.8 mg equivalente ácido gálico (EAG)/g em ambos os casos). Relativamente a *E. resinifera*, as melhores condições de extração foram RPS 1 g/100 mL e 30 minutos e 1 h de extração (7.50 e 7.20 mg EAG/g, respetivamente). Para além disso, os extratos aquosos tinham sistematicamente melhores actividades *in vitro* do que as respetivas amostras de mel.

No que respeita às amostras de própolis, o estudo pretendeu caracterizar o tipo de pólen, os compostos voláteis, os perfis fenólicos e minerais de três amostras de própolis de *Euphorbia* colhidas em Marrocos. Para além disso, também se pretendeu avaliar as suas atividades antioxidantes e antimicrobianas, bem como o potencial de inibição enzimática. A análise de pólen demonstrou que o pólen de *E. resinifera* era dominante na amostra P1 (58%), enquanto o pólen de *E. officinarum* dominava nas amostras P2 e P3 (44%). Os compostos voláteis eram principalmente constituídos por monoterpenos, sendo o α-pineno o mais representativo deste grupo. O cálcio (Ca) foi o elemento mineral predominante em todas as amostras de própolis. Níveis mais elevados de fenóis, flavonóides e di-hidroflavonoides foram detetados na amostra P2 de *E. officinarum*, a que correspondeu também a uma atividade antioxidante superior, determinada pela sua capacidade em sequestrar os radicais livres DPPH, monóxido de azoto (NO) e superóxido, e prevenir a peroxidação lipídica, medida através do método de substâncias reativas ao ácido tiobarbitúrico (SRAT). As amostras P1 e P3 exibiram as melhores atividades inibitórias da atividade de glucosidase, enquanto a amostra P2 demonstrou eficácia mais elevada na inibição das atividades de acetilcolinesterase, lipoxigenase, tirosinase, e xantina oxidase. O valor da concentração mínima inibitória (CMI) variou entre 50 e 450 µL/mL contra bactérias Gram-positiva e Gram-negativa. O própolis de *Euphorbia* promoveu a inibição de quorum sensing no biosensor *Chromobacterium violaceum* CV026 e de disrupção da formação de biofilme bacteriano, incluindo o de bactérias patogénicas resistentes.

Palavra-chave: *Euphorbia*, partes aéreas, ciátio, extratos aquosos, mel, própolis, atividades biológicas *in vitro*.

ملخص

Euphorbia officinarum و *Euphorbia resinifera* نوعان موجودان في المغرب. لقد تم استخدامها في الطب التقليدي. وتشير التقارير إلى استخدام نبات الفربيون *Officinarum* لعلاج الجروح والالتهابات الجلدية والخراجات، كما أن الأجزاء الهوائية من نبات *Euphorbia resinifera* مع العسل أو المستخلصات التي يتم الحصول عليها عن طريق غليها قد استخدمها المرضى في المغرب لعلاج السرطان.

وقد لعب "مخطط المغرب الأخضر" دورا هاما في مجال التنمية المستدامة للقطاع الفلاحي، حيث ساعد على زيادة فوائده، من خلال الترويج لمنتوجات محددة من مختلف الأقاليم. أحد هذه المنتجات هو العسل. بدأ البروبوليس (مادة طبيعية ينتجها النحل من راتنجات نباتية وعصارة ومصادر نباتية أخرى) يجذب اهتمام النحالين المغاربة بفضل خصائصه البيولوجية. لا يركز هذا العمل على اللاتكس، ولكن على الأجزاء الهوائية من *E. officinarum* و *E. resinifera* ، الجزء الإزهاري المسمى cyathium من *E. resinifera* ، والتي لا تفرز اللاتكس؛ العسل أحادي الزهرة من *E. officinarum* و*E.resinifera*؛ وكذلك دنج من *E. officinarum* و*E.resinifera*.

وكان المذيب لاستخراج الأجزاء الهوائية لكلا النوعين هو الماء، لأنه السائل الذي يستخدمه السكان لعلاج أمراضهم. درجة حرارة الاستخراج وقت الاستخراج ونسبة النبات/المذيب هي العوامل المختارة للحصول على مستخلصات تحتوي على أعلى كميات من الفينولات وأفضل خصائص مضادة للأكسدة في المختبر، ونشاط خالبي وقدرة على تثبيط نشاط α-glucosidase باستخدام استجابة منهجية المساحة السطحية (RSM). وكانت أفضل ظروف الاستخلاص المكتشفة هي استخدام 1 جم/ 50 مل خلال 60 دقيقة عند 30 درجة مئوية لـ resinifera و*E. officinarum*؛ وكانت أفضل الظروف هي درجة حرارة 50 درجة مئوية، باستخدام 1 جم/100 مل لمدة 270 دقيقة.

يطلق نبات *E. resinifera* سياثيوم رحيقًا يجمعه النحل ويحوله إلى عسل، ويُصنف حاليًا على أنه مؤشر جغرافي محمي، نظرًا لصفاته. بالإضافة إلى ذلك، تم استخدام عسل زهرة السياثيوم ومستخلصات الماء في الطب التقليدي في المغرب. تم تحديد العديد من المركبات التي تنتمي إلى فئات مستقلبة مختلفة في هذا البحث. فيما يتعلق بالأيضات الأولية: في السداسيات، يسود الجلوكوز يليه الفركتوز؛ في الأحماض العضوية، كانت السيترات وألفا-كيتوجلوتارات هي الأكثر وفرة؛ كما تم العثور على الأحماض الأمينية بكثرة، ولا سيما الأسباراجين (**Asn**)؛ والفيتامينات مثل النياسين، وفيتامين ب3، والثريونات (أحد مستقلبات فيتامين سي)، ومضادات الأكسدة أسكوربات وديهيدرواسكوربات. فيما يتعلق بالأيضات الثانوية: كان اللوتين هو السائد في الأصباغ التي تم تحديدها؛ سيطرت على مركبات الفلافونويد جليكوسيدات الفلافونول كيرسيتين، وكيمبفيرول، وإيسورهامنيتين؛ كما تم التعرف على البوليامينات. قدرة التقاط **DPPH** و تتوافق جذور الأكسيد الفائق بواسطة المستخلصات الزهرية مع ثلثي ونصف سعة المركبات النقية المستخدمة على التوالي كعنصر تحكم إيجابي. من بين سبع عينات من العسل، كانت اثنتان منها من أصل زهري من *E.resinifera* وبعد تحليل حبوب اللقاح، وجد أن الخمس الأخرى تحتوي على نسب عالية من حبوب اللقاح *E. officinarum*.

كان البوتاسيوم (**K**) أهم العناصر المعدنية في جميع العينات بمتوسط محتوى 409 ملجم/كجم. كانت الملامح الكروماتوغرافية التي تم تقييمها بواسطة (**LC-DAD-MS**) لمستخلصات عسل الفربيون مختلفة تمامًا. في جميع العينات، كان من الممكن الكشف عن حمض الغاليك، وحمض 4-هيدروكسي بنزويك وحمض الكوماريك، وإن كان بنسب مختلفة. تم التعرف على نارينجينين في جميع العينات باستثناء عينة واحدة. تم اكتشاف حمض الأبسيسيك في خمس من سبع عينات من العسل، وهو المركب الرئيسي الذي تم اكتشافه في عسل واحد من نوع *E. resinifera*. تم تقييم ومقارنة خصائص مضادات الأكسدة لـ *E.*

officinarum و*E.resinifera* بالإضافة إلى قدرتها على تثبيط إنزيمات الأستيل كولينستراز، والأكسجين الشحمي، والتيروزيناز، والزانثين أوكسيديز.

وفي الوقت نفسه، تم أيضًا تقييم ومقارنة الأنشطة البيولوجية المختبرية للعسل بأكمله والمستخلصات التي تم الحصول عليها من العسل المعني. بشكل عام، سجلت المستخلصات الميثانولية للعسل نشاطًا أفضل في جميع الأنشطة البيولوجية مقارنة بالعسل المقابل. تم تقييم الخصائص المضادة للأكسدة والقدرة على تثبيط بعض الأنشطة الأنزيمية للمستخلصات المائية والعسل *E.resinifera* و*E.officinarum*. تم الحصول على المستخلصات المائية عن طريق ديكوتيون وتم اختيار معلمتين للاستخراج: وقت الاستخراج (1 ساعة، 2 ساعة، و 8 ساعات)؛ و**1 PSR** جم/20 مل؛ 1 جم/50 مل، و1 جم/100 مل. تم أيضًا تقييم الخصائص الفيزيائية والكيميائية للعسل وكانت ضمن نطاق معايير الجودة، وأظهر عسل *E. officinarum* كميات أعلى من إجمالي الفينولات وقدرة أفضل على تفكيك الجذور الحرة لأنيون الأكسيد الفائق والجذور الحرة **DPPH** مقارنة بعسل *E. resinifera*، ولكنه أفقر. القدرة على تثبيط الليبوكسيجيناز، أستيل كولينستراز، التيروزيناز، وأكسيداز الزانثين. علاوة على ذلك، كان للمستخلصات المائية أنشطة أعلى بشكل منهجي في المختبر من عينات العسل المعنية. فيما يتعلق بعينات البروبوليس، هدفت الدراسة إلى توصيف نوع حبوب اللقاح والمركبات المتطايرة والملامح الفينولية والمعدنية لثلاث عينات من دنج الفربيون تم جمعها في المغرب. بالإضافة إلى ذلك، كان الهدف منه تقييم أنشطته المضادة للأكسدة والمضادة للميكروبات، بالإضافة إلى قدرته على تثبيط الأنزيمات. كشف تحليل حبوب اللقاح أن حبوب لقاح *E. resinifera* هيمنت على عينة **P1** (58%)، بينما سيطرت حبوب لقاح *E. officinarum* على عينات **P2** و**P3** (44%). تتكون المركبات المتطايرة بشكل أساسي من هيدروكربونات أحادية التربين، مع ألفا-بينين كونها العنصر الرئيسي. كان الكالسيوم (**Ca**) هو العنصر المعدني السائد في جميع عينات البروبوليس. تم اكتشاف مستويات أعلى من الفينولات والفلافونويدات والديهيدروفلافونويدات في *E. officinarum* والتي تتوافق مع نشاط مضاد للأكسدة متفوق. تم تحديد ذلك من خلال قدرته على تطهير **DPPH** والجذور الحرة لأكسيد النيتريك (**NO**) وجذور أنيون الأكسيد الفائق ومنع بيروكسيد الدهون. أظهرت **P1** و**P3** أفضل التأثيرات المثبطة على نشاط الجلوكوزيداز، في حين أظهرت عينة **P2** أعلى فعالية في تثبيط أنشطة الأسيتيل كولينستراز، وأكسيجيناز الدهون، والتيروزيناز، وأكسيداز الزانثين. أظهر دنج الفربيون القدرة على تثبيط استشعار النصاب في المستشعر الحيوي *Chromobacterium violaceum CV026* وتعطيل تكوين الأغشية الحيوية البكتيرية، بما في ذلك مسببات الأمراض البكتيرية المقاومة.

لكلمات المفتاحية: الفربيون، الأجزاء الهوائية، السياثيوم، المستخلصات المائية، العسل، العكبر، الأنشطة البيولوجية المخبرية.

Résumé

Euphorbia officinarum et *Euphorbia resinifera* sont deux espèces présentes au Maroc; elles ont été utilisées en médecine traditionnelle pour le traitement des plaies, des infections cutanées et des abcès. Les parties aériennes d'*Euphorbia resinifera* en combinaison avec du miel ou des extraits obtenus par décoction ont été utilisées pour traiter le cancer. Le « Plan Vert Maroc » a joué un rôle important en termes de développement durable du secteur agricole, en contribuant à accroître ses bénéfices, à travers la promotion de produits spécifiques issus de différents territoires. L'un de ces produits est le miel. La propolis commence à susciter l'intérêt des apiculteurs marocains grâce à ses propriétés biologiques. Ce travail porte sur les parties aériennes d'*E. officinarum* et d'*E. resinifera*, la partie de l'inflorescence appelée cyathium d'*E. resinifera*, qui ne sécrètent pas de latex ; miel monofloral d'*E. officinarum* et d'*E. resinifera*; ainsi que la propolis d'*E. officinarum* et d'*E. resinifera*. Le solvant utilisé pour extraire les parties aériennes des deux espèces était l'eau. La température d'extraction; temps d'extraction; et le rapport plante/solvant sont les paramètres choisis pour obtenir des extraits avec les plus grandes quantités de phénols et avec les meilleures propriétés antioxydantes *in vitro*, activité chélatante et capacité à inhiber l'activité α-glucosidase en utilisant la réponse méthodologique de surface (RSM). Les meilleures conditions d'extraction détectées étaient l'utilisation de 1 g/50 mL pendant 60 minutes à 30 ºC pour *E. resinifera* et pour *E. officinarum*; les meilleures conditions étaient une température de 50 ºC, en utilisant 1 g/100 mL pendant 270 minutes. *Euphorbia resinifera* cyathium libère un nectar que les abeilles récoltent et transforment en miel, actuellement labellisé Indication Géographique Protégée, en raison de ses qualités. Plusieurs composés appartenant à différentes classes de métabolites ont été identifiés dans la présente enquête. Concernant les métabolites primaires : dans les hexoses, le glucose suivi du fructose prédomine ; dans les acides organiques, le citrate et l'α-cétoglutarate étaient les plus abondants ; les acides aminés ont également été trouvés en abondance, notamment l'asparagine (Asn) ; et des vitamines telles que la niacine, la vitamine B3, le thréonate (un métabolite de la vitamine C) et les antioxydants ascorbate et déhydroascorbate. Concernant les métabolites secondaires : la lutéine était dominante dans les pigments identifiés ; les flavonoïdes étaient dominés par les glycosides de flavonol quercétine, kaempférol et isorhamnétine ; des polyamines ont également été identifiées. La capacité de capture du DPPH et les radicaux superoxydes des extraits floraux correspondaient aux deux tiers et à la moitié de la capacité des composés purs utilisés respectivement comme contrôle positif. Sur sept échantillons de miel, deux étaient d'origine florale provenant d'*E. resinifera* et après analyse du pollen, les cinq autres présentaient des pourcentages élevés de grains de pollen d'*E. officinarum.* Le potassium (K) était l'élément minéral le plus important dans tous les échantillons avec une teneur moyenne de 409 mg/kg. Les profils chromatographiques évalués par (LC-DAD-MS) des extraits de miel d'*Euphorbia* étaient assez différents. Dans tous les échantillons, il a été

possible de détecter de l'acide gallique, de l'acide 4-hydroxybenzoïque et de l'acide p-coumarique, bien que dans des proportions différentes. La naringénine a été identifiée dans tous les échantillons sauf un. L'acide abscissique a été détecté dans cinq des sept échantillons de miel. Les propriétés antioxydantes d'*E. officinarum* et d'*E. resinifera* ainsi que leur capacité à inhiber les enzymes acétylcholinestérase, lipoxygénase, tyrosinase et xanthine oxydase ont été évaluées et comparées. Dans le même temps, les activités biologiques *in vitro* du miel entier et des extraits obtenus à partir du miel ont également été évaluées et comparées. Généralement, les extraits méthanoliques du miel ont enregistré de meilleures activités que le miel entier correspondant. Les propriétés antioxydantes et la capacité à inhiber certaines activités enzymatiques des extraits aqueux et des miels d'*E. resinifera* et d'*E. officinarum* ont été évaluées. Les extraits aqueux ont été obtenus par décoction et deux paramètres d'extraction ont été choisis : la durée d'extraction (1 h, 2 h et 8 h) ; et PSR de 1 g/20 ml ; 1 g/50 ml et 1 g/100 ml. Les caractéristiques physicochimiques du miel ont également été évaluées et se situaient dans la plage des normes de qualité. Le miel d'*E. officinarum* présentait des quantités plus élevées de phénols totaux et une meilleure capacité à piéger les radicaux libres anions superoxydes et les radicaux libres DPPH que le miel d'*E. resinifera*, mais la plus pauvre capacité d'inhiber la lipoxygénase, l'acétylcholinestérase, la tyrosinase et la xanthine oxydase. De plus, les extraits aqueux présentaient des activités *in vitro* systématiquement plus élevées que les échantillons de miel respectifs. Concernant les échantillons de propolis, l'étude visait à caractériser le type de pollen, les composés volatils, les profils phénoliques et minéraux de trois échantillons de propolis Euphorbia collectés au Maroc. De plus, il était prévu d'évaluer ses activités antioxydantes et antimicrobiennes, ainsi que son potentiel d'inhibition enzymatique. L'analyse du pollen a révélé que le pollen d'*E. resinifera* dominait dans l'échantillon P1 (58 %), tandis que le pollen *d'E. officinarum* dominait dans les échantillons P2 et P3 (44 %). Les composés volatils étaient principalement composés d'hydrocarbures monoterpéniques, avec de l'α-pinène étant le composant majeur. Le calcium (Ca) était l'élément minéral prédominant dans tous les échantillons de propolis. Des niveaux plus élevés de phénols, de flavonoïdes et de dihydroflavonoïdes ont été détectés dans *E. officinarum*, ce qui correspondait à une activité antioxydante supérieure. Cela a été déterminé par sa capacité à éliminer les radicaux libres DPPH et oxyde nitrique (NO), les radicaux anions superoxydes et à prévenir la peroxydation lipidique. P1 et P3 ont présenté les meilleurs effets inhibiteurs sur l'activité de la glucosidase, tandis que l'échantillon P2 a démontré la plus grande efficacité pour inhiber les activités de l'acétylcholinestérase, de la lipoxygénase, de la tyrosinase et de la xanthine oxydase. La propolis d'Euphorbia a montré la capacité d'inhiber la détection du quorum dans le biocapteur *Chromobacterium violaceum* CV026 et a perturbé la formation de biofilm bactérien.

Mots clés : Euphorbe, parties aériennes, cyathium, extraits aqueux, miel, propolis, activités biologiques *in vitro*.

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List of Abbreviation and Symbols

AACT	Acetoacetyl-CoA-thiolase
AChE	Acetylcholinesterase
AD	Alzheimer's disease
Adj-R2	Adjusted coefficients of determination
ADP	Adenosine 5’-diphosphate
AMP	5’-Monophosphate
ATCI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
AUC	Area under the curve
BHA	Butylhydroxyanisole
BHI	Brain-heart infusion
BHT	Butylhydroxytoluene
CAE	Caffeic acid equivalent
CAS	Cycloartenol synthase
CAT	Catalase
CCD	Central composite design
CFU	Colony forming units
C-NMR	Carbon 13-nuclear magnetic resonance
CRP	C-reactive protein
DMAPP	Dimethylallyl pyrophosphate
DNP	2,4-Dinitrophenylhydrazine
DPD	Dihydropyrimidine dehydrogenase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
DXP	Deoxyxylulose-5-phosphate
EDTA	Ethylenediaminetetraacetic acid
EE	Eriodictyol equivalent
EEAC	Catechin equivalent antioxidant capacity
EEP	Ethanolic extract of propolis
EIMS	Electron ionization mass spectroscopy
EUCAST	European Society of Clinical Microbiology and Infectious Diseases
FAE	Ferulic acid equivalent
FCA	Freund’s complete adjuvant
FDA	Food and Drug Administration
FPS	Farnesyl diphosphate synthase
FPS	Farnesyl diphosphate synthase
GABA	Gamma amino butyric acid
GC -MS	Gas Chromatography -Mass Spectrometry
GPP	Geranyl diphosphate
GRAS	Generally recognized as safe
HAIs	Healthcare associated infections
HCT 116	Human colorectal cancer
HDL	High -density lipoprotein
HIV-1	Type 1 human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
H-NMR	Proton-nuclear magnetic resonance
HPBMCs	Human peripheral blood mononuclear cells
HPLC	High -performance thin -layer chromatography
HREIMS	High-resolution electron ionization mass spectrometry
IC_{50}	Half maximal inhibitory concentration
IHC	International Honey Commission
IL	Interleukin
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase

Is	Isorhamnetin
ISSRs	The inter-simple sequence repeats
LAS	Lanosterol synthase
LB	Luria -Bertani
LC -MS	Liquid Chromatography -Mass Spectrometry
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LS	Lupeol synthase
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry
MBC	Minimum bactericidal concentration
MEP	Methyl-D-erythritol-4-phosphate
MEP	Methylerythritol 4-phosphate
MIC	Minimum inhibitory concentration
MK	Mevalonate kinase
MRSA	Methicillin -resistant Staphylococcus aureus
MSSA	Methicillin -susceptible Staphylococcus aureus
MVA	The mevalonate pathway
NADH	Reduced nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
NI	Not Indicated
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Oleic acid
OD	Optical density
PBS	Phosphate buffered saline
PDMAB	p -Dimethylaminobenzaldehyde
PGI	Protected Geographical Indication
PHBA	p-hydroxybenzoic acid
PMK	Phosphomevalonate kinase
PMS	Phenazine methosulfate
PNPG	p-Nitrophenyl - β - D -glucopyranoside
PPO	Polyphenol oxidase
PSR	Plant solvent ratio
PT	Portugal
QS	Quorum sensing
R2	Determination coefficient
ROS	Reactive oxygen species
RSM	Response surface methodology
SPSS	Statistical Package for the Social Sciences
SQE	Squalene monooxygenase
SQS	Squalene syntase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TCA cycle	The citric acid cycle
TNF-α	Tumor necrosis factor alpha
TRPV1	Transient receptor potential vanilloid type 1
TYLCV	Tomato yellow leaf curl virus
UHPLC-ESI-MS/MS	Tandem mass spectrometry with an electrospray ionization source
UVB	Short wave ultraviolet B
WoS	Web of science
XO	Xanthine oxidase

Preface and main objectives

In Morocco, *Euphorbia* species are used in traditional medicine for their potential therapeutic benefits. It is important to note that the traditional use of *Euphorbia* spp. in Moroccan medicine has been passed down through generations and may vary by region and community.

Euphorbia resinifera commonly known as resin spurge and locally known as "Zaggoume", and *E. officinarum*, known locally as "Daghmouss", have both very used in traditional Moroccan medicine for decades alone or association with other plants and/or honey. The research has been focused on the latex of these species, although it starts to investigate other plant parts. In *E. officinarum* more than seventy hemisynthesized compounds were obtained from some triterpenes of *E. officinarum* latex in order to obtain compounds with higher insecticide and antimicrobial activity, from the latex of *E. resinifera* can be isolated resiniferatoxin (extremely irritant diterpene) that has been used in some clinical trials as a potential analgesic to relieve cancer and arthritis pain. Scientific research is reduced for the totality of other parts of the plant. The aerial part, the flower, and bee products of *Euphorbia resinifera* and *Euphorbia officinarum* origins are less exploited from a scientific point of view.

With the strategy of Maroc Vert, great attention has been paid to the exploitation of natural products such as plants and development of bee products (honey, propolis...). For that reason, vast scientific research concerning chemical composition, biological properties and pharmaceutical and industrial applications are mandatory for the valorization of the different parts of these two *Euphorbia* species.

The chemical composition and *in vitro* biological properties of some parts of *E. resinifera* and *E. officinarum*, as well as of some bee products of *E. resinifera* and *E. officinarum* origins were the main goals of this thesis.

The main objectives of this work were:

(i) Optimization of the best conditions regarding the solute / solvent (water) ratio (10 - 50 mg/mL), extraction temperature (24 – 50°C) and extraction time (60 – 480 min) of the aerial parts of *E. resinifera* and *E. officinarum* in order to obtain aqueous extracts with higher *in vitro* biological properties;

(ii) Revealing the main primary and secondary metabolites and *in vitro* biological activities of *Euphorbia resinifera* cyathia;

(iii) Evaluation of the chemical composition of Moroccan *Euphorbia* honey and its *in vitro* biological activities and unraveling if the in vitro biological activity found for honey can be attributed to the whole honey or a specific fraction

(iv) Evaluation of the chemical composition of Moroccan *Euphorbia* propolis and its *in vitro* biological activities and its in vitro biological activities, giving particular attention to the antimicrobial activity and the disruption of bacterial biofilm formation, including that of resistant bacterial pathogens.

(v) Unraveling which natural products (aqueous extracts or the respective honey) present better activity.

The goals were generally achieved and the results are compiled in some of the chapters that constitute this Ph.D. thesis.

The Ph.D. thesis is organized into 7 chapters, 4 of which, Chapters 1, 3, 5, and 6 consisting of published peer-reviewed scientific works. The Chapter 1 corresponds to a review article, and Chapters 3, 5, and 6 are regular articles with abstract, introduction, methodology, results, discussion or results and discussion, conclusions, and references. Chapters 2 and 7 correspond to submitted articles to peer-reviewed journals and Chapter 4 corresponds to an article that is under review.

The citations and the reference list criteria were based on the number that appears along the text in ascending order. In the reference sections, this numerical order is also followed. This was made to make uniform the thesis layout since diverse criteria exist depending on the journals. All sections of each chapter start with the number one and follow in ascending order, whereas Tables and Figures follow the number of each chapter.

Chapter 1 and Chapter 2 are review articles on *Euphorbia officinarum* and *E. resinifera*, respectively. They are mainly focused on the chemical composition of latex and its biological properties, although more recently other raw materials and bee products have begun to be of researcher interests. They constitute the Introduction of this Ph.D. thesis.

Chapter 3 focuses on defining the best extraction conditions (temperature, extraction time, and plant-to-solvent ratio) from the aerial parts of *E. officinarum* and *E. resinifera*, without latex and cyathia to obtain higher amounts of total phenols and better *in vitro* biological properties (antioxidant and inhibition of α-glucosidase) from *E. officinarum* and *E. resinifera*.

Chapter 4 focuses on unraveling the primary and secondary metabolites of cyathia of *E. resinifera* that can explain the presence or not of some metabolites in the monofloral honey as well as the biological attributes that lead to the use of this raw material in folk medicine.

Chapter 5 addresses the physicochemical parameters of monofloral *E. officinarum* and *E. resinifera* honey and their antioxidant and inhibition of diverse enzymes' activities, and tries to understand if such activities are due to the entire honey or to some fraction.

Chapter 6 focuses on the total phenol content, antioxidant, and inhibition of diverse enzymes' activities of aqueous extracts obtained from the aerial parts of *E. officinarum* and *E. resinifera* without latex and cyathia and compares with those of the respective honey.

Chapter 7 addresses the mineral and volatile profile of *E. offcinarum* and *E. resinifera* propolis as well as the antioxidant, inhibition of some enzyme and antimicrobial activities, particularly on the ability to inhibit quorum sensing in the biosensor *Chromobacterium violaceum* CV026 and disruption bacterial biofilm formation, including that of resistant bacterial pathogens.

The last part provides global conclusions and future work.

List of publications related to the Ph.D. thesis

Chapter 1

Oumaima Boutoub, Lahsen El Ghadraoui and Maria Graça Miguel. Biological properties of latex, aqueous extracts and bee products of *Euphorbia officinarum* L.: A short review. *Molecules*. **2022**, DOI: 10.3390/molecules27217200.

Chapter 2

Oumaima Boutoub, Lahsen El Ghadraoui and Maria Graça Miguel. *Euphorbia resinifera*: chemical composition and biological properties (short review). Submitted, **2023**.

Chapter 3

Oumaima Boutoub, Smail Aazza, Soukaina El-Guendouz, Lahsen El Ghadraoui, Maria G. Miguel. Response surface methodology (RSM) for optimization of *Euphorbia resinifera* and *Euphorbia officinarum* extracts with antioxidant and anti-diabetic activities. *Pharmacognozy Magazine Journal*. **2022**, DOI: 10.4103/pm.pm_2_22.

Chapter 4

Oumaima Boutoub, Sagar Jadhav, Xiongjie Zheng, Lahsen El Ghadraoui, Salim Al Babili, Alisdair R. Fernie, Ana Cristina Figueiredo, Maria Graça Miguel, Monica Borghi. The biochemical characterization of *Euphorbia resinifera* floral cyathia provides insights into the physiological role of flowers in integrating honeybee nutrition and medicinal properties of plant extracts. Submitted, **2023**.

Chapter 5

Oumaima Boutoub, Soukaina El-Guendouz, Ana Manhita, Cristiana Barrocas Dias, Letícia M. Estevinho, Vanessa B. Paula, Jorge Carlier, Maria C. Costa, Brígida Rodrigues, Sara Raposo, Smail Aazza, Lahsen El Ghadraoui, Maria G. Miguel. Comparative study of the antioxidant and

enzyme inhibitory activities of two types of Moroccan *Euphorbia* entire honey and their phenolic extracts. *Foods*. **2021**, DOI: 10.3390/foods10081909.

Chapter 6

Oumaima Boutoub, Soukaina El-Guendouz, Letícia M. Estevinho, Vanessa B. Paula, Smail Aazza, Lahsen El Ghadraoui, Brígida Rodrigues, Sara Raposo, Jorge Carlier, Maria C. Costa and Maria G. Miguel. Antioxidant activity and enzyme inhibitory potential of *Euphorbia resinifera* and *Euphorbia officinarum* honeys from Morocco and plant aqueous extracts. *Journal: Environmental Science and Pollution Research*. **2020**, DOI: 10.1007/s11356-020-10489-6

Chapter 7

Oumaima Boutoub, Soukaina El-Guendouz, Isabel Matos, Lahsen El Ghadraoui, Maria Clara Costa, Jorge Carlier, Maria Leonor Faleiro, Ana Cristina Figueiredo, Maria Letícia Estevinho and Maria Graça Miguel. Chemical characterization and biological properties assessment of *Euphorbia resinifera* and *Euphorbia officinarum* Moroccan propolis. Submitted, **2023**.

Introduction Générale et objectifs principaux

Au Maroc, les espèces d'Euphorbia sont utilisées en médecine traditionnelle pour leurs potentiels bienfaits thérapeutiques. Il est important de noter que l'utilisation traditionnelle d'*Euphorbia* spp. en médecine marocaine est transmise de génération en génération et peut varier selon les régions et les communautés.

Euphorbia resinifera communément appelée euphorbe résineuse et localement connue sous le nom de "Zaggoume", et *Euphorbia officinarum*, connue localement sous le nom de "Daghmouss", sont toutes les deux très utilisées dans la médecine traditionnelle marocaine depuis des décennies, seules ou en association avec d'autres plantes et/ou du miel. La recherche s'est concentrée sur le latex de ces espèces, même si elle commence à étudier d'autres parties de la plante. Dans *E. officinarum*, plus de soixante-dix composés hémisynthétisés ont été obtenus à partir de certains triterpènes du latex d'*E. officinarum* afin d'obtenir des composés ayant une activité insecticide et antimicrobienne plus élevée. Du latex d'*E. resinifera* peut être isolée la résinifératoxine (diterpène extrêmement irritant) qui a été utilisé dans certains essais cliniques comme analgésique potentiel pour soulager les douleurs liées au cancer et à l'arthrite. La recherche scientifique est réduite pour la totalité des autres parties de la plante. La partie aérienne, la fleur et les produits de la ruche des origines *Euphorbia resinifera* et *Euphorbia officinarum* sont moins étudiés.

Avec la stratégie de Maroc Vert, une grande attention a été portée à la valorisation des produits naturels tels que les plantes et au développement des produits de la ruche (miel, propolis...). Pour cette raison, de vastes recherches scientifiques concernant la composition chimique, les propriétés biologiques et les applications pharmaceutiques et industrielles sont nécessaires à la valorisation des différentes parties de ces deux espèces d'Euphorbia.

La composition chimique et les propriétés biologiques *in vitro* de certaines parties d'*E. resinifera* et *E. officinarum*, ainsi que de certains produits de la ruche d'origine E. resinifera et E. officinarum étaient les principaux objectifs de cette thèse.

Les principaux objectifs de ce travail étaient:

(i) Optimisation des meilleures conditions concernant le rapport soluté / solvant (eau) (10 – 50 mg/mL), la température d'extraction (24 – 50°C) et la durée d'extraction (60 – 480 min) des parties aériennes de *E resinifera* et *E. officinarum* afin d'obtenir des extraits aqueux aux propriétés biologiques *in vitro* plus élevées;

(ii) Révéler les principaux métabolites primaires et secondaires et les activités biologiques *in vitro d'Euphorbia resinifera* cyathia;

(iii) Évaluation de la composition chimique du miel d'Euphorbia marocain et de ses activités biologiques *in vitro* et détermination si l'activité biologique in vitro trouvée pour le miel peut être attribuée au miel entier ou à une fraction spécifique.

(iv) Évaluation de la composition chimique de la propolis Euphorbia marocaine et de ses activités biologiques *in vitro* et de ses activités biologiques in vitro, en accordant une attention particulière à l'activité antimicrobienne et à la perturbation de la formation du biofilm bactérien, y compris celle des pathogènes bactériens résistants.

(v) Déterminer quels produits naturels (extraits aqueux ou miel respectif) présentent une meilleure activité.

Les objectifs ont été globalement atteints et les résultats sont compilés dans certains des chapitres qui constituent ce doctorat thèse.

Le doctorat. La thèse est organisée en 7 chapitres, dont 4, les chapitres 1, 3, 5 et 6 constitués de travaux scientifiques publiés et évalués par des pairs. Le chapitre 1 correspond à un article de synthèse, et les chapitres 3, 5 et 6 sont des articles réguliers avec résumé, introduction, méthodologie, résultats, discussion ou résultats et discussion, conclusions et références. Les chapitres 2 et 7 correspondent aux articles soumis à des revues à comité de lecture et le chapitre 4 correspond à un article en cours de révision.

Les critères de citations et de liste de références étaient basés sur le numéro qui apparaît le long du texte par ordre croissant. Dans les sections de référence, cet ordre numérique est également suivi. Ceci a été fait pour uniformiser la présentation des thèses puisque divers critères existent selon les revues. Toutes les sections de chaque chapitre commencent par le numéro un et suivent par ordre croissant, tandis que les tableaux et figures suivent le numéro de chaque chapitre.

Les chapitres 1 et 2 sont des articles de synthèse sur Euphorbia officinarum et E. resinifera, respectivement. Ils se concentrent principalement sur la composition chimique du latex et ses propriétés biologiques, même si, plus récemment, d'autres matières premières et produits apicoles ont commencé à intéresser les chercheurs. Ils constituent l'introduction de ce doctorat thèse.

Le chapitre 3 se concentre sur la définition des meilleures conditions d'extraction (température, temps d'extraction et rapport plante/solvant) à partir des parties aériennes d'*E. officinarum* et *d'E. resinifera*, sans latex ni cyathia pour obtenir des quantités plus élevées de phénols totaux et une meilleure teneur en propriétés biologiques in vitro (antioxydantes et inhibition de la α-glucosidase) d'*E. officinarum* et d'*E. resinifera*.

Le chapitre 4 se concentre sur la découverte des métabolites primaires et secondaires du cyathia d'*E. resinifera* qui peuvent expliquer la présence ou non de certains métabolites dans le miel

monofloral ainsi que les attributs biologiques qui conduisent à l'utilisation de cette matière première en médecine traditionnelle.

Le chapitre 5 aborde les paramètres physicochimiques du miel monofloral d'*E. officinarum* et d'*E. resinifera* ainsi que leurs activités antioxydantes et inhibitions de diverses enzymes, et tente de comprendre si ces activités sont dues au miel entier ou à une fraction.

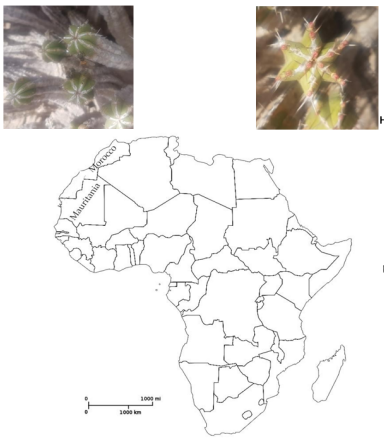
Le chapitre 6 se concentre sur la teneur totale en phénol, l'antioxydant et l'inhibition des activités de diverses enzymes d'extraits aqueux obtenus à partir des parties aériennes d'*E. officinarum* et d'*E. resinifera* sans latex ni cyathia et les compare avec celles du miel respectif.

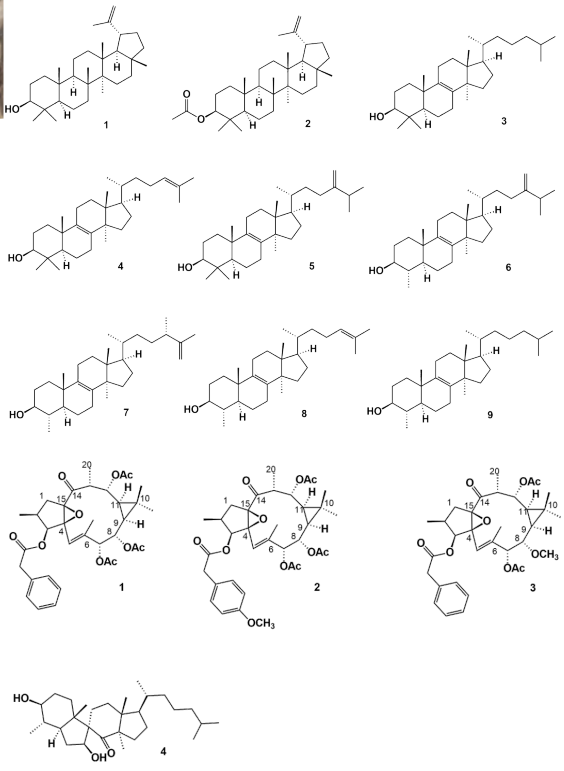
Le chapitre 7 aborde le profil minéral et volatil de la propolis d'*E. offcinarum* et d'*E. resinifera* ainsi que l'antioxydant, l'inhibition de certaines activités enzymatiques et antimicrobiennes, en particulier sur la capacité à inhiber la détection du quorum dans le biocapteur *Chromobacterium violaceum CV026* et la perturbation de la formation de biofilm bactérien. , y compris celle des bactéries pathogènes résistantes.

La derniere partie présente des conclusions globales et des travaux futurs.

Chapter 1

Introduction





1

Chapter I: Biological properties of latex, aqueous extracts and bee products of *Euphorbia officinarum* L.: A short review*

Résumé

Euphorbia officinarum L. est une plante endémique marocaine connue sous le nom de « Tikiout » et « Daghmous » que l'on trouve également en Mauritanie, au Sahara marocain et en Algérie. Dans la présente revue, « *Euphorbia officinarum* », « métabolites » « hémisynthèse » ont été les mots clés utilisés pour la recherche dans le moteur de recherche Web Google Scholar et dans la base de données Web of Science.

Des triterpènes, des phytosterols et des diterpènes d'ingol ont été isolés et identifiés dans le latex d'*E. Officinarum* marocain. Plus de soixante triterpènes ont été obtenus par hémisynthèse à partir de triterpènes naturels. Certains de ces dérivés avaient une activité insecticide et antimicrobienne (bactéries phytopathogènes). La teneur totale en phénols et les activités antioxydante et anti-α-glucosidase dépendaient du temps et de la température des extractions ainsi que du taux de solvant de la plante.

L'activité antioxydante du miel monofloral d'origine *E. officinarum* a été attribuée à la fraction phénolique (cette fraction, préalablement isolée à partir d'échantillons de miel, avait une meilleure activité que le miel entier).

Mots clés : *Euphorbia officinarum* ; Euphorbe; triterpène; les dérivés triterpéniques ; les diterpènes d'ingol; activité insecticide ; activité anti-microbienne; activité antioxydante; activité inhibitrice de l'α-glucosidase

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* Adapté de Oumaima Boutoub, Lahsen El Ghadraoui and Maria Graça Miguel. Biological properties of latex, aqueous extracts and bee products of *Euphorbia officinarum* L.: A short review. *Molecules*. **2022**, DOI: 10.3390/molecules27217200.

1

Biological properties of latex, aqueous extracts and bee products of *Euphorbia officinarum* L.: A short review*

Abstract

Euphorbia officinarum L. is a Moroccan endemic plant known as "Tikiout" and "Dagh- mus" that can also be found in Mauritania, Moroccan sahara, and Algeria. In the present review, "*Euphorbia officinarum*", "metabolites" "hemisynthesis" were the keywords used for the research in the Web search engine Google Scholar and in the database Web of Science. Triterpenes, phytosterols and ingol diterpenes were isolated and identified in the latex of Moroccan *Euphorbia officinarum*. More than sixty triterpenes were obtained by hemisynthesis from natural triterpenes. Some of these derivatives had insecticidal and antimicrobial activity (phytopathogenic bacteria). The total phenol content and the antioxidant and anti-α-glucosidase activities were dependent on the time and temperature of extractions and also on the plant solvent ratio. The antioxidant activity of monofloral honey of *Euphorbia officinarum* origin was attributed to the phenol fraction (this fraction, previously isolated from honey samples, had better activity than the entire honey).

Keywords: *Euphorbia officinarum*; spurge; triterpene; triterpene derivatives; ingol diterpenes; insec-ticidal activity; antimicrobial activity; antioxidant activity; α-glucosidase inhibitory activity

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* Adapted from Oumaima Boutoub, Lahsen El Ghadraoui and Maria Graça Miguel. Biological properties of latex, aqueous extracts and bee products of *Euphorbia officinarum* L.: A short review. *Molecules*. **2022**, DOI: 10.3390/molecules27217200.

1. Introduction

Euphorbiaceae family encloses 6000 species of plants, and genus Euphorbia is the largest in the spurge family. *Euphorbia* are succulent plants that may be found from Africa to the Canary Islands, in Madagascar, India, and the Americas and even Australia (1). *Euphorbia officinarum* L. (Figure 1.1) is a Moroccan endemic plant known as "Tikiout" and "Daghmus". This species can also be found in Mauritania, Moroccan sahara, and Algeria (1) (Figure 1.2). *E. officinarum* belonging to the *Euphorbia* genus has the presence of a milky white latex, which has been studied not only for its chemical characterization but also due to its biological properties (2). Generally, this latex has been used since ancient times in folk medicine although containing harmful compounds. The term *Euphorbia* was named in honour of "Euphorbus", the physician of King Juba II of Mauritania, who paid attention to the medicinal properties of *E. officinarum*, for the first time (3). There is one infra-specific taxon of the species *E. officinarum* L. (*E. officinarum* subsp. *echinus* (Hook.f. & Coss.) Vindt) (4).

Since ancient times, *Euphorbia officinarum* has been used in folk medicine, although currently it has been determined that this is obsolete, at least for some ailments. For example, there are still descriptions that the milky sap of this species is used in earache and as emetic in Buxar district, India (5), although this utilization as emetic has already been considered outdated (6). Anti-diabetic utilization of *E. officinarum* is particularly cited in diverse places in Morocco and almost always under powder obtained from aerial parts of *E. officinarum* subsp. *echinus*. Some local examples of its use include Agadir Ida Outanane region (Southwest Morocco) (7), Tata Province (8), Chtouka and Tiznit (western Anti-Atlas) (9), multiple regions (10) and under decoction in Beni Mellal-Khenifra (11). *E. officinarum* L. is also used as anti-diabetic, according to different authors (10–13).

Figure 1.1. *Euphorbia officinarum* L. in Moroccan fields.

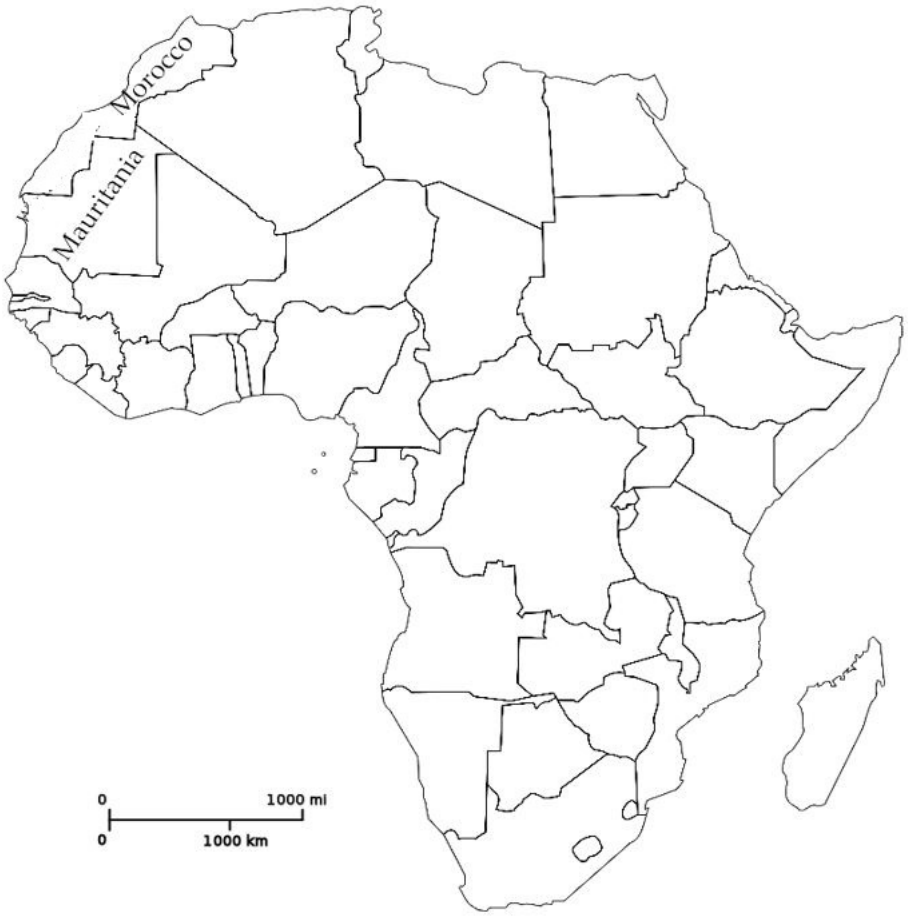


Figure 1.2. Geographical location of Euphorbia officinarum. Adapted from https://en.wikipedia.org/ wiki/File:Blank_Map-Africa.svg (accessed 22 October 2022).

E. officinarum in a mixture with other plants (*Opuntia ficus-barbarica*, *Zea mays* and *Ziziphus lotus*) and honey has been used in the treatment of pyelonephritis and cystitis, in Moroccan Sahara (14); the flowers and roots of *E. officinarum* subsp. *echinus* are also reported in the treatment of pyelonephritis in Central Morocco (15). *E. officinarum* subsp. *echinus* has also been reported in the treatment of wounds, skin infections and abscesses in diverse places in Morocco (16–18) and by the Sahrawi refugees in Algerian refugee camps (19). Although Idm'hand et al. (18) had noted the utilization of that species in the treatment of skin diseases, their work indicated that the specific treatment purpose of *E. officinarum* subsp. *echinus* was the elimination of helminthes.

In Morocco, the root powder of *E. officinarum* subsp. *echinus* has also been reported in the treatment of cancer, although not specifying which type (16,20,21). Through ethno- botanical interviews and vegetation surveys in a Saharan Moroccan village, Blanco and Carrière (22) reported that *E. officinarum* subsp. *echinus* use presented a Smith Salience index:

$$\text{Smith Salience index} = \frac{\sum_{i=1}^{N} \frac{L_i - R_a + 1}{L_i}}{N} \quad (1)$$

N: total number of informants; Li: size of the list for the informant i; Ra: rank of appearance of the ethnospecies and frequency of 0.239 and 38%, respectively. Other uses have also been described for *E. officinarum*, such as for respiratory and circulatory systems (23), and as gum-resin for headache, paralysis and apoplexy (24). In Morocco, *E. officinarum* subsp. *echinus* aerial parts may be chopped and cooked as a vegetable salad (25,26), despite its relative high toxicity (2).

2. Secondary metabolites isolated from Euphorbia officinarum L. of Morocco

In 1985, for the first time, Ben Harref and Lavergne (27) isolated from the methanolic extract of the latex of *E. officinarum*, collected in the North Atlantic coast of Agadir (Morocco), nine compounds with triterpenic (lupeol (**1**), lupeol acetate (**2**)) and steroidal skeleton (lanostenol (**3**), lanosterol (**4**), 24-methylene lanostenol (**5**), 4α,14α-dimethyl-24-methylen- 5α-cholest-8-en-3β-ol or obtusifoliol (**6**), 24(R)-4α,14α,24-trimethyl-5α-cholesta-8,25-dien-3- β-ol (**7**), 4α,14α-dimethyl-5α-cholest-8,24-dien-3β-ol (**8**), and 4α,14α-dimethyl-5 α-cholest- 8-en-3-β-ol (**9**)) (Figure 1.2). In 1999, Daoubi et al. (28) isolated 3 steroidal compounds from a methanolic extract of *E. officinarum* latex collected in the North Atlantic coast of Agadir (Morocco), one of them being 4α,14α-dimethyl-5α-cholest-8-en-3-β-ol, already isolated and identified by Ben Harref and Lavergne (27) and two new ones described for the first time by the authors: 3β,7α-dihydroxy-4α,14α-dimethyl-5α-cholest-8-en-11-one (**10**) and 3β,7β-dihydroxy-4α,14α-dimethyl-5α-ergost-8-en-11-one (**11**) (Figure 1.3). The identification was established on the basis of proton-nuclear magnetic resonance (^{1}H-NMR), carbon 13-nuclear magnetic resonance (^{13}C-NMR), heteronuclear multiple bond correlation (HMBC), electron ionization mass spectroscopy (EIMS), and high-resolution electron ionization mass spectrometry (HREIMS).

Mazoir et al. (29) isolated 2 steroidal compounds, 4α,14α-dimethyl-24-methylen- 5α-cholest-8-en-3β-ol or 4α,14α-dimethyl-5α-ergosta-8,24-dien-3β-ol or obtusifoliol (**6**) and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**), previously identified by Ben Harref and Lavergne (27) from the latex of *E. officinarum* (Figure 1.3) from which they obtained derivatives, by oxidation of the hydroxyl group at C_3 with chromic anhydride. Nevertheless, Mazoir et al. (29) reported 4α, 14α-dimethyl-5α-cholest-8-en-3β-ol as being synonymous of 31-norlanosterol. However, we believe that the identified compound is in fact 4α,14α-dimethyl-5α-cholest-8-en-3β-ol because the chemical structure presented in the article is most definitely the one from this compound and not that of 31-norlanosterol. Beyond the triterpenic and steroidal compounds isolated from *E. officinarum*, Daoubi et al. (2) isolated and identified highly functionalized ingol diterpenes: ingol 7,8,12-triacetate 3-phenylacetate) (**1**), ingol 7,8,12-triacetate 3-(4-methoxyphenyl) acetate (**2**) and 8-methoxyingol 7,12-diacetate 3-phenylacetate (**3**) (Figure 1.4). Along with these three compounds, Daoubi et al. (2) also identified the novel spirotriterpene 3*S*, 4*S*, 5*R*, 7*S*, 9*R*, 14*R*-3, 7-

dihydroxy-4, 14-dimethyl-7(8 9)-abeo-cholestan-8-one (**4**) (Figure 1.4). The structure was established following the same procedures already described by the same team (28). The plant origin was the same as previously reported (2, 28). The authors also proposed a possible biosynthetic pathway of the spirotriterpene compound from 4α, 14α-dimethyl-5α- cholest-8-en-3-β-ol, since they coexist in the same plant. The hypothesis presented by the authors is based on other results observed for *E. supina* and *Ficus microcarpa*.

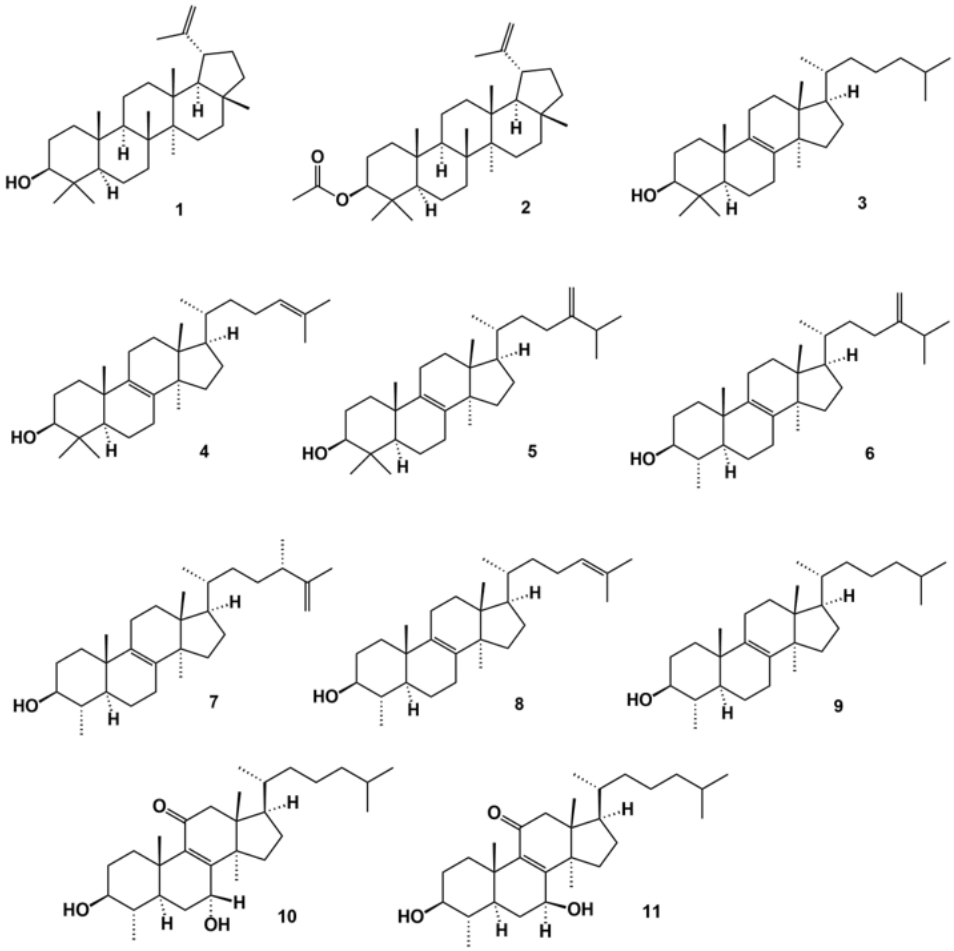


Figure 1.3. Triterpenic (1 and 2) and steroid compounds isolated and identified in the Moroccan latex of *Euphorbia officinarum*.

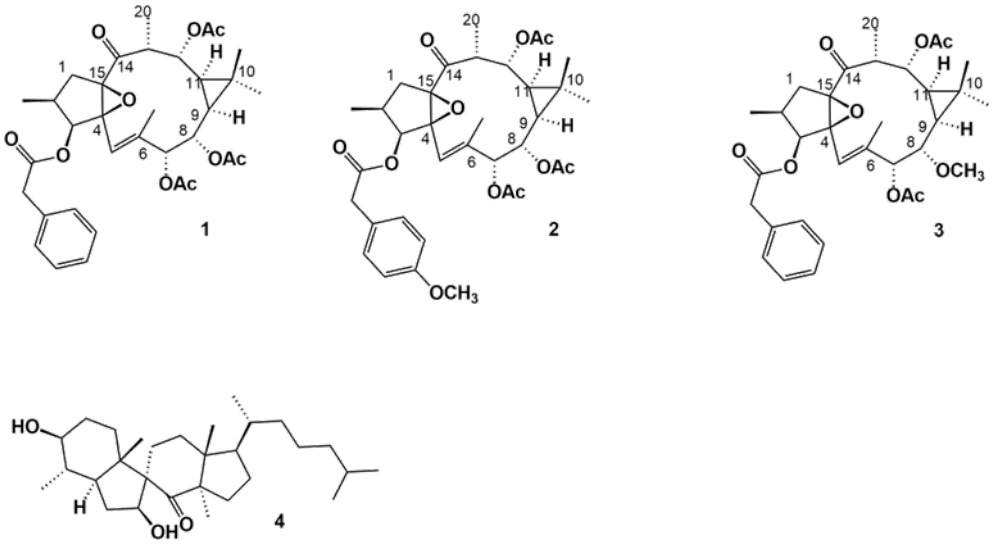


Figure 1.4. Phenylacetylingol derivatives (1, 2, and 3) and spirotriterpenoid (4) isolated from the methanolic extract of *Euphorbia officinarum* latex.

3. Biosynthesis of diterpenes, triterpenes and sterols

Terpenoids are assembled from C_5 units (isoprene-like), and the number of repetitions of this unit, followed by cyclization reactions, rearrangements and oxidation of the terpene skeleton determine the huge diversity of structures (more than 80,000) (30). Nevertheless and concerning the biosynthesis of these natural compounds, they have as central intermediates the isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two distinct pathways generate these C_5 precursors: the mevalonate pathway (MVA), which occurs in cytosol, and the deoxyxylulose-5-phosphate (DXP), more recently known as methyl-D-erythritol-4-phosphate (MEP), which occurs in plastids (Figure 1.5) (31). The MVA pathway provides cytosolic metabolites, such as C_{30} triterpenes and their saponin derivatives, and C_{27}-C_{29} steroids, plus some C_{15} sesquiterpenes, whereas MEP provides C_{10} monoterpenes, C_{20} diterpenes, C_{40} tetraterpenes (carotenoids), some C_{15} sesquiterpenes and the prenyl side chains of chlorophyll and plastoquinones (31). These terpenes can then undergo oxygenation through the activity of cytochrome P_{450} monooxygenases (P_{450}), followed by the introduction of other functional groups, originating more than 80,000 natural products (30).

Many studies have demonstrated that species of the family Euphorbiaceae are producers of unique diterpenoids, isolated from milky latices, which belong to the macrocylic diterpenes with diverse skeletons (jatrophane, lathyrane, terracinolide, ingenane, pepluane, paraliane, and segetane) (Figure 1.6) (32). All skeletons have geranyl geranyl-diphosphate as precursor, as casbene is the precursor of the macrocyclic and polycyclic diterpenes (Figure 1.6) and then proceed through intermediates such as jolkinol C (32).

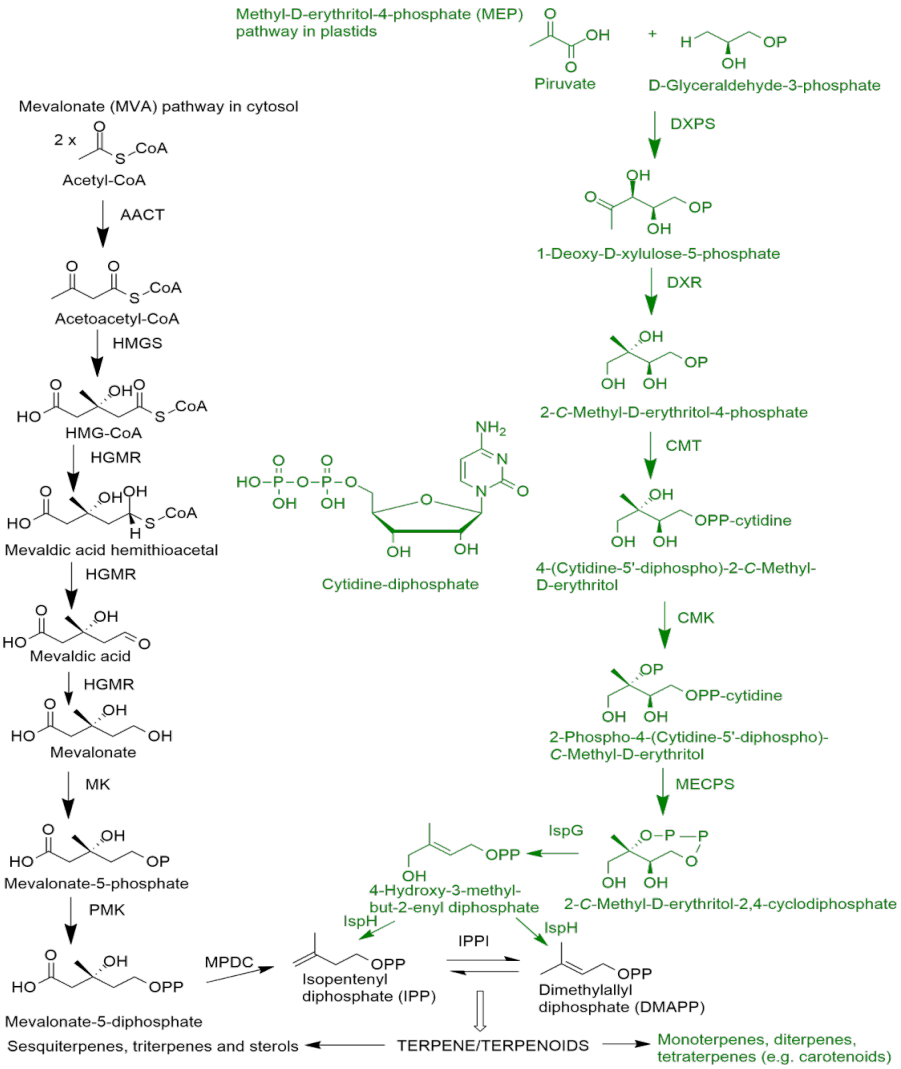


Figure 1.5 Biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by the mevalonate (MVA) and methyl-D-erythritol-4-phosphate (MEP) pathways as well the enzymes involved in such pathways. AACT: Acetoacetyl-CoA-thiolase; CMK: 4-diphosphocytidyl-2-C-methyl- D-erythritol kinase; DXR: 1-deoxy-D-xylulose-5-phosphate reductase; DXPS: 1-deoxy-D-xylulose5- phosphate synthase; HDR: (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate reductase; HMGR: 3- hydroxy-3-methylglutaryl-CoA reductase; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; IPPI: isopentenyl diphosphate isomerase; IspG: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; IspH: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; MDPC: mevalonate-5-diphosphate decarboxylase; MECPS: MEP cytidyltransferase; MK: mevalonate kinase; PMK: phosphomevalonate kinase (adapted from (31)).

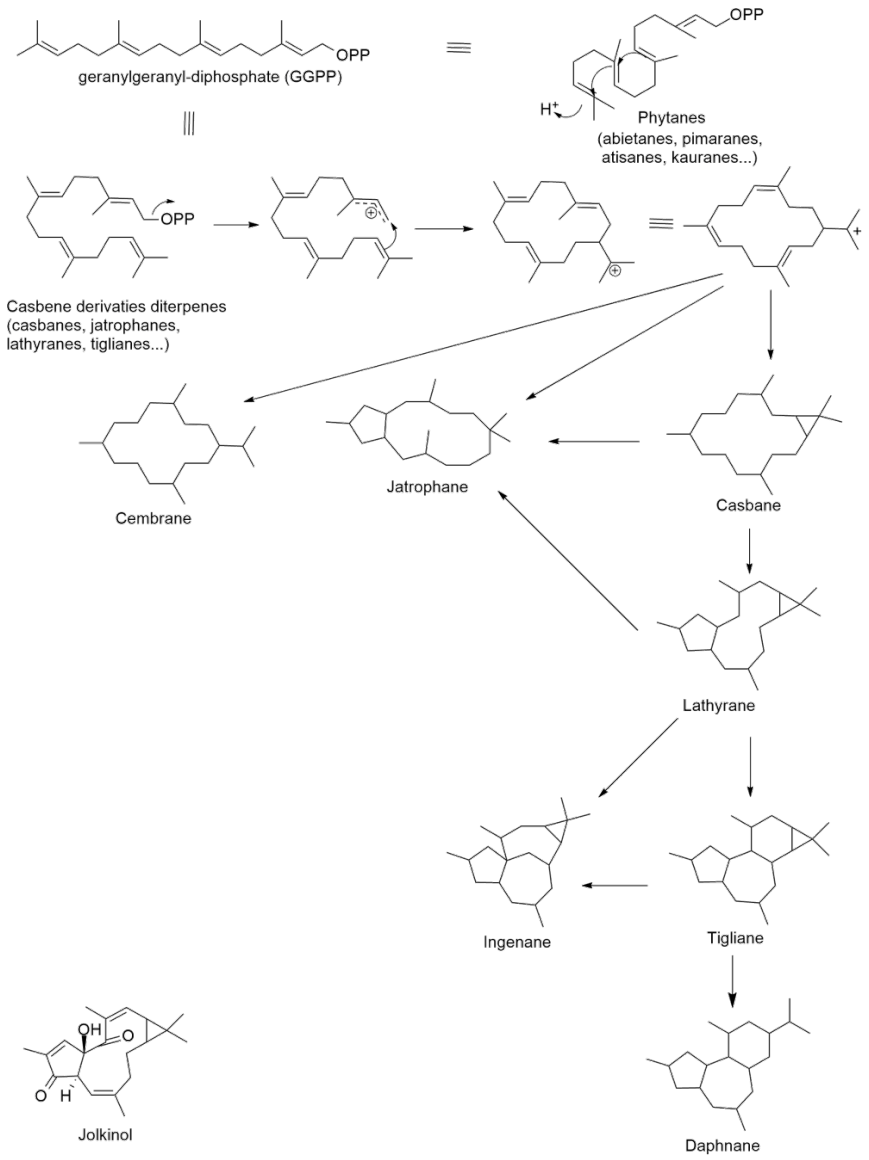


Figure 1.6. Schematic biogenesis of macrocyclic and polycyclic diterpenes derived from casbene (adapted from (32)).

Ingol 7,8,12-triacetate 3-phenylacetate (1), ingol 7,8,12-triacetate 3-(4-methoxy-phenyl) acetate (**2**) and 8-methoxyingol 7,12-diacetate 3-phenylacetate (**3**) have the lathyrane skele-ton, that is, tricyclic diterpenes with a 4/11/3-ring system, an epoxy functionality at C-4 and C-15 and a double bond between C-5 and C-6 (Figure 1.3) (33), which precursors are most likely the ones just mentioned. Further, those ingol diterpenoids underwent redox, etherification and esterification modifications of the 5- and 11-membered rings (Figure 1.3). The presence of the phenylacetyl group is also frequent in lathyrane diterpenoids (34). Although the biological properties of those lathyrane diterpenes have not been evaluated, Vela et al. (34) in their review work noted that terpenes belonging to this group have biological properties with clinical potential (cytotoxicity, multidrug resistant reversal ability, antiviral and anti-inflammatory properties, and capability to induce neural progenitor cell proliferation or differentiation into neurons).

As far as we know there are not any biosynthetic studies on the diterpenes, triterpenes and sterols in *E. officinarum*, only a hypothesis for the biosynthetic pathway of the spirotriterpene, 3*S*,4*S*,5*R*,7*S*,9*R*,14*R*-3,7-dihydroxy-4,14-dimethyl-7(8 9)-abeo-cholestan- 8-one proposed by Daoubi et al. (2) (Figure 1.7).

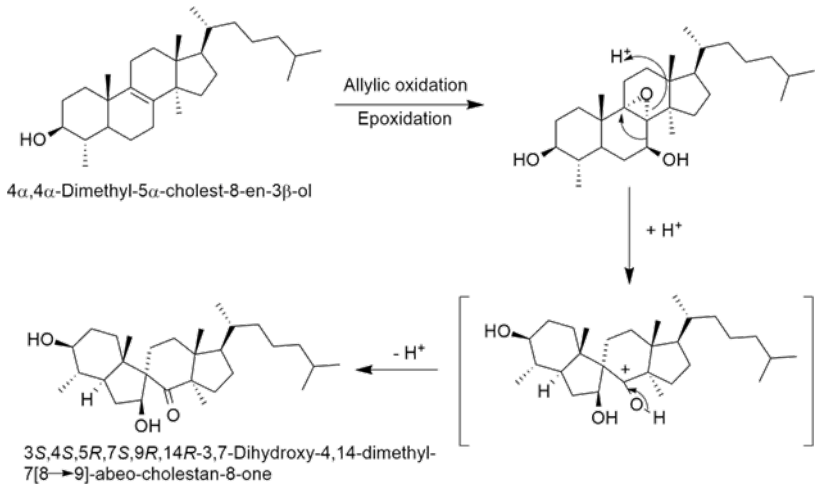


Figure 1.7. Hypothesis for the biosynthesis of the spirotriterpene (adapted from (2)).

Sterols and triterpenes are synthesized via the mevalonate pathway as aforementioned and the condensation tail-to-tail of two units of FPP originates the squalene (Figure 1.8). After the formation of this intermediate, there is the formation of 2,3-oxidosqualene, in animals, fungi and plants, which after cyclization leads to the sterols and triterpenes. In sterol biosynthesis, 2,3-oxidosqualene in the conformation chair-boat-chair is cyclized to lanosterol, in animal and fungi, whereas in plants, the cyclization originates cycloartenol (Figure 1.8) (35). In the triterpenes, 2,3-oxidosqualene is folded into the chair-chair-chair conformation, which originates diverse structures such as lupeol, after cyclization, as depicted in Figure 1.8, a triterpenoid found in the *E. resinifera* latex (27).

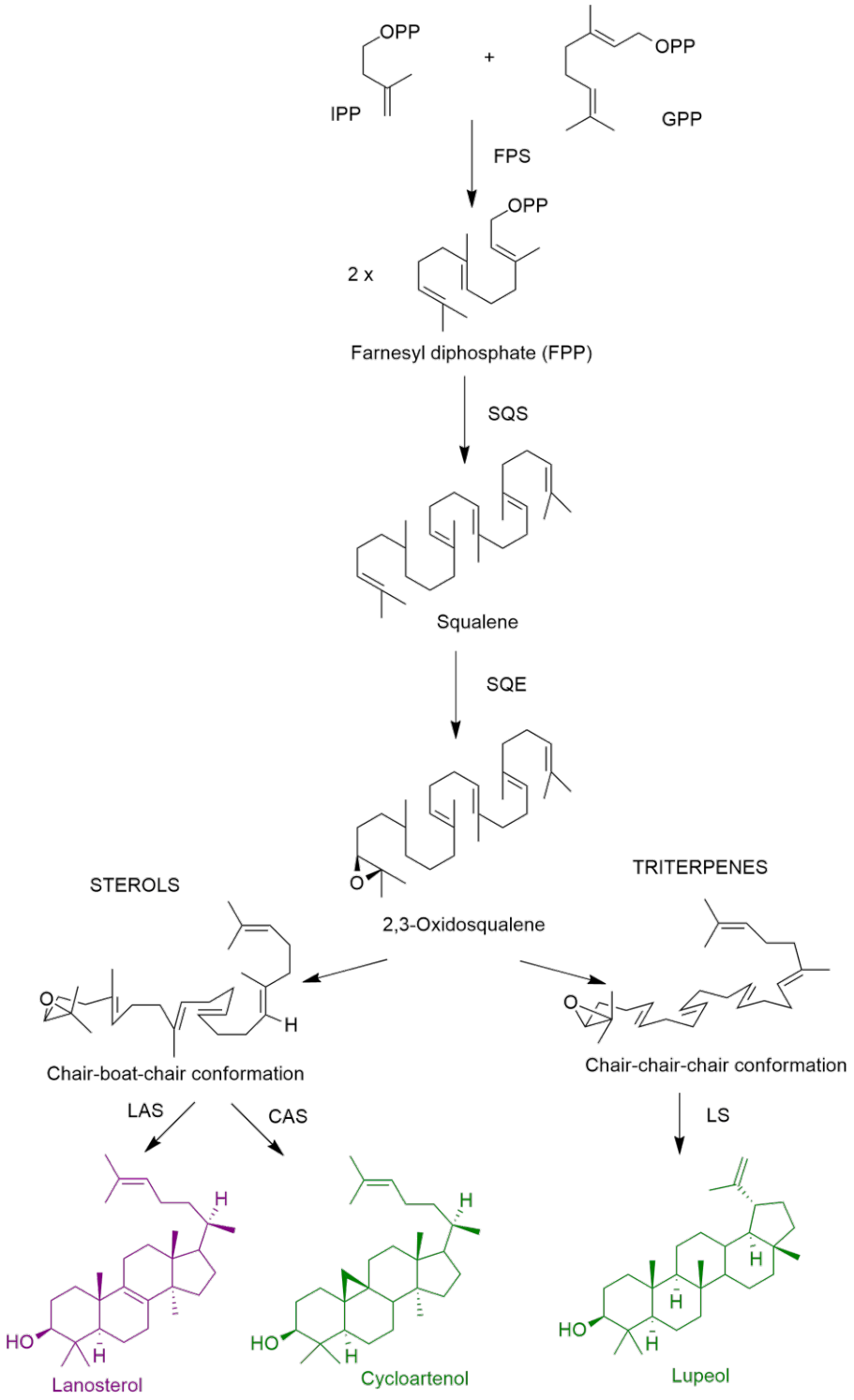


Figure 1.8. Schematic biosynthesis of sterols and triterpenes. CAS: cycloartenol synthase; FPS: farnesyl diphosphate synthase; GPP: geranyl diphosphate; LAS: lanosterol synthase; LS: lupeol synthase; IPP: isopentenyl diphosphate; SQE: squalene monooxygenase or epoxidase; SQS: squalene syntase (adapted from (35)).

Obtusifoliol is a phytosterol, which means the presence of an extra one-carbon on the side-chain, attached at C_{24} and the substrate for alkylation is cycloartenol (Figure 1.9) (36). Generally, the substrate for the biosynthesis of phytosterols is cycloartenol, whereas in fungi it is lanosterol (36). However, in *E. lathyris*, Forestier et al. (37) reported lanosterol synthase in the cytoplasm of laticifers, in combination with cycloartenol synthase and butyrospermol synthases. Therefore, further studies are needed in order to better understand the role of cycloartenol and lanosterol in the biosynthesis of phytosterols in Euphorbiaceae.

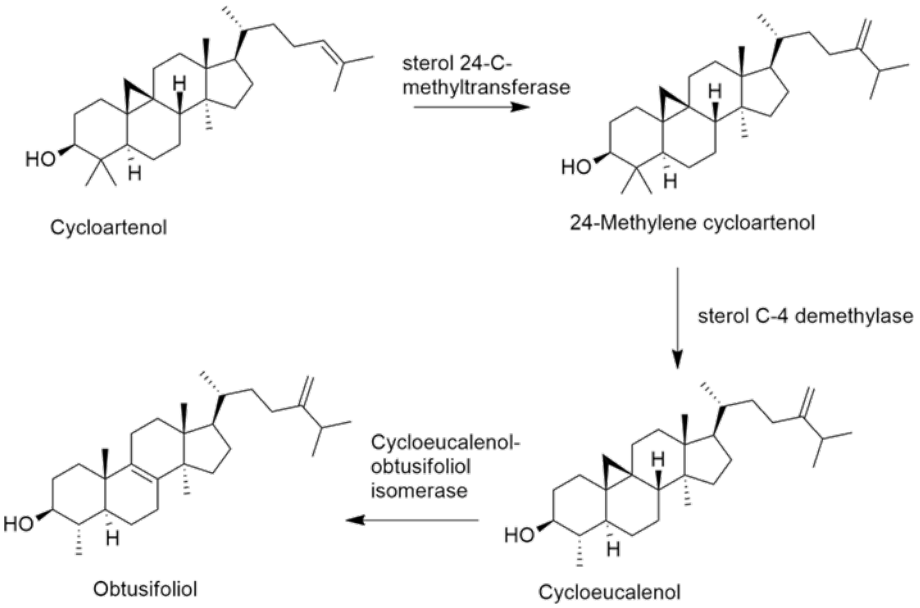
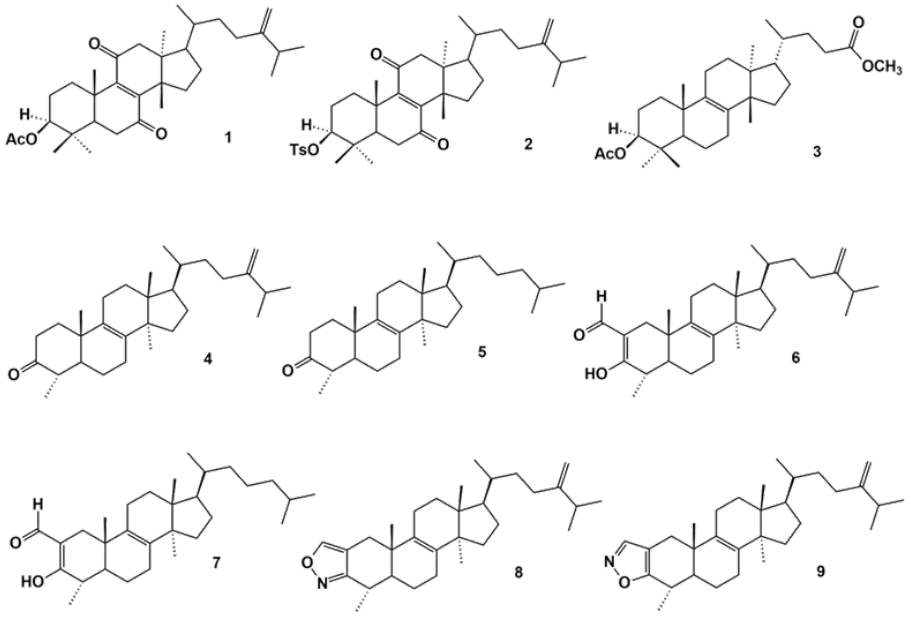


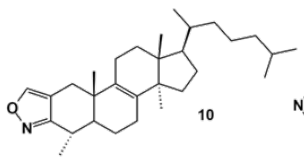
Figure 1.9. Schematic biosynthesis of obtusifoliol (adapted from (36)).

4. Hemisynthesis of triterpene derivatives isolated from E. offlcinarum latex and their biological properties

The biological properties found for latex samples or some of their isolated compounds (diterpenic, triterpenic or steroidal compounds) of *Euphorbia* species (cytotoxic, antimicrobial, human immunodeficiency virus type 1 reactivation, among others) (1, 2, 38–41), including *E. officinarum*, have led chemical modifications to obtain derivative compounds with the aim of improving the active properties. For example, Mazoir et al. (see below) published several works in order to obtain oxygenated triterpenic compounds that show good pharmacological activities. Generally, the procedures used were based on the oxidation of diverse triterpenic or steroidal compounds isolated from *E. officinarum*: from 24-methylene lanostenol to (3*S*)-acetyl-24-methyl-elemo-lanosta -8,24-diene-7,11- dione (**1**) (Figure 1.10), using chromic anhydride and acetone (42); (3*S*)-tosyl-24-methyl-elemo-lanosta-8,24-diene-7,11-dione (**2**) from 3(*S*)-tosyl-24-methylene lanostenol using the same reagents (43); (3*S*,5*S*,10*S*,13*S*,14*S*,17*S*)-methyl-3β-acetyl-25,26,27-trisnorlanost-8-en-24-oate (**3**) from eupholanosta-8,24-dien-3β-ol or lanosterol after

oxidation by ruthe- nium(III) chloride trihydrate, followed by esterification and acetylation reactions (29); 4α,14α-dimethyl-5α-ergost-8,24-dien-3-one (**4**) and 4α,14α-dimethyl-5α-cholest-8-en-3-one (**5**) from 4α,14α-dimethyl-5α-ergosta-8,24-dien-3β-ol and 4α,14α-dimethyl-5α-cholest-8- ene-3β-ol, respectively, with oxidation carried out using chromic anhydride and acetone; 2-formyl-4α,14α-dimethyl-5α-ergost-2,8,24-trien-3-ol (**6**) and 2-formyl-4α,14α-dimethyl-5α- cholesta-2,8-dien-3-ol (**7**) from the compounds (**4**) and (**5**), respectively, after treatment with ethyl formate, benzene and sodium methoxide (36). From (**6**), Mazoir et al. (44) obtained (1,2)isoxazolo (4,3-b)-4α,14α-dimethyl-5α-ergosta-8,24-diene (**8**) after treatment of (**6**) with acetic acid and hydroxylamine hydrochloride, and (1,2)isoxazolo (4,5-b)-4α,14α-dimethyl-5α-ergosta-8,24-diene (**9**) after reaction of (**6**) with pyridine and hydroxylamine hydrochloride; and from 2-formyl-4α,14α-dimethyl-5α-cholesta-2,8-dien-3-ol (**7**), Mazoir et al. (44) obtained (1,2)isoxazolo (4,3-b)-4α,14α-dimethyl-5α-cholesta-8-ene (**10**) and (1,2)isoxazolo (4,5-b)-4α,14α-dimethyl-5α-cholest-8-ene (**11**) (Figure 1.10) using the same reagents for obtaining the compounds (**8**) and (**9**), respectively. Mazoir et al. (45) also obtained by hemisynthesis, from the derivative 3β-tosyl-5α-ergost-8,24-diene, the oxidized compound (3*S*,4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-4α,14α-dimethyl-3β-tosyl-5α-ergost-8-ene-7,11,24-trione (**12**) after reaction with ruthenium trichloride followed by allylic oxydation with chromic anhydride. The same authors (46) also obtained the derivative (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**) (Figure 1.10), that is a triterpene functionalized with an oxirane bridge, from 4α,14α-dimethyl-5α-cholest-8-en-3β-ol, using the reagent chromic anhydride followed by epoxidation with a stoichiometric quantity of meta-chloroperbenzoic acid. This derivative could also be obtained from another deriva- tive, 8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3β-ol (**14**), after reaction with chromium anhydride (47).





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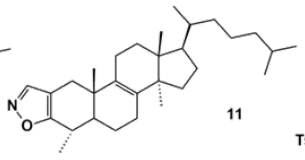

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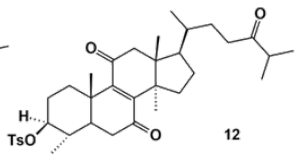

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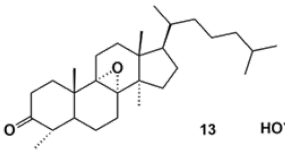

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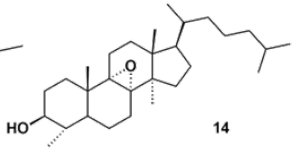

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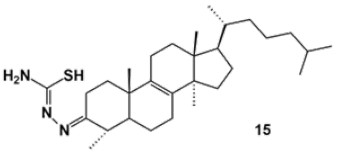

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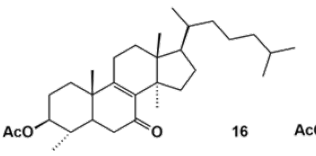

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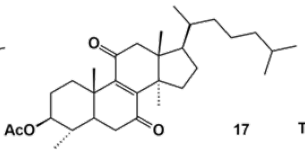

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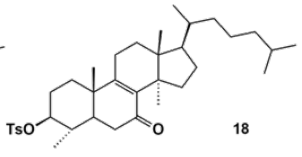

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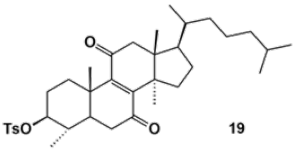

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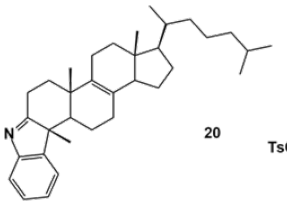

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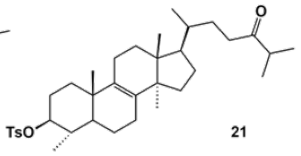

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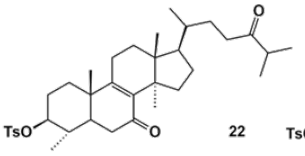

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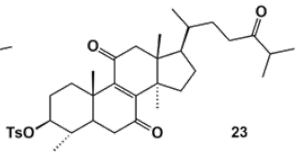

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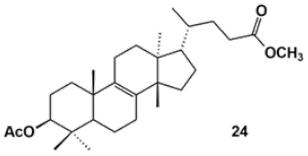

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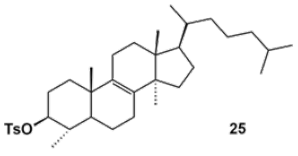

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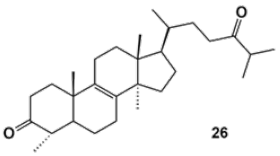

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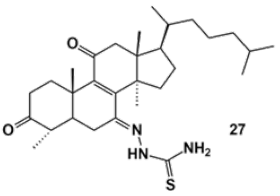

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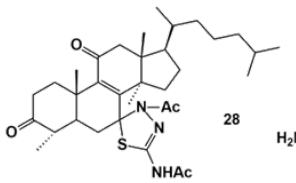

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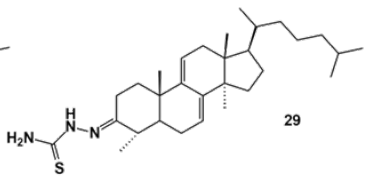

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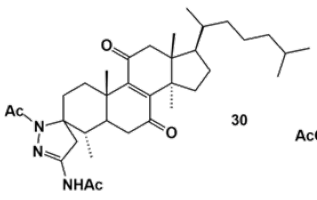

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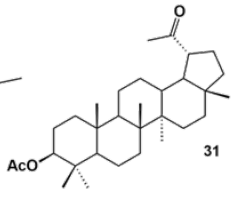

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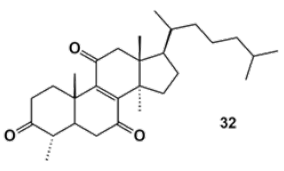

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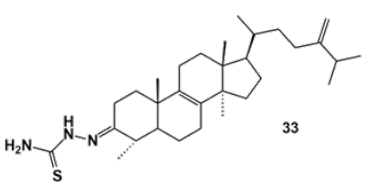

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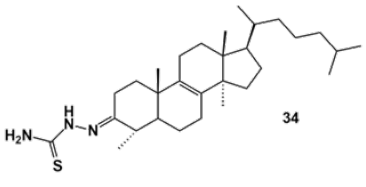

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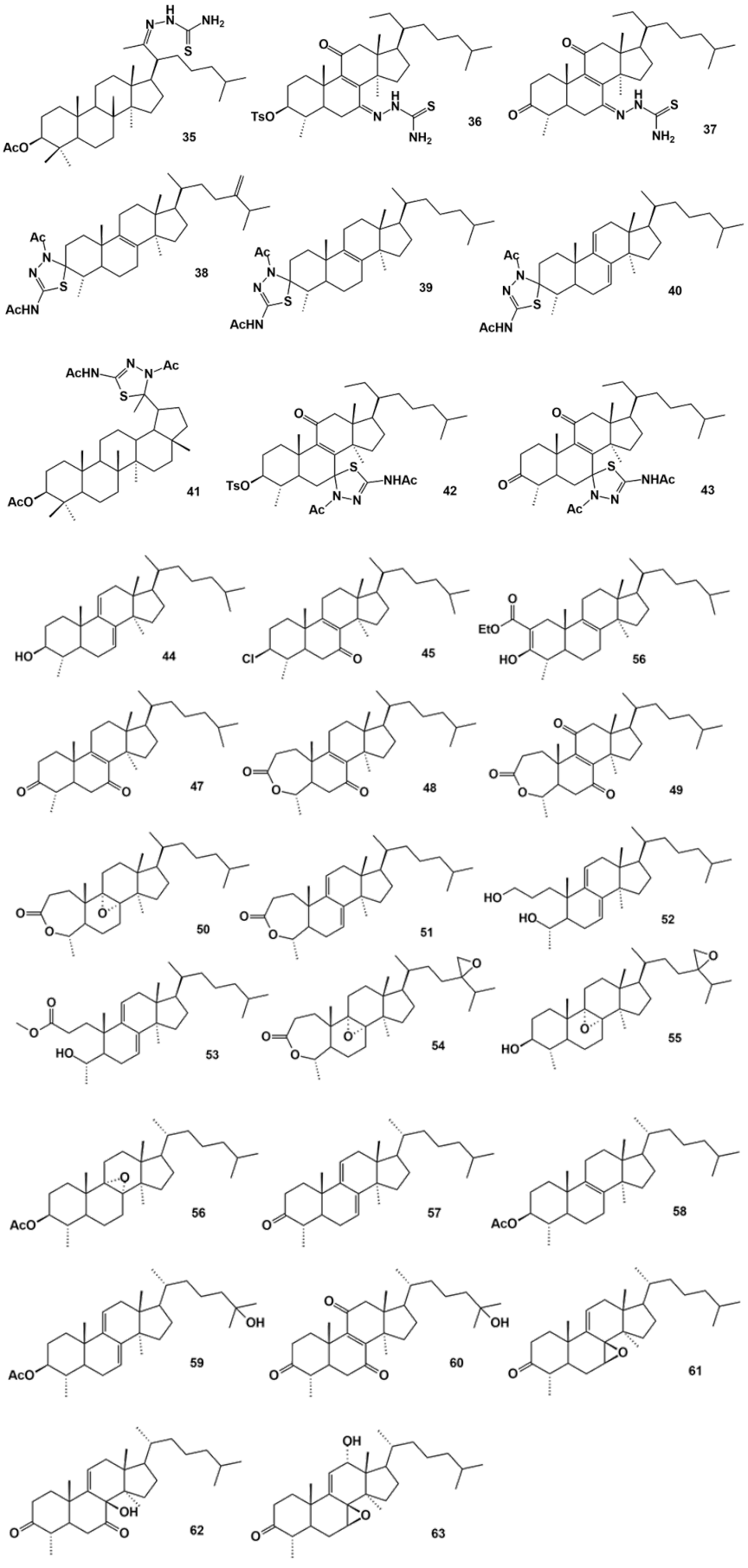
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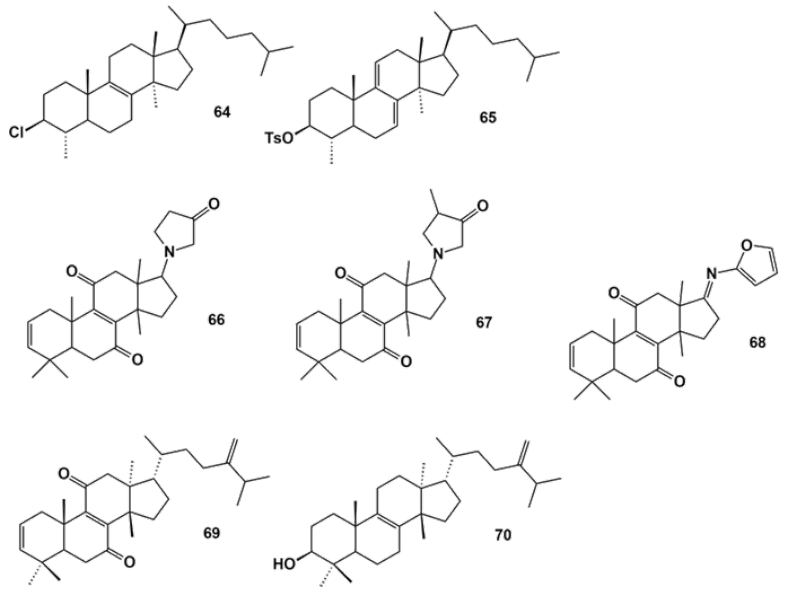

Figure 1.10. Triterpene derivatives obtained from natural triterpenes isolated from *Euphorbia officinarum* latex. Compounds 66, 67 and 68 according to the structures presented in Figure 1.9 of Daoui et al. (48).

The oxidation of 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**) with chromic anhydride produced the oxidized derivative 4α,14α-dimethyl-5α-cholest-8-en-3-one, which in the presence of thiosemicarbazide dissolved in ethanol and some drops of concentrated sulphuric acid produced the thiosemicarbazone derivative (**15**) (49). From 4α,14α-dimethyl- 5α-cholest-8-en-3β-ol (**9**), a major triterpene isolated from *E. officinarum* latex, it was also possible to obtain, by hemisynthesis, the derivatives 3β-acetoxy-4α,14α-dimethyl- 5α-cholest-8-en-7-one (**16**) and 3β-acetoxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**17**), after acylation and oxidation processes (47). The acylation can be replaced by a treatment with tosyl chloride followed by a similar oxidation condition, giving rise to the new derivatives 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-en-7-one (**18**) and 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**19**) (47). From the same steroidal compound isolated from *E. officinarum* latex, 4α,14α-dimethyl-5α-cholest-8-en-3β-ol, López-Rodríguez et al. (50) obtained by hemisynthesis 1-(1,5-dimethylhexyl)-3a,5b,12a,14a-tetramethyl- 2,3,3a,4,5,5a,5b,11,12,13,14, 14a-dodecahydro-1H,12aH-cyclopenta(1,2)-phenanthro(7,8-b)indole(**20**) (Figure 1.10), after oxidation with chromic anhydride in acetone at 273 K, in the presence of phenylhydrazine and acetic acid.

Tosylation of 4α-14α-dimethyl-5α-ergost-8-en-3β-ol, another terpene isolated from *E. officinarum* latex, Mazoir et al. (47) obtained by hemisynthesis 3β-tosyloxy-4α,14α-dimethyl- 5α-ergost-8-en-24-one (**21**), which after oxidation originated the derivatives 3β-tosyloxy- 4α,14α-dimethyl-5α-ergost-8-ene-7,24-dione (**22**) and 3β-tosyloxy-4α,14α-dimethyl-5α- ergost-8-ene-7,11,24-trione (**23**) (Figure 1.10). Oxidation of eupho-lanost-8,24-dien-3β-ol, another metabolite found in *E. officinarum* latex, with the system sodium periodate-ruthenium (III) chloride

trihydrate ($NaIO_4$-($RuCl_3$,$3H_2O$)) followed by esterification and acetylation reactions, originated (3*S*,5*S*,10*S*,13*S*,14*S*,17*S*)3β-acetyl-25,26,27-trisnorlanost-8-en-24-oate (**24**) (39) (Figure 1.10).

Beyond the production of triterpenoid derivatives obtained by hemisynthesis from triterpenoids isolated from the latex of *E. officinarum*, the same Moroccan team and others also from Morocco started to test the biological properties of the derivatives obtained or new derivatives along with the determination of their respective biological activities, with the purpose of obtaining compounds with better biological activities than the natural triterpenoids. This will fill the gap in studies on biological attributes of the natural triterpenes from *E. officinarum* latex or their derivatives—a gap that exists despite their known interesting pharmacological properties, including anti-inflammatory, antimicrobial and antiplasmodial activities (51).

With modifications on positions 3,7, 11 and 24 of obtusifoliol and 4α,14α-dimethyl-5α- cholest-8-en-3β-ol, isolated from *E. officinarum* latex, Mazoir et al. (52) obtained ten derivatives and evaluated the antifeedant effect on several insect species (*Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*), toxic effects on insect Sf9 and mammalian CHO cells, and phytotoxic effects on *Latuca sativa*. Out of the tested compounds, 4 had been already obtained: 3β-tosyloxy-4α,14α-dimethyl-5α-ergost-8-en-24-one (**21**), 4α,14α-dimethyl- 5α-ergost-8,24-dien-3-one (4), 3β-acetoxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**17**), and 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-en-7,11-dione (**19**). The remaining six compounds were β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene (**25**), 4α,14α-dimethyl-5α- ergost-8-en-3,24-dione (**26**), 4α,14α-dimethyl--5α-cholest-8-en-3,11-dione-7-thiosemicarbazone (**27**), 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7-thiadiazoline (**28**), 4α,14α-dimethyl-5α-cholesta-7,9-diene-3-thiosemicarba-zone (**29**), and 4α,14α-dimethyl-5α-cholest-8-ene- 7,11-dione-3-thiadiazoline (**30**) (Figure 1.10). The compounds 4α,14α-dimethyl-5α-cholest-8- en-3β-ol (**9**), 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**19**), 4α,14α-dimethyl-5α- ergost-8-en-3,24-dione (**26**) and 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7-thiosemicarbazone (**27**) were active in relation to *Myzus persicae*; 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**), 3β-acetoxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**17**), and 4α,14α-dimethyl-5α-ergost-8,24-dien-3-one (**4**) were active in relation to *Rhopalosiphum padi*; higher number of compounds were active in relation to *Spodoptera littoralis*, affecting insect growth, the C-3 substituent (C-3 hydroxyl is not essential for the insect growth) and C-7 substituent (52) being important. In addition, Mazoir et al. (52) also observed that the insect cells Sf9 were more sensitive to these 10 terpene derivatives than mammalian CHO cells, which could be explained by the differences in membrane composition and/or receptor affinity between insect and mammalian cells (52). All of these derivative compounds had non selective moderate phytotoxic effects on radicle elongation of *Lactuca sativa*. The *in vitro* activity on *Leishmania infantum* promastigotes and *Trypanosoma cruzi* epimastigotes of these terpenoid derivatives was also evaluated by the same Moroccan team

(53). The choices made by the authors relied on the fact that Leishmaniosis and Chagas' disease are still major worldwide health problems, with some medicines being ineffective or, in some cases, producing important side effects. The activities found for all compounds were moderate on both parasites, although some of them showed better activities. Thus, and in descending order of activity, the following compounds stand out: 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7-thiadiazoline (**28**), 3β-acetoxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**17**), 4α,14α-dimethyl-5α-ergost-8-en-3,24-dione-(**26**), 4α,14α-dimethyl-5α-ergosta-8,24-dien-3- one (**4**), and 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7-thiosemicarbazone (**27**). All of them had ED_{50} values (the effective dose to give 50% cell viability) lower than 10 µg/mL. The activity of the terpene derivatives on *Trypanosoma cruzi* was less effective. Only 4α,14α- dimethyl-5α-ergost-8-en-3,24-dione (**26**), 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7- thiosemicarbazone (**27**) and 4α,14α-dimethyl-5α-cholest-8-ene-3,11-dione-7-thiadiazoline (**28**) had ED_{50} values lower than 10 µg/mL (53). The activities of these terpene derivatives on *L. infantum* and *T. cruzi* were associated with low to moderate effects on mammalian CHO cells, revealing a desirable selective toxicity (53). The anti-parasite activities were higher in the tetracyclic triterpenes highly oxygenated with ketone/OH substituents at C-3 and C-7 and or C-11 and/or the presence of a substituent epoxy- or ketone group at C-24 in the lateral chain (53).

4α,14α-Dimethyl-5α-cholest-8-ene-3,11-dione-7-thiosemicarbazone (27) had moderate antileishmanial and antitrypanosomal activity (44). Later, Mazoir and Benharref (54) obtained new thiosemicarbazone derivatives by hemisynthesis, treating hemisynthesized mono-, di-, and tricarbonyl compounds (e.g., 4α,14α-dimethyl-5α-ergost-8,24-dien-3-one (**4**), 4α,14α-dimethyl-5α-ergosta-8-en-3-one (**5**), (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**), 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione (**19**), 3-acetoxy-30-nor-20-oxolupane (**31**), and 4α,14α-l-5α-cholest-8-ene-3,7,11-trione (**32**)) (Figure 1.10) from latex of Moroccan *Euphorbia officinarum* , with thiosemicarbazide and oxidation by chromic anhydride, with the purpose of finding new thiosemicarbazones derivatives with good yield and high regioselectivity. The same hemisynthesized mono-, di-, and tricarbonyl compounds were used for obtaining thiadiazolines since they possess several biological properties (53,55). The new thiosemicarbazone derivatives obtained were 4α,14α- dimethyl-5α-ergost-8,24-dien-3-one thiosemicarbazone (**33**), 4α,14α-dimethyl-5α-cholest-8-en-3-one thiosemicarbazone (**34**), 3β-acetoxy-28-norlup-20-one thiosemicarbazone (**35**), 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione-7-thiosemicarbazone (**36**), and 4α,14α-dimethyl-5α-cholest-8-ene-3,7,11-trione-7-thiosemicarbazone (**37**) (Figure 1.10) (54). 4α,14α-Dimethyl-5α--cholesta-7,9-dien-3-one thiosemicarbazone (**29**) was reported as a new compound, nevertheless, Mazoir et al. (52) had already obtained this triterpenic compound. Concerning the thiadiazoline derivatives, the compounds obtained were 4α,14α-dimethyl-5α-ergost-8,24-dien-3-one thiadiazoline (**38**),

4α,14α-dimethyl-5α-cholest-8-en-3-one thiadiazoline (**39**), 4α,14α-dimethyl-5α-cholest-7,9-diene-3-one thiadiazoline (**40**), 3β-aceto-xy-28-norlup-20-one thiadiazoline (**41**), 3β-tosyloxy-4α,14α-dimethyl-5α-cholest-8-ene-7,11-dione-7-thiadiazoline (**42**), and 4α,14α-dimethyl-5α-cholest-8-ene-3,7,11-trione-7-thiadiazoline (**43**) (Figure 1.10) (55).

Triterpene derivatives of *E. officinarum* latex have demonstrated toxic effects on two protozoan species *L. infantum* and *T. cruzi* (53), antifeedant and toxic effects on *S. littoralis*, an important crop pest, and selective cytotoxicity on insect and mammalian cells (52), as aforementioned. Bailen et al. (56) continued this work, studying the same biological properties previously reported (52,53) but hemisynthesizing new triterpene derivatives from obtusifoliol and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**), major latex components of *Euphorbia officinarum* from the semi-arid regions of Morocco. From 4α,14α-dimethyl-5α-cholest-8- en-3β-ol (**9**), Bailen et al. (56) have obtained 14 derivatives, 4 being already hemisynthetized. 4α,14α-dimethyl-5α-cholest-8-ene-3,7,11-trione (**32**), (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**), 8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3β- ol (**14**) and 4α,14α-dimethyl-5α-cholest-8-en-3-one (**5**). 4α,14α-Dimethyl-5α-cholest-7,9- dien-3β-ol (**44**), 3-chloro-4α,14α-dimethyl-5α-cholest-8-en-7-one (**45**), 2-carbomethoxy-4α,14α-dimethyl-5α-cholest-2,8-dien-3-ol (**46**), 4α,14α-dimethyl-5α-cholest-8-en-3-one (**47**), 4α,14α-dimethyl-7-oxo-5α-cholest-8-en-3,4-lactone (**48**), 4α,14α-dimethyl-7,11-dioxo-5α-cholest-8-en-3,4-lactone (**49**), 8α,9α-epoxy-4α,14α-dimethyl-5α-cholest-3,4-lactone (**50**), 4α,14α-dimethyl-5α-cholest-7,9-dien-3,4-lactone (**51**), 4α,14α-dimethyl-3,4-seco-5α-cholest-7,9-dien-3,4-diol (**52**), 3-carbomethoxy-4-hydroxy-4α,14α-dimethyl-3,4-seco-5α-cholest-7,9- diene (**53**) were new derivatives (Figure 1.10). 4α,14α-dimethyl-5α-cholest-7,9-dien-3β-ol (**44**), 3-chloro-4α,14α-dimethyl-5α-cholest-8-en-7-one (**45**) and (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**) were selective toxicants being most effective against *Leishmania*, although 3-carbomethoxy-4-hydroxy-4α,14α-dimethyl-3,4-seco-5α-cholest-7,9-diene (**53**) was the strongest antiparasitic with activity levels similar to or better than the reference drugs against *L. infantum* and *T. cruzi*, respectively. (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-Epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**), 3-chloro-4α,14α-dimethyl-5α-cholest-8-en-7-one (**45**). 4α,14α-Dimethyl-7-oxo-5α-cholest-8-en-3,4- lactone (**48**) were not cytotoxic to mammalian CHO cells, which showed that they were selective to the parasites and therefore could be considered molecular leads for selective insecticides (56). Compounds 3-carbomethoxy-4-hydroxy-4α,14α-dimethyl-3,4-seco-5α-cholest-7,9-diene (**53**) and 8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3β-ol (**14**) had the strongest cytotoxic effects on insect-derived Sf9 cells. The compounds with an epoxide group such as (4*S*,5*S*,10*S*,13*R*,14*R*,17*R*)-8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3-one (**13**), 8α,9α-epoxy-4α,14α-dimethyl-5α-cholestan-3β-ol (**14**), 8α,9α-epoxy-4α,14α-dimethyl-5α- cholesta-3,4-lactone (**50**), and 8α,9α,24,28-diepoxy-4α,14α-dimethyl-5α-ergost-3,4-lactone (**54**) had selective

cytotoxic effects on Sf9 cells compared with mammalian CHO cells. 8α,9α,24,28-Diepoxy-4α,14α-dimethyl-5α-ergosta-3,4-lactone (**54**) as well as 8α,9α,24,28- diepoxy-4α,14α-dimethyl-5α-ergost-3β-ol (**55**) were hemisynthesized from obtusifoliol (56).

Epoxidation of the double bonds and hydroxylations of non-activated C–H groups of semisynthetic functionalized triterpenes 4α,14-dimethyl-5α,8α-8,9-epoxy-chole-stan-3β-yl acetate (**56**); 4α,14-dimethyl-5α-cholest-8-ene-3,7,11-trione (**32**); 4α,14-dimethyl-5α- cholesta-7,9-dien-3-one (**57**) and 4α,14-dimethyl-5α-cholest-8-en-3β-yl acetate (**58**), previously prepared from 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (a natural insecticide present in *E. officinarum* latex) were performed by Mazoir et al. (57) with the purpose of obtaining optimized derivatives with high region selectivity and insecticidal activity. Several approaches had already been followed by this team and aforementioned. In the work presented in 2020, Mazoir et al. (57) used as reagents hydrogen peroxide (H_2O_2) and iodosobenzene (PhIO) catalyzed by porphyrin complexes (cytochrome P-450 models). Under these conditions, the compounds obtained were: 25-hydroxy-4α,14-dimethyl-5α-cholest-7,9-dien-3β-yl acetate (**59**), 25-hydroxy-4α,14-dimethyl-5α-cholest-8-ene3,7,11-trione (**60**), 4α,14-dimethyl-5α,7β-7,8-epoxychol-est-9-en-3-one (**61**), 8-hydroxy-4α,14-dimethyl-5α-cholest-9-ene-3,7-dione (**62**), 12α-hydroxy-4α,14-dimethyl-5α,7β-7,8-epoxycholest-9-en-3-one (**63**), and 4α,14-dimethyl-5α,8α-8,9-epoxycholestan-3β-yl acetate (**64**). The antifeedant and post-ingestive effects of these terpenoid derivatives were investigated for the insects *M. persicae*, *R. padi* and *S. littoralis* and Mazoir et al. (57) concluded that none of the compounds tested had significant antifeedant effects. All were more effective post-ingestive toxicants on *S. littoralis* larvae than the natural 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**), with 4α,14-dimethyl-5α,8α-8,9-epoxychole-stan-3β-yl acetate (**64**) being the most active. The authors also concluded that overall, the substituents at C-3 and C-7/C-8 modulated the insecticidal activity of the derivatives: acetylation at C-3/epoxidation at C-8 gave the maximum insecticidal effect (56), followed by carbonyl groups at C-3/C-7 with additional hydroxy groups (C-8, C-25) (60, 62) or C-3 carbonyl and C-7 epoxide with C-8 unsaturation (61, 63). The most active IGRs (insect growth regulators) had a C-3 carbonyl group and a C-8 epoxide or C-3 β-OH/C-7 unsaturation (56,57).

Beyond the insecticidal and antiparasitic activities aforementioned for some terpene derivatives obtained after hemisynthesis of terpenes isolated from *E. officinarum* latex, other biological activities have been reported. For example, Smaili et al. (58) reported that 3β-acetoxy-norlup-20-one (**31**) and 3-chloro-4α,14α-dimethyl-5α-cholest-8-ene (**64**), obtained by hemisynthesis from lupeol acetate (**2**) and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**), respectively, were able *in vitro* to greatly reduce conidia formation and germination of *Verticillium dahlia*, and *Fusarium oxysporum* fsp. *melonis*, both causal agents of wilt, and *Penicillium expansum*, which is responsible for post-harvest rot that infects diverse fruits (e.g., tomato and apple). Such results

indicate that those two derivatives act as fungistatic compounds (58). The antibacterial activities of these derivatives were also evaluated *in vitro*. The phytopathogenic bacteria used were *Agrobacterium tumefaciens*, causal agent of crown gall disease, *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae*, which cause wild fire disease of tobacco, and diseases of various monocot and dicot plants, respectively. The results showed that compound 3-chloro-4α,14α-dimethyl-5α--cholest-8-ene (**64**) was more effective in inhibiting the growth of *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae*, even being similar to the positive control used (chloramphenicol). Concerning *Erwinia amylovora*, the causal agent of fire blight disease of pear and apple trees, 3-chloro-4α,14α-dimethyl- 5α-cholest-8-ene (**64**) was also the only one of these compounds that showed antibacterial activity but at a moderate level and significantly lower than that recorded with the positive control (**58**).

Since 3β-acetoxy-norlup-20-one (**31**) and 3-chloro-4α,14α-dimethyl-5α-cholest-8-ene (**64**) were able to reduce *in vitro* conidia formation and germination of *Verticillium dahlia*, one of the most important vascular diseases reported on tomato plants, Smaili et al. (59) studied the effect of two triterpene derivatives (4α,14α-dimethyl-5α-cholest-7,9-dien-3β-ol (**44**) and 3β-tosyloxy4α,14α-dimethyl-5α-cholest-7,9-diene (**65**)), obtained after oxidation of 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**9**) isolated from *E. officinarum* latex, on the protection of tomato plants against *V. dahliae* in a greenhouse as well as in tomato plants derived from seeds that germinated in the presence of low concentrations of those two triterpenic derivatives. The results showed that they were able to significantly reduce disease severity at 10 µg/mL (e.g., reduction of leaf alteration index and of stunting index ranged from 52 to 68% and from 43 to 67%, respectively, while vessel discoloration was reduced by at least 95%) (59). Moreover, the compounds were also able to elicit H_2O_2 accumulation before and after fungal inoculation, and enhance peroxidase and polyphenol oxidase activities. According to Smaili et al. (59), induction of protection against plant diseases by triterpenes of plant origin was reported for the first time.

Previously, Smaili et al. (58) concluded that 3-chloro-4α,14α-dimethyl-5α-cholest- -8- ene (**64**) was effective in inhibiting the growth of *P. syringae* pv. *tabaci* in *in vitro* studies. Later, Smaili et al. (60) treated seeds of *Nicotiana benthamiana* with three hemisynthetic triterpenes, including 3β-acetoxy-norlup-20-one (**31**) and 3-chloro-4α,14α-dimethyl-5α-cholest-8-ene (**64**) derived from the latex of *E. officinarum*, in order to evaluate their ability to enhance resistance to *P. syringae* pv. *tabaci*. Smaili et al. (60) observed that soaking seeds in the triterpene derivatives did not harm germination and significantly reduced the diameter of the lesions in inoculated leaves, when compared to the control; bacterial growth was also significantly reduced in plants previously treated with the triterpenic derivatives by at least 0.54 logarithmic units when compared to the control. At the same time, the mock-inoculated leaves of plants that germinated in the presence of the triterpenic derivatives showed enhanced ascorbate peroxidase and catalase activities (two

antioxidant enzymes). An increase of guaiacol peroxidase and polyphenol oxidase inoculated plants with *P. syringae* pv. *tabaci* was observed when pre-treated with the triterpenic derivatives.

Continuing the study on the effect of some triterpene derivatives obtained from triterpene isolated from *E. officinarum* latex, Smaili et al. (61) evaluated the effect of direct application of the 3β-acetoxy-norlup-20-one (**31**) and 3-chloro-4α,14α-dimethyl-5α-cholest-8-ene (**64**) on the growth of tomato seedlings under normal conditions or in the presence of the pathogens *V. dahliae* and *Agrobacterium tumefaciens*. After foliage spraying with these two derivatives, the authors observed a significant improvement of growth rate, fresh weight, dry weight and leaf area, an increased content of chlorophylls a and b, carotenoids, proline, and the activity of nitrate reductase (an enzyme which is correlated with growth and plant yield) (61). In the presence of the infection by *V. dahliae*, triterpene derivatives reduced leaf alteration indexes induced by *V. dahliae*, particularly for 3-chloro-4α,14α-dimethyl-5α-cholest-8-ene (**64**). The browning index of the vessels caused by this phytopathogen was also much reduced, with the percentage of protection being 97–99% (61). The diameter of lesions caused by the oncogenic strain C58 of *A. tumefaciens* was also reduced when pre-treated with those triterpenic derivatives. Moreover, these compounds also induced H_2O_2 accumulation and increased the activity of several antioxidant enzymes such as catalase, ascorbate peroxidase, and guaiacol peroxidase (61). According to these results, Smaili et al. (61) concluded that the two derivatives are able to mediate resistance of tomato plant against bacterial and fungal diseases through improvement of antioxidant defences.

So far, diverse hemisynthesized triterpenoids obtained from isolated triterpenes of *E. officinarum* latex have been demonstrated to possess insecticidal and antimicrobial activities. Daoui et al. (48) have as purpose the improvement of these biological activities using in silico studies based on 3D molecular modelling techniques applied on 27 semisynthetic triterpene derivatives obtained from triterpenes isolated from *E. officinarum* and *E. resinifera* latices. To achieve this objective, Daoui et al. (48) have developed the three dimensional quantitative structure property relationships (3D-QSAR) based on Comparative Molecular Similarity Indices Analysis (CoMSIA) and Comparative Molecular Field Analysis (CoMFA) techniques. Such an approach enabled the authors to design 38 new derivatives and also to predict their pLD_{50} ($\log_{10}(1/LD_{50})$), where LD_{50} is the amount of sample which causes the death of 50% of the living beings of a group. Studies taking into account the absorption, distribution, metabolism, excretion (ADME), and toxicity (ADME-Tox) of the designed molecules led the authors to select four molecules as promising antibacterial and insecticidal molecules. The molecular docking test predicting the referential interactions that occur between the molecular structures and the receptors made it possible to find 3 molecules (**66**), (**67**), (**68**) (Figure 1.10) that were able to inhibit the MurE (PDB code: 1E8C) and EcR (PDB code: 1R20) proteins involved in the process of antibacterial and insecticidal activities and had greater stability than the reference molecule 24-methylen-elemo-lanosta-

2,8,24- trien-7,11-dione (**69**) inside the MurE and EcR receptors pocket. The reference used was a triterpene derivative obtained through chemical modifications of the major component of *E. resinifera* , α-euphorbol (**70**) (53). According to Daoui et al. (48), such observations may permit adoption of these molecules as references for the synthesis of insecticidal and antimicrobial activities.

5. Extracts and bee products from Euphorbia origin

Although *E. officinarum* is used in folk medicine as antidiabetic or in the treatment of diseases of the respiratory and circulatory systems, pyelonephritis, treatment of wounds, skin infections and abscesses, headache, paralysis, apoplexy, among other ailments (see Introduction), very few works can be found that confirm these abilities. In fact, only the antimicrobial and insecticidal activities of triterpene derivatives from *E. offcinarum* latex have been extensively evaluated (see previous Section). Nevertheless, some works start to approach other attributes of *E. officinarum* extracts or their monofloral honeys. For example, El-Hawary et al. (41) tested the cytotoxic potential of fifteen *Euphorbia* species and concluded that methanolic extracts of the aerial parts of *E. officinarum* presented the highest activity against human colon adenocarcinoma (Caco2) cell line (IC_{50} = 7.2 μM). Chemical analysis through liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HR-ESIMS) and dereplication strategies using the Dictionary of Natural Products (DNP) database, followed by chemotaxonomic filtration, resulted in the characterization of 44 natural compounds from the 15 *Euphorbia* studied. For *E. officinarum*, two compounds were characterized but without activity (3β,7α-dihydroxy-4α,14α-dimethyl-5α-cholest-8-en-11-one (**10**) (Figure 1.2) and 8-methoxyingol 7,12-diacetate 3-phenylacetate (**3**) (Figure 1.3). After an orthogonal partial least square discrimination analysis (OPLS-DA), the authors concluded that the metabolite highly correlated with the Caco2 cytotoxicity was at *m/z* $(M)^+$ 281.272 (time retention = 29.16), and was not yet isolated and identified in this species.

Beyond the antimicrobial and insecticidal activities of the derivatives obtained from natural triterpenes of the *E. officinarum* latex, Daoubi et al. (2) demonstrated that 8-methoxyingol 7,12-diacetate 3-phenylacetate (**3**) (Figure 1.3) was able to induce both G0/G1 cell-cycle arrest and human immunodeficiency virus type 1-long terminal repeat (HIV-1-LTR) promoter activation in a concentration-dependent manner, in the leukaemia cell line Jurkat-LTR-green fluorescent protein (Jurkat-LTR-GFR). According to these results, Daoubi et al. (2) suggested that 8-methoxyingol 7,12-diacetate 3-phenylacetate may be important for the development of therapies against HIV-latency. In fact, to eradicate HIV-1 has been difficult since the virus can be in reservoirs of latently infected cells and within them, the proviral DNA is integrated in the host's

genome but it does not actively replicate. In such a situation, the virus remains invisible to the host immune system and is not affected by antiviral drugs (62).

In the work developed by Boutoub et al. (63), they compared the antioxidant activity of aqueous plant extracts and monofloral honey of *E. officinarum* origin. They demonstrated that the ability of plant extracts for scavenging some free radicals such as 2,2-diphenyl- 1-picrylhydrazyl (DPPH) and nitric oxide (NO) of plant extracts was more than 20 times greater than the activity of honey; likewise, the capacity for scavenging superoxide radical anions or antiacetylcholinesterase and anti-lipoxygenase were approximately 10 times higher in the plant extracts than in the honey samples. However the detailed chemical composition was not reported, only the total phenol, and in this case all aqueous extracts had higher amounts of these metabolites than the honey samples. The extraction conditions (time, temperature and plant solvent ratio) were determinant of the total concentration of phenols as well the antioxidant activity and inhibition of the α-glucosidase activity of the *E. officinarum* aqueous extracts (64). The same team (65) also concluded that the activities found for honey samples could be attributed to the phenol fraction since the phenols extracts isolated from the monofloral honey of *E. officinarum* origin had better activities than the entire honey. In this case, Boutoub et al. (65) presented a preliminary chemical composition of the extracts (gallic acid (**1**), p-hydroxybenzoic acid (**2**), caffeic acid (**3**), *p*-coumaric acid (**4**), abscisic acid (**5**), luteolin (**6**), quercetin (**7**), apigenin (**8**), naringenin (**9**) and kaempferol (**10**)) (Figure 1.11) (65). The authors (63,65) studied monofloral honey because in Morocco the *Euphorbia* honey is considered the most precious by the consumers but it has been scarcely studied, although the physico-chemical and palynological characteristics, generally needed for honey characterisation, have already been found (63,66–69). Many times, beekeepers labelled the honey as being only of *Euphorbia* origin, although three honey types of this genus are produced (*E. officinarum* subsp. *echinus*, *E. resinifera* and *E. regis-jubae*) (48,70). The work of Abderrahim et al. (71) demonstrated the antioxidant, synergistic antimicrobial and burn wound healing activities of monofloral honey of *Euphorbia* origin (without any specification of the species) mixed with *Allium sativum*. This mixture had higher wound healing activity, since shorter epithelialization and wound contraction time was observed, as well as better histological recovery of the treated tissues (71).

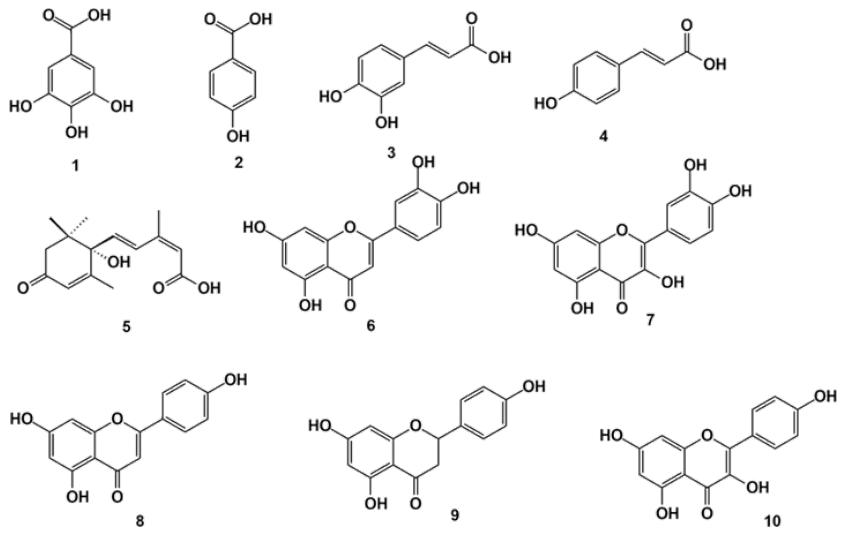

Figure 1.11. Phenols identified in the phenol fraction of *Euphorbia officinarum* honey from Morocco.

Another bee product is propolis, a plant-derived product that bees collect from resins and exudates from diverse parts of the plants, and subsequently transport to the hive, mixing it with beeswax. Bees use propolis to protect the hive against intruders and pathogenic microorganisms (72). The detailed chemical composition and antimicrobial activity of propolis from a semi-arid region of Morocco were evaluated by Chimshirova et al. (72). Fifteen compounds were isolated and identified, some of them being already reported as constituents of plants in the genus *Euphorbia*, particularly the macrocyclic diterpenes and triterpenoids, as well as other groups of known compounds (e.g., coumarins, phenolic acids) and new ones (e.g., 29-norlanost-3β-hydroxy-8-ene-7,11-dione). The macrocyclic diterpenes, particularly ingol diterpenes containing a phenylacetyl group were only found in the latex of *E. resinifera* and *E. officinarum*. However, the ingol diterpenes found in propolis of this work were those isomers characteristic of the *E. resinifera* latex. Such results may indicate the utilization of latex of *E. resinifera* by bees for making propolis, but also from *E. officinarum*, since obtusifoliol is generally present in the *E. officinarum* latex. *p*-hydroxybenzoic acid was reported in this propolis sample, being also observed in honey of *E. officinarum* and *E. resinifera* origins (65).

6. Conclusions

Euphorbia officinarum is geographically limited to Morocco, Moroccan sahara, Algeria and Mauritania. This species has been used in folk medicine in various ways: as anti-diabetic; in the treatment of skin diseases, although later it was concluded that the main treatment purpose was the elimination of helminths; when associated with other plants (*Opuntia ficus-barbarica*, *Zea mays* and *Ziziphus lotus*) and honey, in the treatment of pyelonephritis and cystitis. So far, fifteen compounds (diterpenes, triterpenes and sterols) have been isolated and identified in the *E. officinarum* latex from Morocco. The *E. officinarum* honey is considered the most precious; nevertheless, many times it is mixed with other Euphorbiaceae honeys. The chemical composition of the phenolic fraction of the monofloral honey was found to include ten compounds. More than seventy hemisynthesized compounds were obtained from some triterpenes of *E. officinarum* in order to obtain compounds with higher insecticide and antimicrobial activity. The *in silico* studies indicated that three hemisynthesized compounds were able to inhibit proteins involved in the process of antibacterial and insecticidal activities and also presented great stability inside the protein receptors pocket. They are, therefore, of interest for possible adoption as references for the synthesis of antibacterial drugs and insecticides. This review shows an insufficiency of knowledge on the chemical composition of latex, flow- ers and other organs of *E. officinarum*, and the need for further investigation. Greater insight into the relationship between the chemical structures of the natural compounds or normalized extracts and biological properties is also needed. A possible relationship of the chemical composition of flowers and that of honey is another field requiring investigation. Since honey is appreciated by consumers and in order to increase the commercial value of a monofloral *E. officinarum* honey, it would be important to find one or more specific markers for this type of honey. Since many hemisynthesized compounds have been obtained, it is necessary to assay more biological activities of such compounds and not only antimicrobial and insecticidal activities.

Author Contributions: O.B. and M.G.M. wrote the first draft of the manuscript; L.E.G. performedthe first revision. All authors revised the manuscript for the second time before submission. Allauthors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FCT (Fundação para a Ciência e a Tecnologia, Portugal) under the project UIDB/05183/2020.

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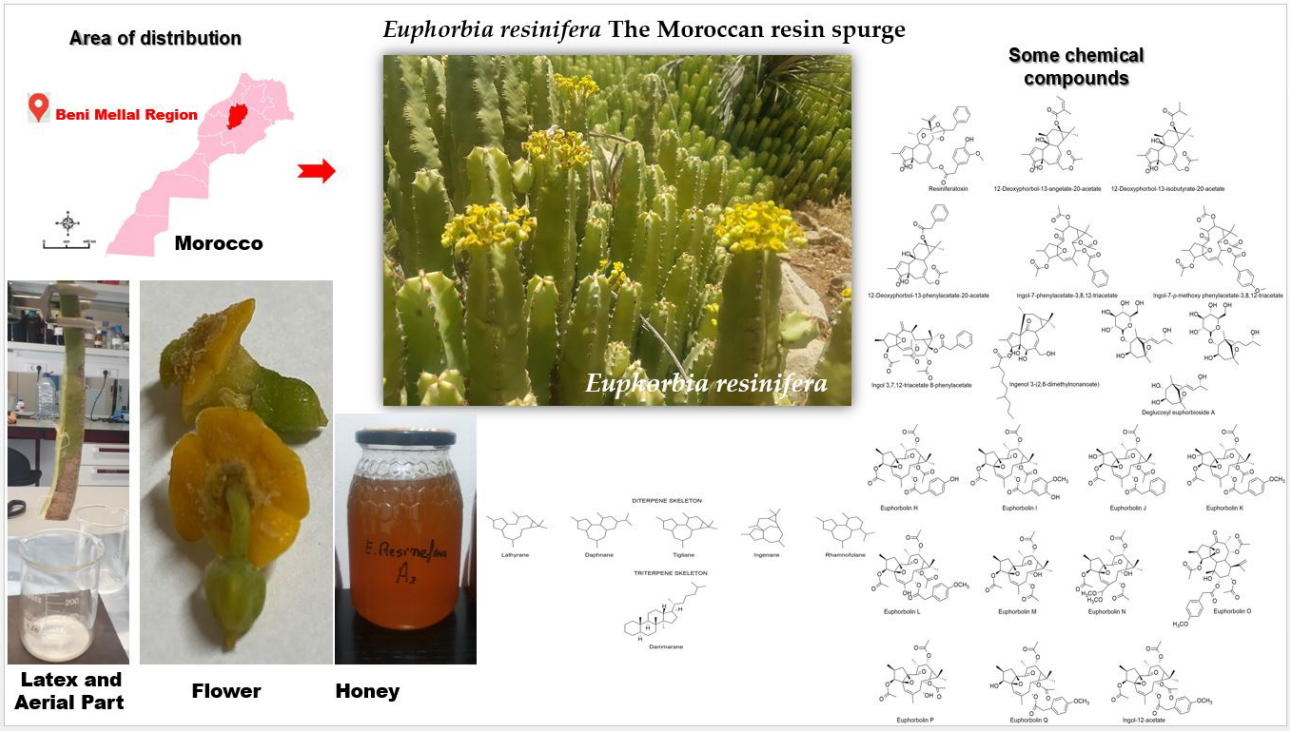
Chapter 2

Introduction



2

Chapter II: Euphorbia resinifera: chemical composition and biological properties (Short review) *

Résumé

Cette revue met à jour les informations sur la composition chimique du latex *d'Euphorbia resinifera* (résine d'euphorbe) et leurs propriétés biologiques ainsi que celles des parties aériennes et du miel, généralement sans latex. À partir de cet examen et selon les études développées jusqu'à présent, il a été possible de confirmer que la composition chimique des composés latex et non-latex diffère. Dans le latex, les diterpènes, les norsesquitepernes, les triterpènes et les sérine protéases (EuRP-61) de 61 kDa prédominent, malgré la présence d'autres composés mineurs. La protéase identifiée avait des propriétés anticoagulantes, antiplaquettaires et inhibitrices de l'agrégation des cellules du sang périphérique. Les phénols, dont les flavonoïdes et les tanins, à activité antioxydante, dominaient dans les parties aériennes d'*Euphorbia resinifera*. Des acides phénoliques et des flavonoïdes, en particulier des flavanones, des flavones et des flavonols et leurs glycosides, ont été identifiés dans le miel de résine d'euphorbe. En général, les échantillons de miel se situaient dans la limite acceptable des normes internationales. Une activité antioxydante a été rapportée pour ce miel monofloral.

Mots clés : Latex, parties aériennes, terpènes, sérine protéases, phénols, miel

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* Oumaima Boutoub, Lahsen El Ghadraoui, Maria Graça Miguel. *Euphorbia resinifera*: chemical composition and biological properties: Short review. Article soumis, **2023**

2

Euphorbia resinifera: chemical composition and biological properties (Short review) *

Abstract

This review updates the information on the chemical composition of *Euphorbia resinifera* (spurge resin) latex and their biological properties as well as those from the aerial parts and honey, generally without latex. From such review and according to the studies developed so far, it was possible to confirm that the chemical composition of latex and non-latex compounds differs. In the latex, diterpenes, norsesquitepernes, triterpenes, and serine proteases (EuRP-61) with 61 kDa pre-dominate, in spite the presence of other minor compounds. The identified protease had anticoagulant, antiplatelet, and peripheral blood cell aggregation inhibitory properties. Phenols including flavonoids and tannins, with antioxidant activity, dominated in the aerial parts of *Euphorbia resinifera*. Phenolic acids and flavonoids, particularly flavanones, flavones, and flavonols and their glycosides, were identified in the spurge resin honey. Generally, honey samples were within the acceptable limit of international standards. Antioxidant activity was reported for this monofloral honey.

Keywords: Latex, aerial parts, terpenes, serine proteases, phenols, honey

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* Oumaima Boutoub, Lahsen El Ghadraoui, Maria Graça Miguel. *Euphorbia resinifera*: chemical composition and biological properties: Short review. Submitted, **2023**

1. Introduction

The family Euphorbiaceae is known as the spurge family and is one of the largest families of flowering plants with over 300 genera and 8,000 species. The genera in the spurge family are widely used in traditional medicine for the treatment of several diseases, such as respirational infections, digestion complaints, nutritional diseases, body pain, body and skin irritations, inflammatory infections, snake or scorpion bites, endocrine, pregnancy/birth/puerperium disorders, genitourinary syndromes, and sensory disorders. The genus *Euphorbia* contains several other subgenera and sections with over 2,000 species, characterized by the production of milky irritant latex (1).

Euphorbia resinifera Berg. (Libana maghrabi in Arabic or "Zaqqum" or "Zaggoume" in local language), a large, leafless cactus-like perennial, is endemic to Morocco, generally distributed in the center of the country, in the regions of Azilal and Beni Mellal (Middle Atlas), with some scattered populations in the High Atlas Mountains and the Anti-Atlas (2-4). *E. resinifera* appears on sunny slopes, mainly in the rocky and arid rocks of the low limestone mountains between 600 to 1,500 m. In 1966, the Ministry of Agriculture and Agricultural Reform considered the geographical area of this species to be the Haouz, Middle Atlas, Umm-er-Rbia, Central High Atlas (M'Goun), and JbelLkest, although currently, that species occurs mainly in the Beni Mellal-Khenifra region (5).

Euphorbia resinifera has a thick woody stem originating to a number of fleshy, quadrangu-lar, spiny green branches. Their specialized flowering structure is called cyathium, which consists of a cup-like pseudanthial inflorescence. Cyathia appear in small groups at the top of branches forming clusters of yellow pseudo-inflorescences (6). *E. resinifera* forms large, cushionlike, glaucous green, 0.80-1.50 m high shrubs, with stems branched only at the base, forming dense bushes of 0.5-2 min diameter (7,8).

After an analysis of variance it was possible to detect significant differences between the populations of *E. resinifera* reflecting the existence of a high phenotypic variability within this species. This conclusion was obtained after analysis of seventeen qualitative and quantitative morphological characters related to the bush, stem, spine, flower, and fruit of the plant of twelve natural populations collected from its geo-graphical range in Morocco (8). The same team, in another publication (9), also studied the genetic diversity and structure of *E. resinifera* populations in Morocco. In the assay, the authors used twelve populations of this species from diverse altitudes and geographical areas, and through molecular markers, the inter-simple sequence repeats (ISSRs) primers (fourteen), they concluded that there exists high genetic diversity, and the this variation mainly occurs within populations. The generation of the secondary metabolites by spurge resin was tried to obtain through *in vitro* propagation methods for the production of

undifferentiated (*callus* and cell suspension cultures) as well as the micropropagation *of E. resinifera* (10). This genetic diversity may require a previous selection of plants to achieve adequate yields.

Euphorbia resinifera can be target of infections; for example, Muntañola-Cvetković and Gómez-Bolea (6) described plants with pale brown lesions extending upwards toward the branches and surrounded with brownish margins, found in the desert of Marrakech. This appearance was attributed to an infection by a new sporodochial hyphomycete, *Pseudostilbella euphorbia*. These diseases along with natural fires or promoted by local populations for agriculture or forage, utilization for domestic burning, and urban stress have led to a reduction of its healthy population (7,8,11). *Euphorbia resinifera* has been used in folk medicine in which Moroccan patients mixed the aerial parts with honey or using extracts obtained by decoction method in the treatment of general cancer, whereas fresh latex is used for poisonous punctures, bites, and dental pains. Flower infusion or water latex have also been used as antidiabetic in spite of its toxicity, since the exposure to latex induces oral, dermal, and ocular symptoms (1,12-14). In Algeria, this plant is used to treat rheumatism, cyst, and snakes bite poisoning (12,15-17).

Currently, *E. resinifera* is used in the melliferous field because the resin spurge (*E. resinifera*) honey is considered a local product with Protected Geographical Indication (PGI) in the Tadla-Azilal region (5), and considered among the best honey with a unique peppery taste and pungent and powerful aroma (18). This monofloral honey has also been reported as possessing antibacterial and antifungal activity and, therefore, considered interesting from medical and cosmetic points of view (4). This review intends to compile the chemical and biological data of latex and non-latex extracts and those of honey, a local Moroccan product with PGI in the Tadla-Azilal region.

2. Methodology

The approach of this review was made by seeking the Web of Science (WoS) and PubMed databases. The keyword was "*Euphorbia resinifera*". In the WoS, 62 results were obtained, whereas in PubMed 38 results were found. In the WoS database, the first results date from 1973, and the last ones are of 2023. Still in this database, the results included 2 meeting abstracts and 60 complete articles. The first meeting abstract dates from 1973 and the second one from 2014. The Figure 2.1 depicts the evolution of the publication over time, making it clear that there is a significant increase in the number of publications from 2016. In the same Figure, it is depicted the evolution of the publications found in the PubMed database using the same keyword: "*Euphorbia resinifera*". The first publication dates from 1969. Three articles were in Chinese language, and only the abstracts were in English language, so they are referred to in the present manuscript but only consulting the abstracts.

The lower number of publications (36) found in the PubMed database with the sole keyword "*Euphorbia resinifera*" does not mean that the other ones found in the WoS database cannot be observed, such only means that the WoS database has a larger search window than PubMed when the same keyword is used during the seeking process.

Eleven findings in the PubMed database were not found in the WoS one. All findings in the PubMed database and 58 in the WoS one were reported in the present review; therefore four references are not cited. The main reasons are the difficulty to have access to these references. In addition to these references, 14 others not found in these databases were mentioned in this work, as we considered that they would enrich the review work.

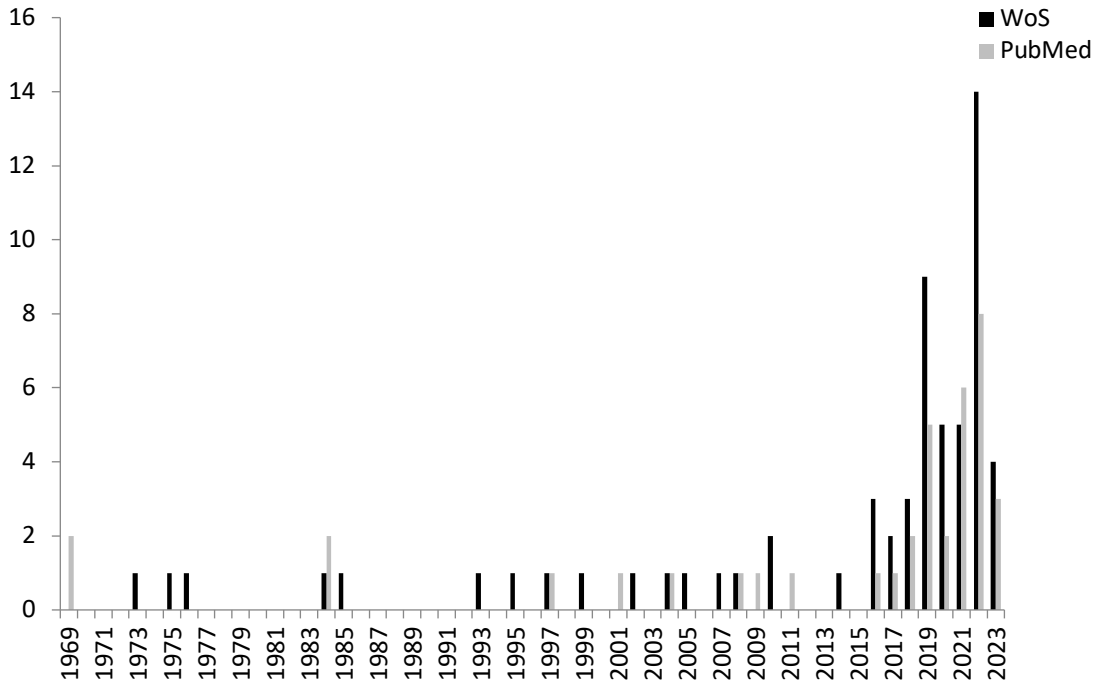


Figure 2.1. Evolution of the publications on *Euphorbia resinifera* over time registered in the WoS and PubMed databases.

3. Chemical composition of latex

3.1. Diterpenes and norsesquiterpenes and their biological properties

There are several diterpenes isolated from *E. resinifera* and other species of the same genus that possess medical interest due to their anticancer activity (ingenol 3-angelate) or potent analgesic properties (resiniferatoxin). There were homoeopathic mother tinctures of *E. resinifera* in which the ingenol content could range from 0.5 to 16.7 µg/mL, determined by high performance liquid chromatography, and after hydrolysis with KOH in methanol (18). Despite this possible medical

interest by these compounds, there has been little information about the biosynthesis of these *Euphorbia* diterpenes. Kirby et al. (18) investigated terpene biosynthesis and reported on the distribution of diterpene synthases within Euphorbiaceae family. The authors discovered genes encoding putative casbene synthases in all of selected Euphorbiaceae species (*Homalanthus nutans*, *E. resinifera*, *E. esula*, *Sapium sebiferum* and *Ricinus communis*); and a neocembrene synthase in *R. communis*. The authors did not consider gibberellins' biosynthesis (19). The carbene synthase is important in the synthesis of phorbol esters (20). These authors concluded that carbene synthase from four *Euphorbia* species (*E. esula*, *E. resinifera*, *E. peplus*, and *E. fischeriana*) was highly identical; nonetheless, carbene synthase from (family Eu-phorbiaceae) derives from a different origin than the remaining Euphorbiaceae plants. In a review made by Fattahian et al. (21), it was clear that two mechanistically different biogenetic pathways are known in diterpene biosynthesis: one of them will originate the phytanes such as abietanes, kauranes, atisanes, etc; and the second one will originate the macrocyclic and polycyclic diterpenes such as jatrophane-, casbane-, lathyrane-, tigliane-, ingenane- and daphnane-type. For example, the biosynthesis of 12-deoxyphorbol 13-phenylacetate (tigliane-type) (Figure 2.2), it seems to be derived either from cytosol-localized mevalonic acid (MVA) or plastid-localized methylerythritol 4-phosphate (MEP) pathways, though MEP pathway playing a dominant role, according to the next-generation sequencing technologies to build a transcriptome dataset assayed by Zhang et al. (22). The same type of core structures of diterpenes was present in the *E. officinarum*, as would be expected and reviewed recently (23).

Resiniferatoxin, a daphnane diterpene (Figure 2.2), was isolated and identified for the first time by Hergenhahn et al. (24) in the fresh latex of *E. resinifera*. The authors verified that during the drying process of latex, the compound concentration decreased due to oxidation. This observation might explain the non-utilization of young samples for medicinal purposes (25). The dried resinous preparation is called euphorbium and is one of the oldest drugs in the Western medicinal tradition due to its pharmacological properties despite its emetic, powerfully purgative and sternutatory effects (26,27). Euphorbium is used in the chronic pain and articular tuberculosis and to mitigate the toothache (27).

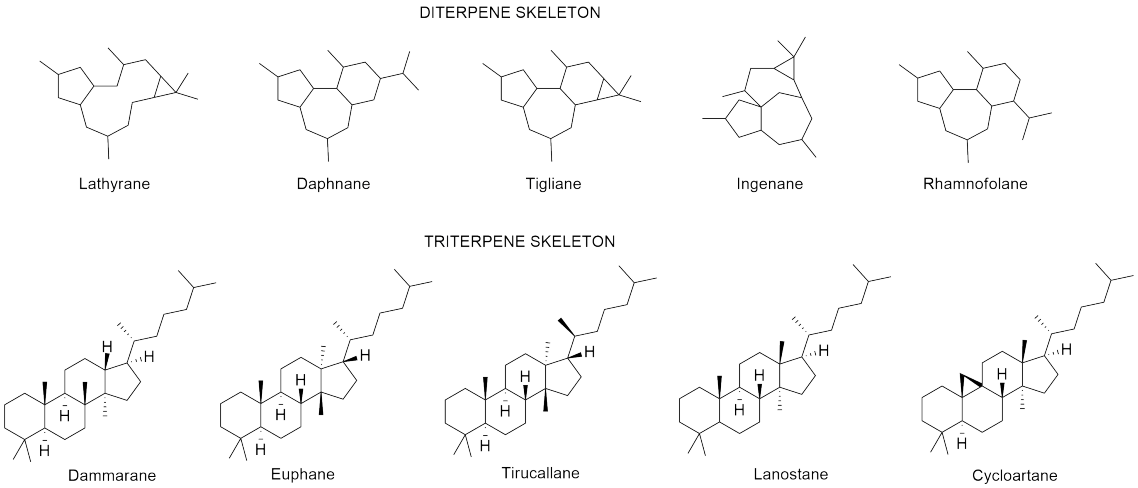
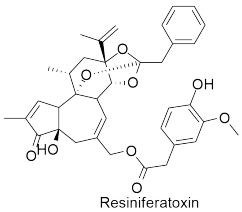
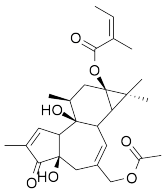


Figure 2.2. Main diterpene (28) and triterpene skeletons present in the latex of *Euphorbia resinifera*.

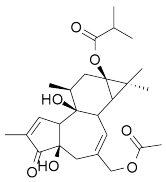
From euphorbium, Hergenhahn et al. (29) isolated 3 diterpene fractions being two of them irritant on the mouse ear. In these two fractions, the authors isolated and identified the following tigliane diterpenes: 12-deoxy-phorbol-13-angelate-20-acetate, 12-deoxyphorbol-13-isobutyrate-20 acetate, 12-deoxyphorbol-13-phenylacetate-20 acetate (Figure 2.3), and a mixture of the ingenane diterpenes ingenol-3-acylates. The lathyrane diterpenes ingol-7-phenylacetate-3,8,12-triacetate and ingol-7-*p*-methoxy-phenylacetate-3,8,12-triacetate did not present irritant activity. The absence of physical and spectroscopic data of ingol-7-phenylacetate-3,8,12-triacetate being the acylation pattern of the positional isomer deduced from partial hydrolysis experiments makes difficult to be sure about the presence of this isomer and not another positional isomer (27). Ingol 3,7,12-triacetate 8-(phenylacetate) was spectroscopically confirmed by Fattorusso et al. (27) in the fresh latex of *E. resinifera* along with other diterpenes such as ingenol 3-(2,6-dimethylnonanoate), and 12-deoxyphorbol 13-isobutyrate 20-acetate (Figure 2.3). This identification approach of the spurge resin diterpenoids, including the determination of optical rotations, was essential for the confirmation of the presence of 12-deoxy phorbol 13-isobutyrate 20-acetate in other species of Euphorbia growing in different latitudes (30). Fattorusso et al. (27) also isolated and identified three new bisnorsesquitepenes combining spectroscopic data and chemical reactions: the bisnorsesquitepenes glycosides euphorbioside A, and euphor-bioside B, as well as the alycone of euphorbioside A (deglucosyl euphorbioside A) (Figure 2.3) (27). Later on, Ourhzif et al. (31) also isolated euphorbioside monohydrate from *E. resinifera* latex.



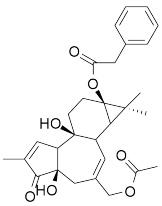
Resiniferatoxin



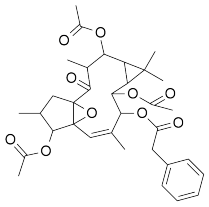
12-Deoxyphorbol-13-angelate-20-acetate



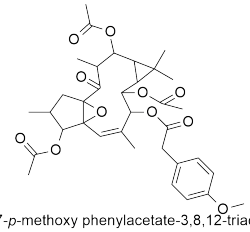
12-Deoxyphorbol-13-isobutyrate-20-acetate



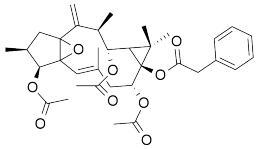
12-Deoxyphorbol-13-phenylacetate-20-acetate



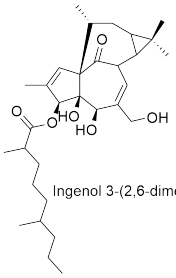
Ingol-7-phenylacetate-3,8,12-triacetate



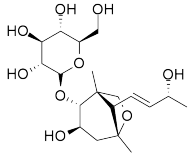
Ingol-7-*p*-methoxy phenylacetate-3,8,12-triacetate

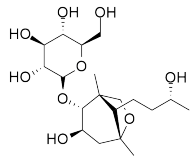


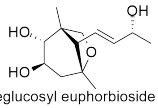
Ingol 3,7,12-triacetate 8-phenylacetate



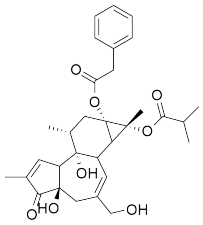
Ingenol 3-(2,6-dimethylnonanoate)



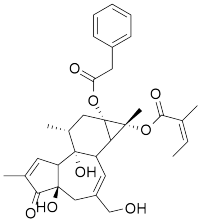




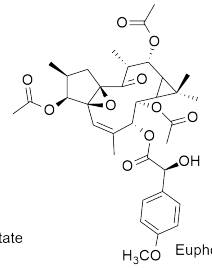
Deglucosyl euphorbioside A



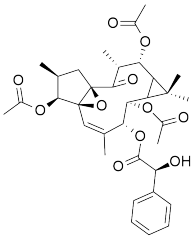
12-Deoxy-16-hydroxyphorbol 13-phenylacetate 16-isobutyrate



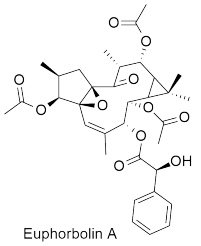
12-Deoxy-16-hydroxyphorbol 13-phenylacetate 16-tigliate



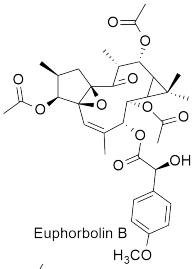
Euphoresin A



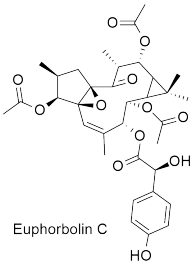
Euphoresin B



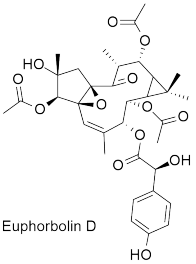
Euphorbolin A



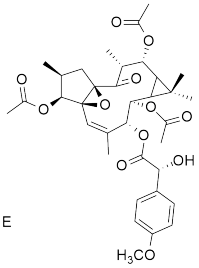
Euphorbolin B



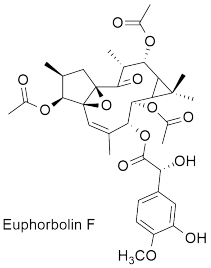
Euphorbolin C



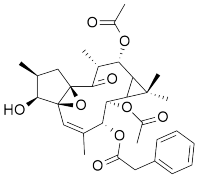
Euphorbolin D



Euphorbolin E



Euphorbolin F



Euphorbolin G

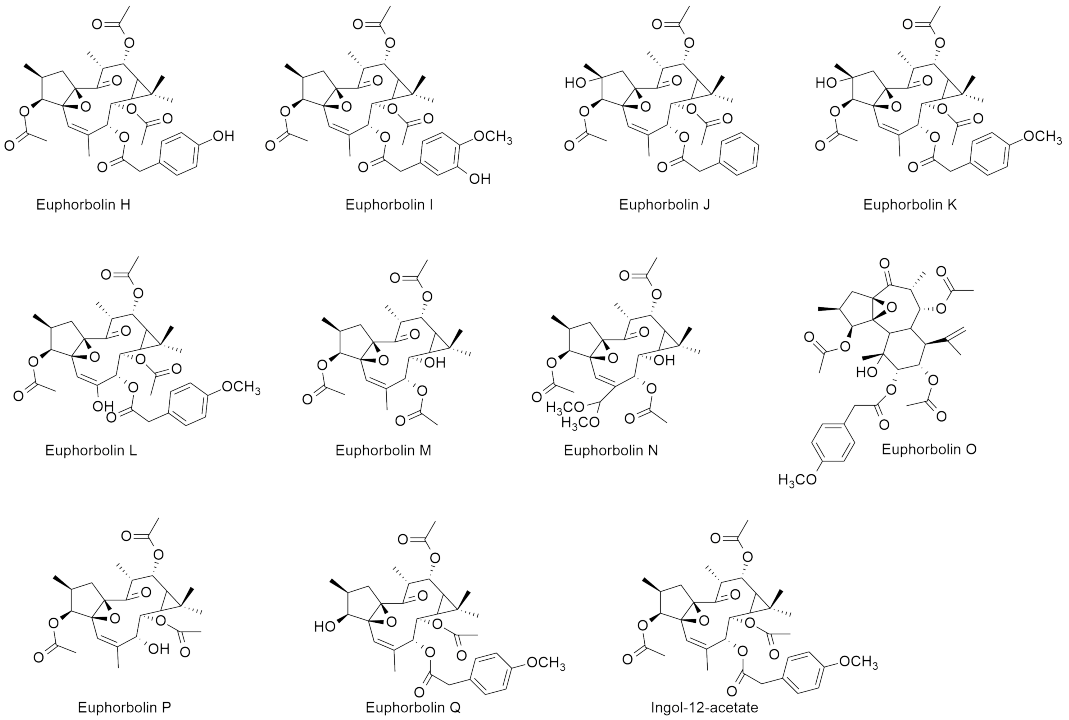


Figure 2.3. Chemical structures of some diterpenes present in the latex of *Euphorbia resinifera*.

12-Deoxyphorbol-13-angelate-20-acetate and 12-deoxyphorbol-13-isobutyrate-20 acetate were also detected by Ourhzif et al. (32) in the dichloromethane fraction of the latex of *E. resinifera*, along with and ingol-7-*p*-methoxy-phenylacetate-3,8,12-triacetate and resiniferatoxin (Figure 2.3). The norsesquiterpenoids deglucosyl euphorbioside A and euphorbioside A were also reported by the authors but in the *n*-butanol fraction. 12-Deoxyphorbol-13-isobutyrate-20-acetate inhibited the growth of *Aspergillus carbonarius*, whereas 7-*p*-metoxyphenylacetate-3,8,12-triacetate ingol and deglucosyl euphorbioside A had a cytotoxic effect on breast cancer cell line MCF7 with an increase in the level of intracellular reactive oxygen species (ROS), but not on MCF10A normal breast cells (32).

From the latex of *E. resinifera*, Hergenhahn et al. (26) isolated 20 factors (RL1-20), which some of these ones or some compounds (RL1, RL2, RL9, RL14, RL20) had been previously identified and reported by the same team (24), and from the resin (euphorbium) they isolated 4 factors (RR1-RR4) or compounds. Hergenhahn et al. (26) reported that many of these factors of tigliane-, ingenane-, or daphnanae-types sharing some chemical particularities had strong irritant properties. For example, tigliane-type 12-deoxyphorbol esters bearing, in the 13 position, either long-chain, partially me-thylsubstituted acyl residues (10-16 carbon atoms) or short-chain acyl residues (4 or 5 carbon atoms) or a (substituted) phenylacetyl group with a 20-acetoxy group had strong irritant properties; or long-chain 3-esters of ingenane-type ingenol with similar acyl residues (10-16 carbon atoms, partially methyl-substituted) were also irritant; or the daphnane

type: 9,13,14-orthophenylacetate of resinifer-onol-20-(4-hydroxy-3-methoxy)phenylacetate (resiniferatoxin), and 9,13,14-orthophenylacetate of resiniferonol. Nevertheless, along these irritant diterpenes other ones were nonirritant, which were esters of the tigliane-type 12,20-dideoxyphorbol; and of the lathyrane type ingol (26). Therefore, naturally-occurring diterpene esters with ingenane, tigliane and daphnane skeletons (Figure 2.2) have irritant properties, but they also present tumour promoting activities, and other biological effects on normal and tumour cells. The most active diterpene ester tumour promoters (esters of phorbol and ingenol) have long-chain acids (33,34). However, some of these diterpenes may present low tumor promoting activity. One of the RL factors isolated by Hergenhahn et al. (26) was identified as 12-deoxyphorbol 13-phenylacetate, a non-tumor promoting phorbol ester, since it does not present a long-chain acid. However, 2-deoxyphorbol 13-phenylacetate was able to induce type 1 human immunodeficiency virus (HIV-1) gene expression in latently infected ACH-2 T cells, and much more po-tent than prostratin (12-deoxyphorbol 13-acetate), probably due to its more lipophilic side chain structure (35).

Resiniferatoxin was isolated by Hergenhahn et al. (26) from *E. resinifera* was de-scribed by the authors as being extremely irritant. This daphnane diterpene is an analog of capsaicin and a potent activator of transient receptor potential cation channel subfamily V member 1 (TrpV1), also known as capsaicin receptor and vanilloid receptor 1. TrpV1 is a non-selective cation channel with high Ca^{2+} permeability composed up to six transmembrane segments with a pore region between the fifth and the sixth segment, which is mainly expressed on peripheral nociceptive C-fibers and at lesser extent on Aδ-fibers, but also in cortex, hippocampus, amygdala, and periaqueductal grey of the central nervous system. The channel pore can be opened by endocannabinoids, capsaicin, resiniferatoxin, and temperatures above 43˚C. The activation of TrpV1 leads to a painful and burning sensation, nevertheless upon prolonged exposure to capsaicin or resiniferatoxin desensitization occurs, a phenomenon that leads to an analgesic effect, but remaining unaffected the perception of cold, touch-sense and locomotor function (36,37). The molecular key is in the residues Tyr511, Met547, and Thr550 of the transmembrane regions of TrpV1, being its key regulatory site where capsaicin or resiniferatoxin establish bonds (38).

Resiniferatoxin is about 1,000-fold more potent than capsaicin. For this reason, resiniferatoxin has been used in some clinical trials as a potential analgesic to relieve cancer and arthritis pain (39,40) and in some cases a pain reliever by desensitizing cardiac sensory fibers expressing TrpV1, which could improve chronic heart failure (41), nevertheless, there are bioanalytical limitations in the quantification of resiniferatoxin in plasma due to the minimal effective dose that is in the range of few nanograms (39). These authors developed a method using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry with an electrospray ionization source (UHPLC-ESI-MS/MS) in multiple reaction monitoring modes. With this

approach, Sharma et al. (39) reported to be possible to have an efficient, rapid, and reliable method to quantify a low concentration of resiniferatoxin, which make it easier to de-termine its pharmacokinetic profile, data important in therapeutics.

The analgesia promoted by the resiniferatoxin, isolated from the latex of *E. resinifera*, is due to the interaction with the TrpV1, nevertheless, the latex isolated from a relative of *E. resinifera*, *E. bicolor* Engelm. & A. Gray, also demonstrated analgesic properties through the same action mechanism of that of the spurge resin, assessed through *in vivo* assays (male and female rats). The authors isolated some compounds which belonged to coumestans, diterpenes, and flavonoid groups. However, they did not present a relationship between any compounds identified in the latex and the agonist action on the TrpV1 (42).

Ziglioli et al. (36) reported that apoptosis in prostate cancer cells by the vanilloids, such as capsaicin or resiniferatoxin, can be mediated by a TrpV1-1-dependent (indirect pathway) and a TrpV1-1-independent (direct pathway) mechanism. In the former case, the vanilloids need to interact with the receptor TrpV1-1, which leads to an intracellular calcium increase and late elements of apoptosis; whereas in the last mechanism, the vanilloids inhibit the electron transport chain because they are analogs of the coenzyme Q, with the consequent electron transport chain inhibition, and great delivery of ROS. Finally and still in this mechanism, vanilloids also induce apoptosis by interacting with caspase 1 and 3.

Two tigliane diterpenes (Figure 2.3) were isolated from *E. resinifera* latex from Demnate, Beni Mellal-Khenifera Province (Morocco), and identified as 12-deoxy-16-hydroxyphorbol 13-phenylacetate-16-isobutyrate and 12-deoxy-16-hydroxyphorbol 13-phenylacetate-16-tigliate. The former compound facilitated the transforming growth factor alpha (TGFα) release and promoted neural progenitor cell proliferation, at nanomolar levels (43). Two novel ingol-type diterpenes have been isolated from the methanol extract of the latex of *E. resinifera* after separation by diverse chromatographic methods: euphoresin A and euphoresin B. These compounds had weak cytotoxic effects against MCF-7 (breast cancer cell line), U937 (pro-monocytic model cell line) and C6 (rat glial tumor) cells, with values of IC_{50} (half maximal inhibitory concentration) at least ten times higher than those found for taxol (44).

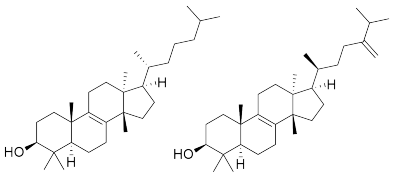
Zhao et al. (45) identified 18 diterpenes in the *E. resinifera* latex, including 14 new ingol-type (Figure 2.2) diterpenoids (euphorbolins A-N) (Figure 2.3), a new rhamnofolane diterpenoid (euphorblin O), and three known analogues (euphorblin P and Q and ingol-12-acetate) (Figure 2.3). The physical data of euphorblin P and Q were reported for the first time (45). Euphorbolins B and D and ingol-12-acetate were able to induce lysosomal biosynthesis through the upregulation of the lysosomal genes lysosomal-associated membrane protein 1 (LAMP1), cathepsin B (CTSB), cathepsin A (CTSA), and ATPase H^+ transporting V0 subunit E1 (ATP6 V0E1). According to the

authors such compounds must be deeper studied for being used for Alzheimer's disease since in this disease, lysosome lysis occurs and the proteases are released, giving rise β-amyloid accumulation and neuron death (46).

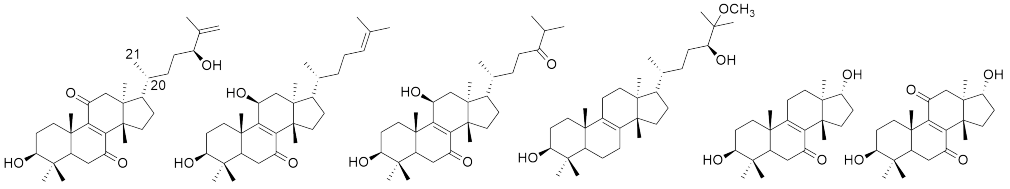
3.2. Triterpenes and their biological properties

In the latex of *E. resinifera* have been isolated triterpenes with dammarane, euphane, tirucallane, lanostane, cycloartane (Figure 2.2), and nortriterpenes in which the carbon loss generally occurs at the side chain.

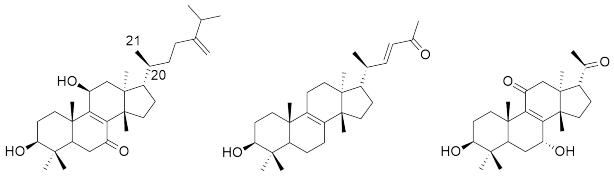
As aforementioned, from the euphorbium, Fattorusso et al. (27) isolated diterpenes, new bisnorsesquitepenes, and the triterpenes euphol and euphorbol (Figure 2.4). Such compounds were also isolated and identified from the resinified latex euphorbium by Newbold and Spring (47), who had already reported that euphorbol had also previously been isolated from euphorbium but in very small amounts. The pharmacological properties (anti-inflammatory, antimicrobial, antiplasmodial, and anti-insecticidal properties) described for dammarane triterpenes have led to the development, by hemisynthesis, of new triterpenoid derivatives, generally through oxidation methods, giving rise to much more oxygenated derivatives, that according to some authors, present remarkable biological properties (48-53). Nevertheless, Zhou et al. (54) without any triterpenoid derivative demonstrated that euphorbium total triterpenes constituted by euphol (47.03% and euphorbol (18.77%) was able to decrease signifi-cantly the serum tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP), interleukin (IL)-1β, IL-6, IL-18 and rheumatoid factor (RF) levels in rats in a concentration-dependent manner; and the swelling degree and histopathological change of the hind paws in rats and arthritic score were significantly improved when arthritis had been induced by Freund's complete adjuvant (FCA). In addition, up to 12 g/kg of the euphorbium total triterpenes any acute toxic symptoms (abnormal state of respiration, secretion, excretion or mortality) were detected.



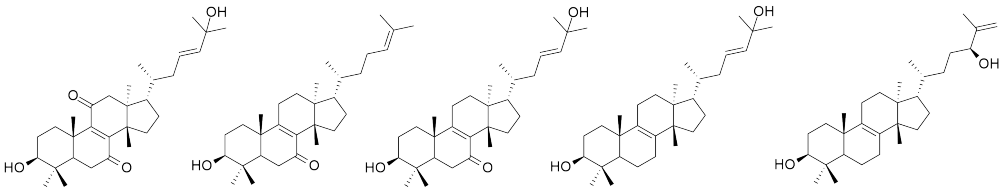
Euphol α-Euphorbol



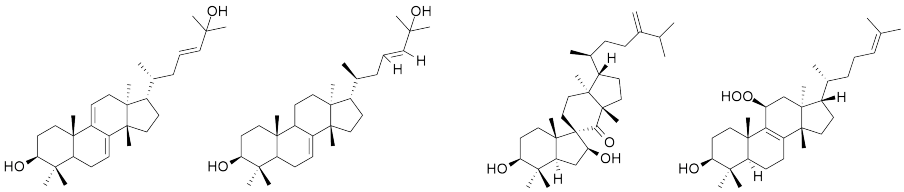
Euphorol A Euphorol B Euphorol C Euphorol D Euphorol H Euphorol I



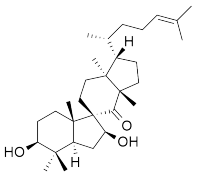
Euphorol E Euphorol F Euphorol G



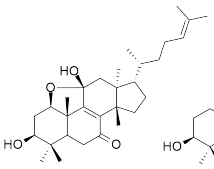
11-oxo-Kansenonol Kansenone Kansenonol (20*R*,23*E*)-Eupha-8,23-diene-3β,25-diol (24*R*)-Eupha-8,25-diene-3β,24-diol



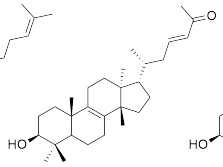
Kansenol 3β,25-dihydroxy-Tirucalla-7,23-diene Euphorol K Euphorol J



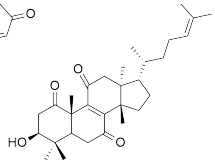
Kansuinone



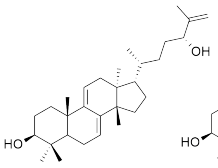
Euphatexol A



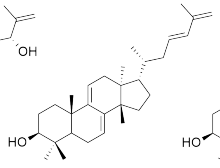
Euphatexol B



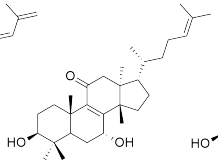
Euphatexol C



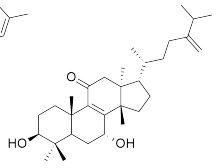
Euphatexol D



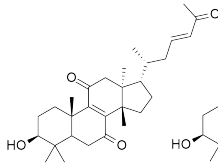
Euphatexol E



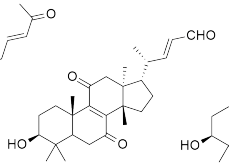
Euphatexol F



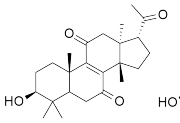
Euphatexol G



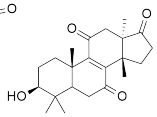
Euphorbiumrin A



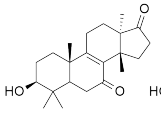
Euphorbiumrin B



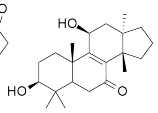
Euphorbiumrin C



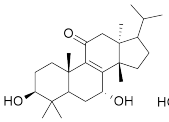
Euphorbiumrin D



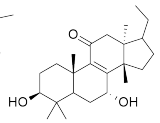
Euphorbiumrin E



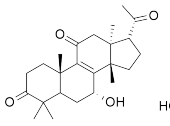
Euphorbiumrin F



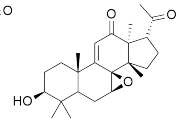
Euphorbiumrin G



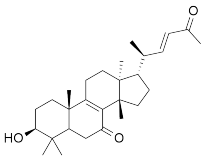
Euphorbiumrin H



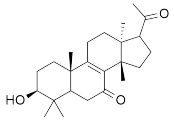
Euphorbiumrin I



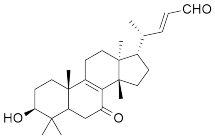
Euphorbiumrin J



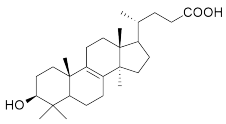
Euphorol F



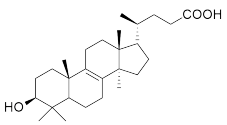
Sooneuphanone C



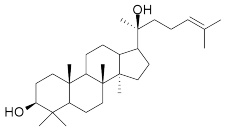
Sooneuphanone A



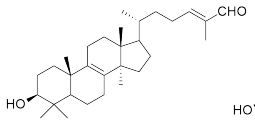
3 β–Hydroxy-25,26,27-trinor eupha-8-ene-24-oate



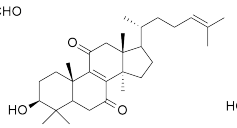
3 β–Hydroxy-25,26,27-trinortirucall-8-ene-24-oate



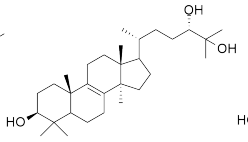
Dammarenediol II



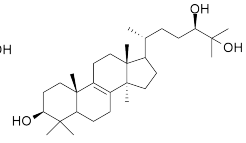
Eupha-8,24-diene-3-ol-26-al



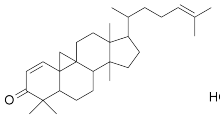
Eupha-8,24-diene-3b–ol-7,11-dione



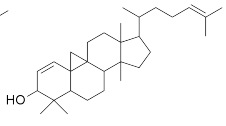
Inoterpene A



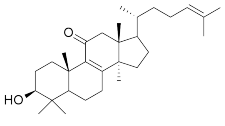
Inoterpene B



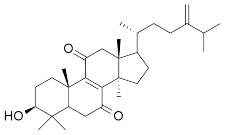
Cycloartane-1,24-diene-3-one



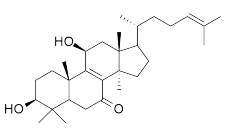
Cycloartane-1,24-diene-3-ol



3 β–Hydroxy-lanosta-8,24-diene-11-one



Eupha-24-methylene-8-ene-3β-ol-7,11-dione



Eupha-8,24-diene-3β,11β-diol-7-one

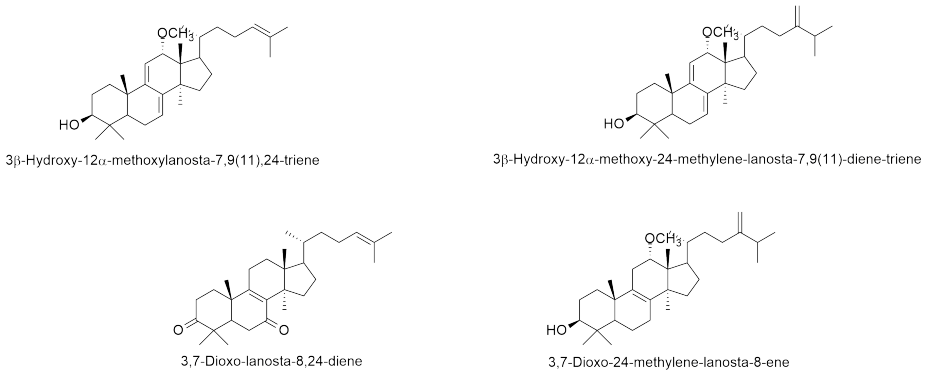


Figure 2. 4. Chemical structures of some triterpenes present in the latex of *Euphorbia resinifera*. From 3β-hydroxy-25,26,27-trinor eupha-8-ene-24-oate , the chemical structures were written only according to name of the compounds described in the abstracts (55-57). The authors did not access to the main text. In addition, the structure of lnonotusane C is not provided because it is unknown for the authors.

From the air-dried latex of *E. resinifera*, Wang et al. (58) isolated six new euphane triterpene derivatives (Figure 2.4) (euphorol A-D, H and I), three new tirucallane (20S-stereoisomer of euphane) triterpene derivatives (euphorol E-G) and seven known compounds (11-oxo-kansenonol, kansenone, kansenonol, (20*R*, 23*E*)-eupha-8,23-diene-3β,25-diol, (24*R*)-eupha-8,25-diene-3β,24 diol, kansenol, and 3β,25-dihydroxy-tirucalla-7,23-diene), but isolated in other species of the Euphorbiaceae, Melicaceae and Orchidaceae families. All triterpenes isolated and identified were tetracyclic-type but with different grade of oxidation, number and position of double bonds, obtained via oxidation, epoxidation, reduction, hydrolyzation, and dehydration from the same 8-ene dammarane triterpene precursor. According to this observation, the authors (58) proposed a biogenetic pathway for all of those compounds. The cytotoxicity of all compounds against MCF-7, U937 and C6 cancer cell lines were assayed and (24*R*)-eupha-8,25-diene-3β,24-diol revealed to be the most effective with IC_{50} values of 56.2 μM, 34.6 μM and 49.6 μM, respectively, but 5-9 times lower than the positive control taxol (58). Later on, other team described one new tirucallane triterpene with a spiro (5,6) ring system (euphorol K), and a novel euphane triterpene hydroperoxide (euphorol J), along with kansuinone (Figure 2.4) (59).

The activity of these compounds against the same tumor cell lines previously studied were investigated and the authors found that euphorol J had better activity than the remaining compounds, presenting the following IC_{50} values (37.36 μM, 47.17 μM, and 46.89 μM, respectively) (59).

From the latex of *E. resinifera* the authors Qi et al. (60) isolated and identified an unsual euphane triterpenoid due to the presence of a tetrahydrofuran ring formed via C-1 and C-11 (euphatexol A) (Figure 2.4). An unusual 27-noreuphane triterpenoid was also isolated (euphatexol B) (Figure

2.4). Both had cytotoxic activity against HepG2, with 87.0% and 87.4% inhibition rate at 1 μM, respectively. However, the positive control, cisplatin, showed 78.6% inhibition rate at 0.3 μM, that is, better activity than the triterpenes (60). The same team and as a part of their ongoing program for searching bioactive compounds, they reported five new triterpenoids isolated from the latex of *E. reisinifera* (euphatexols C-G) (Figure 2.4) with anti-inflammatory activity because they were able to inhibit nitric oxide production induced by lipopolysaccharide (LPS) in RAW264.7 cells. The IC_{50} values ranged from 20.35 μM (euphatexol E) to 48.04 μM (euphatexol D). The IC_{50} for the positive control (dexamethasone) was 20.35 μM (61).

Beyond euphatexol B, a 27-noreuphane triterpenoid previously identified (60), Zhao et al. (62) isolated and identified other nortriterpenoids from euphorbium. They named the ten new nortriterpenoids as euphorbiumrin A-J (Figure 2.4). Along with these components, the authors also found the known compounds euphorol F (58), sooneuphanone C and sooneuphanone A (Figure 2.4) (63). The nortriterpenoids had a dammarane skeleton, with the main differences at C-17 side chain moiety. The authors proposed a possible biosynthetic pathway for the nortriterpenoids. In general, they consider that euphol is the compound from which all nortriterpenoids identified are biosynthesized after degradation of its side chain at C-17, C-22, C-23, C-25, and C-27, which may also undergo epoxidation, oxidation, reduction, hydrolyzation, and dehydration reactions (62), as already suggested by Wang et al. (58). The inhibitions on tomato yellow leaf curl virus (TYLCV) were evaluated and the compound euphorbiumrin E exhibited the best activity with an inhibition rate of 71.7% at concentration of 40 μg/mL (62).

Wang et al. (55) isolated and identified ten triterpenes from a methanolic extract of *E. resinifera* latex: 3β-hydroxy-25,26,27-trinoreupha-8-ene-24-oate, isomaticadienediol, 25,26,27-trinortirucall-8-ene-3β-ol-4-acid, dammarendiol II, eupha-8,24-diene-3-ol-26-al, lnonotusane C, eupha-8,24-diene-3β-ol-7,11-dione, inoterpene A, inoterpene B, and eupha-24-methylene-8-ene-3β-ol-7,11-dione (Figure 2.4). According to the authors (55), 3β-hydroxy-25,26,27-trinoreupha-8-ene-24-oate was a new natural product, whereas isomaticadienediol, 25,26,27-trinor-tirucall-8-ene-3β-ol-4-acid, dammarendiol II were firstly described in the Euphorbiaceae, and eupha-8,24-diene-3-ol-26-al, lnonotusane C isolated for the first time from the genus *Euphorbia*. The structures were confirmed identified by spectroscopic methods and physicochemical properties after separation of the compounds through chromatographic methods using silica gel, octadecysilyl groups (ODS groups or C18 groups), and semi-preparative high performance liquid chromatography (HPLC). The cytotoxic properties of these compounds were also assayed against MCF-7, U937, and C6 cell lines (55).

Using the same methodologies of extraction, but with ethanol as extraction solvent, separation and identification, Li et al. (56) reported seven triterpenoids in the latex of *E. resinifera*. Two cycloartane triterpene structures (Figure 2.2) are new compounds (cycloartan-1,24-diene-3-one and, cycloartan-1,24-diene-3-ol) (Figure 2.4), and one lanostane triterpene structure (Figure 2.2) was obtained from nature for the first time (3β-hydroxy-lanosta-8,24-diene-11-one): The remaining compounds were: lnonotusane C, eupha-8,24-diene-3β-ol-7,11-dione, eupha-24-methylene-8-ene-3β-ol-7,11-dione, and eupha-8,24-diene-3β,11β-diol-7-one. In this case, any biological activity was reported in the abstract, the sole document consulted in this work.

Beyond the lanostane triterpenoids aforementioned, four new ones were reported by Li et al. (57): 3β-hydroxy-12α-methoxylanosta-7,9(11),24-triene, 3β-hydroxy-12α-methoxy-24-methylene-lanost-7,9(11)-diene, 3,7-dioxo-lanosta-8,24- diene, and 3,7-dioxo-24-methylene-lanost-8-ene (Figure 2.4) in the latex of *E. resinifera*. The two first compounds and the last one were those with moderate inhibition activity of LPS-induced nitric oxide (NO) production by RAW264.7. Their IC_{50} values were 30.4, 37.5, and 28.3 μmol/L, respectively.

3.3. Other compounds in the latex and plant extracts and their biological properties

Beyond the diterpene and triterpene aforementioned as being present in the crude drug Euphorbium, Bøe et al. (64) found (-)-quinic acid, D(+)-α-hydroxyglutaric acid, *myo*inositol, L(-)-inositol and probably vanillic acid (Figure 2.5), nevertheless quinic acid 1,3-lactone although present in one experiment, the authors considered that it was most likely an artifact resulting from the experimental procedure. Phorbic acid (Figure 2.5) was also found by Nordal and Benson (65) in the crude drug Euphorbium. The authors also concluded that this acid is formed in the latex producing cell system and not in the photosynthetic or chlorophyll-free tissues of *E. resinifera*. Its conclusion was achieved through a radioactive biosynthetic experiment: a branch of *E. resinifera* was kept in a $^{14}CO_2$ atmosphere with 12 hours of light-dark periods for 2 days and then kept under natural conditions in the air outside for 3 days. In these conditions, radioactive phorbic acid was detected in the latex. According to these results, the authors hypothesized that phorbic acid synthesis can be seen as independent of the photosynthetic and respiratory activities of the plant, only achieved if the latex of *E. resinifera* contains enzymes directly involved in the biosynthesis of phorbic acid. Phenolic compounds such as catechol, protocatechuic acid and 3,4-dihydroxy-phenylacetic acid were also described by Ourhzif et al. (32) for the first time in the ethyl acetate fraction of the latex of *E. resinifera*.

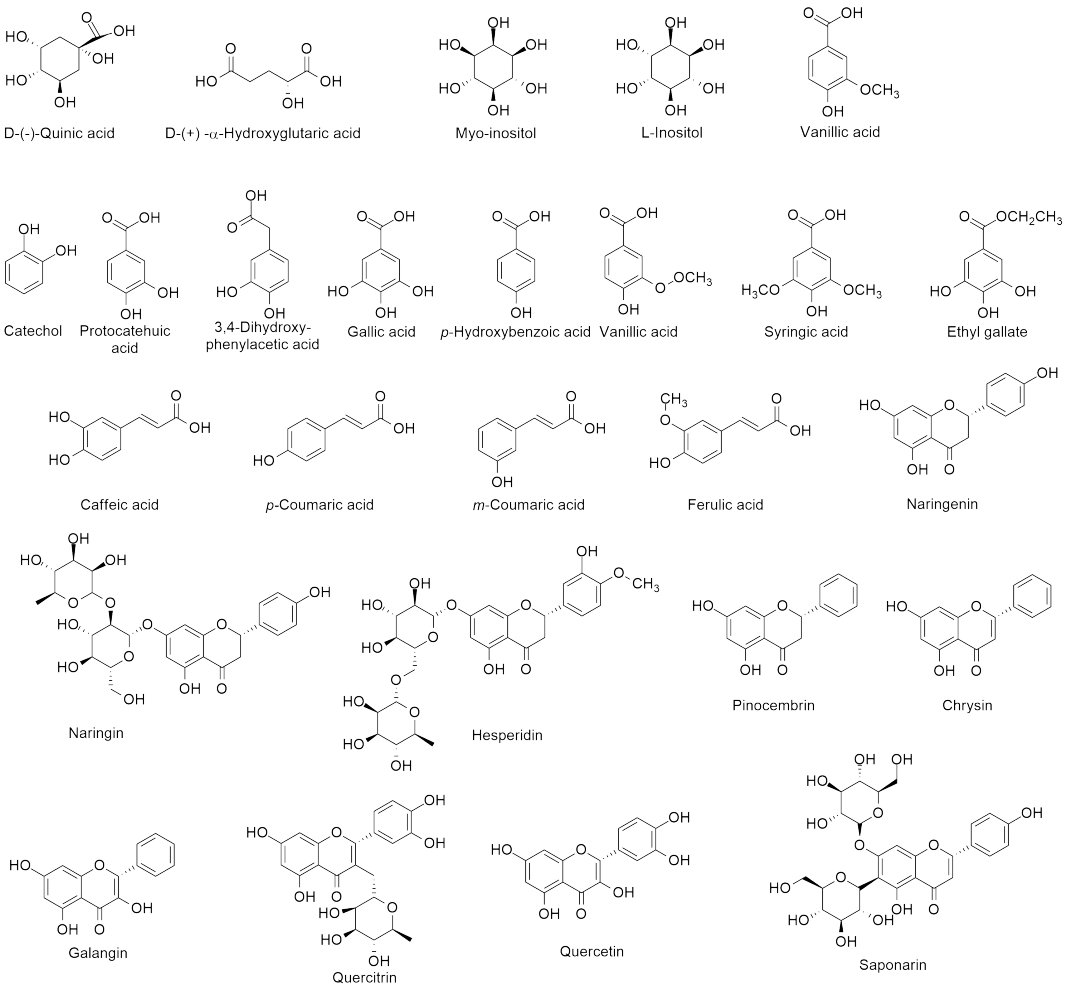


Figure 2.5. Some phenols identified in the *Euphorbia resinifera* latex (catechol, protocatechuic acid, and 3,4-dihydroxy-phenylacetic acid), in the extract (saponarin), and in honey. Other non-phenolic compounds identified in the latex of *Euphorbia resinifera* (-)-quinic acid, D-α-hydroxyglutaric acid, myo-inositol, L-inositol. See references along this section.

Saponarin, a flavone glycoside (Figure 2.5), was reported to be present in the aqueous extracts of the aerial parts of *E. resinifera*. No diterpene or triterpene were found in the same extracts (3). Whether the aerial parts used by the authors did not include the latex and only the green part of the plant this can explain such observation. The extracts also presented antioxidant (DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid, and H_2O_2 scavenging activities, and inhibition of xanthine oxidase activity), hypoglycemic activity (inhibition of α-amylase and α-glucosidase activities), and anti-hyperlipidemic effects (inhibition of lipase activity), with very low acute toxicity (lethal dose 50: LD_{50}) higher than 2 g/kg in treated rats) (3). However, in other study made by Issiki et al. (15), they reported that a concentration higher than 2.5 g/kg of the aqueous extract of *E. resinifera* can cause liver and kidney toxicity.

In this study, the authors revealed the presence of flavonoids and tannins, and there were no saponins or alkaloids.

The aqueous extract of *E. resinifera* latex presented antioxidant activity and antidepressant effect by reducing the latency of sleeping and increased sleeping time significantly at 75 mg/kg, reduced the immobility time and increased swimming significantly at all doses assessed (25, 50, and 75 mg/kg) in mice. For these properties, the authors believe the possible involvement of the α2-adrenoreceptors, 5-hydroxytryptamine receptor 2 (5HT2), dopamine receptor D2, and γ-aminobutyric acid type A (GABAA) receptors, because after pretreatment with antagonists there was a reverse of those effects. The extract contained flavonoid and condensed tannins (66).

Methanolic extracts of *E. resinifera* presented phenols, flavonoids, and condensed tannins, although the chemical profile has not been performed, their quantification was made by spectrophotometric method, and this extract had higher amounts of total phenols than *E. echinus*, both species collected in Morocco (67). The antioxidant activity, measured through the capacity for scavenging the DPPH free radicals, was revealed to be better than the well-known antioxidant ascorbic acid. The authors (67) also described that there was a positive correlation between antioxidant activity and total phenolic content. The antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* was found for the methanolic extract, but an absence of inhibitory activity against *Escherichia coli*. Previously, Boutoub et al. (68) through the Response Surface Methodology (RSM) using a full three-level factorial design were able to optimize the conditions for the extraction of antioxidants and α-glucosidase inhibitors. For this, they made aqueous extracts and then studied the effect of temperature, time, and plant-to-solvent ratio (PSR) and their linear and quadratic interactions on total phenol concentration, total flavonoid concentration, DPPH trapping activity, and α-glucosidase inhibiting activities. The results showed that the best extraction temperature was between 30°C and 35°C. Extraction using PSR of 20 mg/mL for 1 hour at 30°C yielded extracts with an optimal phenolic content and optimal values of the studied activities for *E. resinifera*. However, for aqueous extracts of *E. officinarum*, other results were obtained: extractions for 270 min, at 50°C, using PSR of 10 mg/mL were needed to yield extracts with better activities. Such results reveal the importance of determining the best conditions of extraction to obtain extracts with the best biological activities (68).

Plant latex is a source of proteases exhibiting anticoagulant and fibrinolytic activity such as a cysteine protease (ficin) from *Ficus carica* (69). From the *E. resinifera* crude latex, Siritapetawee et al. (70) isolated and purified a new protease, designated as EuRP-61. This is a serine protease because its amino acid sequence had homology (between 50 and 70% identities) with the subtilisin-like proteases of other plants, with molecular weight estimated at 61 kDa analyzed by Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF

MS). The enzyme had a broad range of pH stability from 1 to 14 and tolerance to denaturation up to a temperature of approximately 65 - 66 ºC. The protein sequence and native crystal structure of EuRP-61 were characterized (70) and the main structural components of EuRP-61 were composed of three domains: catalytic, protease-associated, and fibronectin type III (Fn3)-like domains. The Fn3-like domain may provide flexibility to the enzyme to bind with various substrates and cell receptors. The active site of EuRP-61 consisted of the catalytic triad of Ser434,His106, andAsp32, similar to other serine proteases. The enzyme has anticoagulant, antiplatelet, and peripheral blood cell aggregation inhibitory properties being not toxic to human red blood cells in the 4 common blood groups (A, B, O and AB) (all Rh^+) or human peripheral blood mononuclear cells (hPBMCs) (70,71). These properties of EuRP-61 led Siritapetawee et al. (72) to develop a method for improving the antithrombogenicity of a blue nylon monofilament suture (USP 3/0) by coating it with that protease and by using a combination of dipping and ultrasonication methods. The results were promising since the enzyme-coated nylon presented fibrinogenolytic and fibrinolytic activities. Comparing the enzyme-coated and bare nylon, the enzyme-coated nylon prevented the human fibrin clot from adhering to its surface. Analyses of the fibrinogenolytic and fibrinolytic activities were performed by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and microwell plate assays. The results revealed that the adequate concentration of protease would be 1 mg/mL for each coating of 20 strips of 10-mm nylon. According to these results, the authors hypothesize the utilization of this EuRP-61-coated nylon as a surgical suture, particularly for vessel anastomosis (72).

3.4. *Euphorbia resinifera honey*: chemical composition and their biological properties

In Morocco, *E. resinifera* honey (resin spurge) is highly appreciated by consumers due to its unique peppery taste, pungency, and powerful aroma, medical and cosmetic properties, mainly due to their antibacterial and antifungal activity (4,73). For these reasons, several teams have developed their works searching for the chemical composition of resin spurge as well as their biological activities and sanitary quality. It is very important to guarantee a good sanitary quality of honeys because they need to be safe. Moujanni et al. (74) performed a microbiological profile of thirty-seven samples of *E. resinifera* honeys collected in a Protected Geographical Indication area of Tadla-Azilal region (Morocco). They reported that in these samples, Coliforms (Total and fecal Coliforms), *Salmonella* spp., *Shigella* spp., sporus of *Bacillus cereus* and *Clostridium perfringens* were not detected. The molds and yeasts were present in 32% and 40% of samples, respectively. However, no samples showed a higher value than recommended limit (102 CFU/g), so the samples can be considered of good commercial quality parameters (74). Other aspect very important in a safe food is the absence of antibiotics and this was confirmed in 37 samples of *E. resinifera* honeys, in which only one presented Trimethoprim at 6.48 μg/kg (75). According to the authors (75) the antibacterial activity found in the honey samples against *Staphylococcus*

aureus and *Escherichia coli*, can only be attributed to their physicochemical properties (high osmotic nature, low pH, content of phenolic compounds, hydrogen peroxide and also to its content of methylglyoxal) and not to the possible presence of antibiotic residues.

In contrast to the absence of antibiotics in *E. resinifera* honey, Massous et al. (76) found diverse pesticides, plasticizers and bisphenols in samples collected in the Béni Mellal-Khénifra region (Morroco). In what concerns pesticides, the authors found carbaryl, diazinon, metalaxyl-M, acephate and cyromazine at concentrations higher than 10 µg/kg. The plasticizers diethyl phthalate, diethanolamine and dibutyl adipate were present at concentrations higher than 1 mg/kg. Although dibutyl phthalate is lower than 1 mg/kg (0.84 mg/kg), this concentration is substantially higher than the relative specific migration limits, according to the Reg. (EU) No. 10/2011 (77), which is 0.3 mg/kg. Bisfenol A, bisfenol B, and bisfenol AF were present in honey samples, but at concentrations lower than the regulatory specific migration limits, particularly for bisfenol A migration from food contact material which is 600 ng/g (76). According to the authors the presence of plasticizers and bisphenols can be attributed to the plastic components that were released from the honey production equipment, such as honey extractor and uncorkers, although the presence of these compounds cannot be ruled out as environmental contamination, for example, from the nectar used by bees to make honey.

Owing to the importance of resin spurge honey their botanical and geographical characterization are needed. For this reason, Terrab et al. (4) made a palynological and geographical characterization of 29 monofloral resin spurge honeys along the protected geographic indication area in the Middle Atlas Mountains (Morocco) and concluded that they presented low to very low pollen content. For this reason, Terrab et al. (4) suggest a revision of the established threshold proposing to lower it to 20% of pollen of *E. resinifera* to be considered as monofloral honey. The other pollen species also detected in the samples were *Ceratonia siliqua*, *Echium plantagineum*, *Olea* sp., *Plantago coronopus* and *Quercus*. However, Boutoub et al. (78) found two resin spurge honeys with higher than 45% of *E. resinifera* pollen, both collected in Beni Mellal-Khénifra (Morocco). In the same work, the authors studied the *E. officinarum* honeys and their extracts for comparison. All honey samples were within the acceptable limit of the international standards, as already reported by Moujanni et al. (79) for a total of 29 honey samples, nevertheless *E. resinifera* honeys presented lower moisture, HMF but higher reducing sugar percentage than *E. officinarum* honeys.

In what concerns the secondary metabolites, gallic acid, 4-hydroxybenzoic acid and *p*-coumaric acid (Figure 2.5) were detected in all samples, although in different ratios. The methanolic extracts of honeys presented higher biological activities (antioxidant activity measured through DPPH radical-scavenging capacity, nitric oxide scavenging activity and scavenging ability of superoxide

anion radical; and inhibition of acetylcholinesterase, lipoxygenase, tyrosinase and xanthine oxidase activities) than the entire honeys. Nevertheless, *E. resinifera* honeys had better anti-inflammatory activities (anti-lipoxygenase activity) than the *E. officinarum* honeys. Both teams (78,79) found that potassium (K) was the most abundant mineral element in *E. resinifera* honey samples. The best activity of the honey extracts would be expected because Boutoub et al. (80) previously found that the *E. resinifera* or *E. officinarum* extracts generally had better biological properties than the entire honeys, which can support the hypothesis that the antioxidant activity, for example, can be mainly attributed to the secondary metabolites of the plants visited by honeybees.

A much higher number of phenolic compounds (17) were found by Hernanz et al. (81) in *E. resinifera* honey than Boutoub et al. (78): gallic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, ethyl gallate, caffeic acid, *p*-coumaric acid, m-coumaric acid, ferulic acid, naringenin, naringin, hesperidin, pinocembrin, chrysin, galangin, quercitrin, and quercetin (Figure 2.5). Some of these compounds were also reported by Boutoub et al. (78): gallic acid, *p*-hydroxybenzoic acid, caffeic acid (in only one sample), *p*-coumaric acid, quercetin and naringenin. Hernanz et al. (81) also reported a correlation between the chromatic attributes and phenolic acids, total phenolic compounds, caffeic acid, *p*-and *m*-coumaric acids and hesperidin.

4. Conclusion

Euphorbia resinifera is a large, leafless cactus-like perennial, endemic to Morocco, generally distributed in the center of the country, in the regions of Azilal and Beni Mellal (Middle Atlas). This species has been used in folk medicine, which many times, Moroccan patients mixed the aerial parts with honey or using extracts obtained by decoction in the treatment of general cancer. Fresh latex is used for poisonous punctures, bites, and dental pains. The last utilization can be attributed to the presence of resiniferatoxin (daphnane diterpene), which is an analog of capsaicin by acting with capsaicin receptor. That diterpeneis 1,000-fold more potent as an analgesic than that alkaloid, nevertheless it is extremely irritant. For this reason, resiniferatoxin has been used in some clinical trials as a potential analgesic to relieve cancer and arthritis pain, nevertheless there are bioanalytical limitations in its quantification in plasma due to the minimal effective dose that is in the range of few nanograms. Some studies have been developed to achieve an efficient, rapid, and reliable method to quantify low concentrations of resiniferatoxin, to make easier the determination of its pharmacokinetic profile, data important in therapeutics. Other diterpenes have been also identified in the latex along with norsesquiterpenes and triterpenes with diverse biological attributes, such as anti-inflammatory, proliferation inhibition of some cancer cell lines, antimicrobial, antiplasmodial, and anti-insecticidal properties. In the latex was also reported the presence of serine proteases named as EuRP61, with 61 kDa showed anti-coagulant, antiplatelet,

and peripheral blood cell aggregation inhibitory properties being not toxic to human red blood cells in the 4 common blood groups (A, B, O and AB) (all Rh+) hPBMCs. These attributes led the development of antithrombogenicity blue nylon monofilament suture coated with EuRP61. The positive results show that can be possible the utilization of this EuRP-61-coated nylon as a surgical suture, particularly for vessel anastomosis. The aerial parts of *E. resinifera* without latex are constituted by other group of compounds such as polyphenols, although the detailed chemical profile of such samples have not been done so far, in contrast to the *E. resinifera* honey in which there is already some chemical detail. In this case, there are several phenolic acids, flavanones, flavones, flavonols and some flavonoids glycosides identified. The *in vitro* biological properties of honey include free radical scavenging activity (superoxide anion radical), anti-inflammatory activity through the inhibition of lipoxygenase activity. Other enzyme inhibition activities were described such as inhibition of acetylcholinesterase, tyrosinase, and xanthine oxidase activities. These activities were attributed to the secondary metabolites present in the honey because the extracts where these compounds were present had better activity than the entire honey. For aqueous extracts, it was demonstrated that the extraction conditions are important to improve the amounts of phenols and antioxidant activity. The best conditions found were: extraction temperature between 30°C and 35°C, plant solvent of 20 mg/mL, and extraction time of 1 h if the extraction is made at 30 ºC.

Author Contributions: M.G.M: conceptualization, methodology, writing –review, validation; L.E.G. Writing –review; O.B. methodology, writing –original draft.

Funding: This research was funded by the FCT (Fundação para a Ciência e a Tecnologia, Portugal) under the project UIDB/05183/2020.

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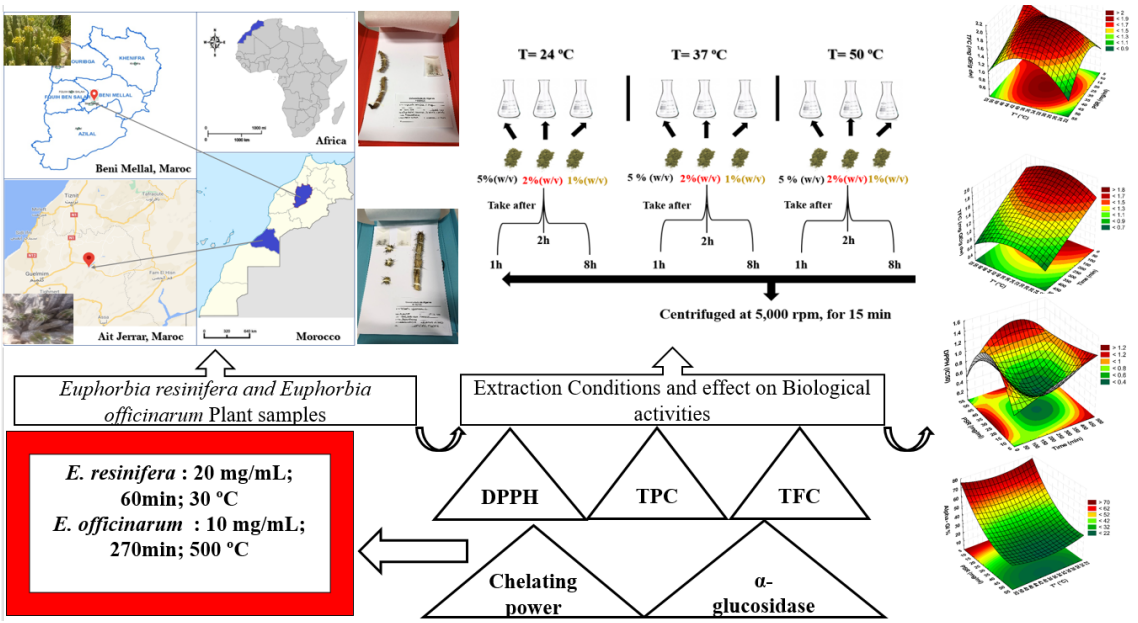
Chapter 3

Response surface methodology (RSM) for optimization of *Euphorbia resinifera* and *Euphorbia officinarum* extracts with antioxidant and anti-diabetic activities



3

Chapiter III: Response surface methodology (RSM) for optimization of Euphorbia resinifera and Euphorbia officinarum extracts with antioxidant and anti-diabetic activities*

Résumé

L'Euphorbe est l'une des plantes les plus utilisées par les herboristes et les thérapeutes au Maroc. Objectifs : La partie aérienne de deux échantillons de plantes (*Euphorbia resinifera et Euphorbia officinarum*) collectés au Maroc a été examinée pour l'effet solvant, le temps d'extraction et la concentration de la plante afin de déterminer les meilleures conditions d'extraction. Pour atteindre cet objectif, une méthodologie de surface de réponse (RSM) utilisant un plan factoriel complet à trois niveaux a été utilisée pour optimiser les conditions d'extraction des antioxydants et des inhibiteurs de l'α-glucosidase. Température, temps et rapport plante/solvant (PSR) et leurs interactions linéaires et quadratiques sur l'activité de piégeage du TPC (concentration totale de phénol), du TFC (concentration totale des flavonoïdes), du DPPH (2, 2-diphényl-1-picrylhydrazyl), et les activités d'inhibition de l'α-glucosidase ont été étudiées.

Résultats : Selon les fonctions de désirabilité, les conditions opératoires optimales pour obtenir un rendement d'extraction plus élevé des phénols et une activité antioxydante et antidiabétique plus élevée ont été trouvées en utilisant une extraction pendant 60 min à 30 °C en utilisant un PSR de 20 mg/mL, alors qu'un un temps d'extraction plus long (270 min) était nécessaire pour E. resinifera et une température d'extraction plus élevée (50 °C), avec un PSR plus faible (10 mg/mL) pour E. officinarum. Afin de trouver les meilleures conditions d'extraction des métabolites secondaires à activité biologique et application en phytothérapie, il convient d'utiliser le solvant approprié généralement utilisé par les populations, l'eau en l'occurrence, mais il faut trouver les meilleures conditions d'extraction afin d'améliorer l'extraction actions pharmacologiques.

Mots clés : activité inhibitrice de l'α-glucosidase, plan factoriel, méthodologie de surface de réponse, composés phénoliques totaux, flavonoïdes totaux.

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* Adapté de Oumaima Boutoub, Smail Aazza, Soukaina El-Guendouz, Lahsen El Ghadraoui, and Maria G. Miguel. Response surface methodology (RSM) for optimization of *Euphorbia resinifera* and *Euphorbia officinarum* extracts with antioxidant and anti-diabetic activities. *Pharmacognosy Magazine Journal*. **2022**, DOI: 10.4103/pm.pm_2_22.

3

Response surface methodology (RSM) for optimization of *Euphorbia resinifera* and *Euphorbia officinarum* extracts with antioxidant and anti-diabetic activities*

Abstract

Euphorbia is one of the plants most used by herbalists and therapists in Morocco. Objectives: The aerial part of two plant samples (*Euphorbia resinifera* and *Euphorbia officinarum*) collected in Morocco was examined for the solvent effect, extraction time, and plant concentration in order to determine the best extraction conditions. To achieve this goal, a response surface methodology (RSM) using a full three-level factorial design was used to optimize the conditions for the extraction of antioxidants and α-glucosidase inhibitors. Temperature, time, and plant-to-solvent ratio (PSR) and their linear and quadratic interactions on TPC (total phenol concentration), TFC (total flavonoid concentration), DPPH (2, 2-diphenyl-1-picrylhydrazyl) trapping activity, and α-glucosidase inhibiting activities were studied. Results: According to desirability functions, the optimum operating conditions to achieve a higher extraction yield of phenols and higher antioxidant and anti-diabetic activity were found by using extraction during 60 min at 30 °C using a PSR of 20 mg/mL, whereas a longer extraction time (270 min) was needed for *E. resinifera* and a higher extraction temperature (50 °C), with a lower PSR (10 mg/mL) for *E. officinarum*. In order to find the best conditions to extract secondary metabolites with biological activity and application in phytotherapy, the appropriate solvent generally used by populations, water in this case, should be used, but the best extraction conditions have to be found in order to enhance the pharmacological actions.

Keywords: α-glucosidase inhibitory activity, factorial design, response surface methodology, total phenolic compounds, total flavonoids

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* Adapted from Oumaima Boutoub, Smail Aazza, Soukaina El-Guendouz, Lahsen El Ghadraoui, and Maria G. Miguel. Response surface methodology (RSM) for optimization of *Euphorbia resinifera* and *Euphorbia officinarum* extracts with antioxidant and anti-diabetic activities. *Pharmacognosy Magazine Journal*. **2022**, DOI: 10.4103/pm.pm_2_22.

1. Introduction

Euphorbia resinifera O. Berg, with the local name "Zaggoume, Takiout", is an endemic melliferous species of Morocco well adapted to drought. Currently, it is considered as a "terroir" product with a Protected Geographical Indication (PGI) (1). In habitat, this plant forms densely branched, compact shrubs of some meters in diameter and until 1.50 m height. Since ancient times, the North-African Berber tribes have used this species as a remedy (2). In Morocco, where the use of traditional medicine is a widespread practice, it is one of the oldest plant drugs in folk medicine much used by Moroccan herbalists and therapists. The leaf stem's decoction (3) or one drop of latex in a glass of water once a day (4) has been used orally to treat diabetes in Morocco.The second most important species in Morocco, belonging to the family of Euphorbiaceae, is *E. officinarum* L.; "Daghmouss" is the local name. This endemic plant is generously found over the north of the Souss river until Moroccan sahara reaching the region of Zemmour (5). In Morocco, this species has been largely used in folk medicine over the years for the treatment of various diseases such as diabetes because of the presence of several secondary metabolites such as phenolic compounds (6). According to Idm'hand et al. (7), prolonged uncontrolled diabetes leads to an increase in the activation of oxidative stress.

The extraction of phenolics or other secondary metabolites from plant sources is the first step involved in their analysis. In spite of several works on the analysis of the plant and phenolics, there is still no available standardized procedure for sample preparation and extraction (8). It is necessary to optimize the solid–liquid extraction process to obtain the highest biological activities of the extracts, which are generally associated with the polyphenol yield (9). Response surface methodology (RSM) is an important tool in the experimental design of analytical chemistry procedures offering a broad range of information ranging from the significance of independent variables to the interaction between them (10).

The objective of this study was the optimization of the best conditions, namely, solute/solvent (water) ratio (10–50 mg/mL), extraction temperature (24–50°C), and extraction time (60–480 min), in order to obtain aqueous extracts with higher antioxidant and anti-α-glucosidase activities obtained from the aerial part of *E. resinifera* and *E. officinarum*.

2. Experimental

2.1. Plant samples

Euphorbia resinifera and *E. officinarum* aerial parts were collected directly from the fields of Beni Mellal, Morocco, and Ait Jerrar, Tiznit, Morocco, respectively (Figure 3.1). Dried plant material was deposited as authenticated vouchers in the Herbarium of the Universidade do

Algarve (acronym ALGU), with the accession numbers 15745/ALGU and 15746/ALGU, respectively.

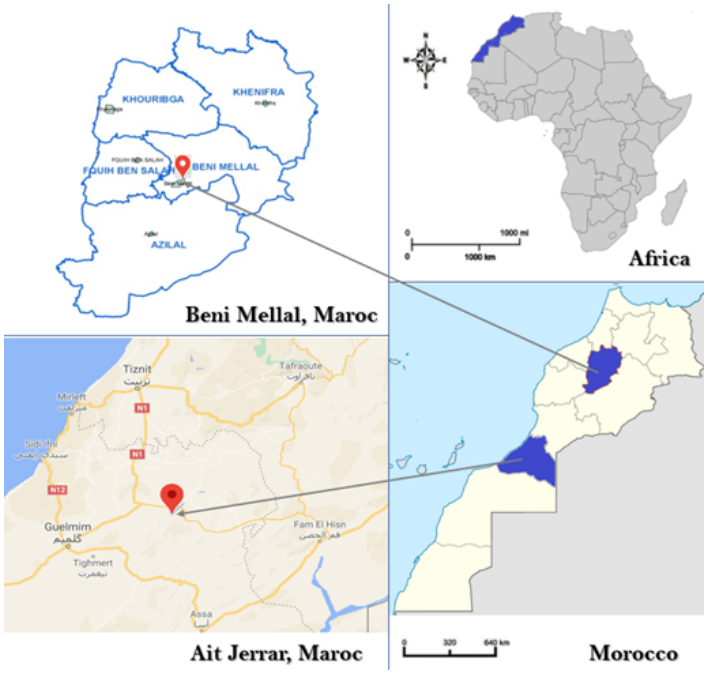


Figure 3.1. Moroccan map showing the harvesting locations of *Euphorbia resinifera* and *Euphorbia officinarum*.

2.2. Sample preparation

Euphorbia resinifera and *E. officinarum* were well dried at room temperature in the dark and grinded. Afterwards one gram from the aerial parts was extracted by maceration with 20 mL, 50 mL and 100 mL of distilled water (w/v) in four different temperatures (24 ºC, 37 ºC, and 50 ºC) during 1 h, 2 h and 8 h to yield 27 extracts for each plant. Each extract was centrifuged at 5,000 rpm, for 15 min. The supernatant was recovered and kept in –20 ºC until further analysis.

2.3. Total phenol content (TPC)

The total phenolic content was determined using Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (11), with some modifications. Each extract (50 µL) was mixed with 125 µL Folin-Ciocalteu's phenol reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3. After 1 h of incubation at room temperature the absorbance was measured at 765 nm. The total polyphenol content was expressed as mg Gallic Acid Equivalents (GAE) per g for plant extract.

2.4. Total flavonoid content (TFC)

The amounts of flavones and flavanols in extracts were determined according to the method described by Miguel et al. (12) with minor modification. An amount of 100 µL of $AlCl_3$ (20%) was added to 100 µL of extract, and after 1h incubation at room temperature, the absorbance was

measured at 420 nm. Total flavones and the flavanols content were calculated as quercetin equivalents (QE) (mg per g) using a calibration curve. Tests were carried out in triplicate.

2.5. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals' scavenging ability

DPPH free radical scavenging activity was assessed as described by Miguel et al. (13) with some modifications. Plant extracts (25 µL) were added to 250 µL of DPPH solution (63.4 µM) and incubated for 30 min at room temperature, the absorbance was measured at 517 nm. The result was calculated using the following formula: Inhibition = $((A_0-A_1)/A_0 \times 100)$; with A_0 is the absorbance of the control and for A_1 is the absorbance of the sample. The sample concentration providing 50% inhibition (IC_{50}) was achieved by plotting the inhibition percentage against sample's concentrations. Butylated hydroxytoluene (BHT) was used as standard (0.03 – 1 mg/mL).

2.6. α-Glucosidase inhibition activity

α-Glucosidase inhibition assay was carried out according to El-Guendouz et al. (14) with some modifications. The total assay mixture consisted on adding 70 µL of plant extract, mixed with 50 µL of yeast α-glucosidase (2.4 U/mL) prepared in phosphate buffer (100 mM; pH 6.8), the mixture was incubated for 10 min. After this period, 100 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in phosphate buffer was added. The reaction solution was incubated at room temperature for 30 min, at the end 80 µL of sodium carbonate solution (0.4 mM) was added to stop the reaction. The reading was done at 405 nm, the assay was done in triplicate. The activities were presented as IC_{50} values and determined as reported for the antioxidant activity. Acarbose was used as standard (0.002- 1 mg/mL)

2.7. Chelating metal activity

The degree of chelating ferrous ions of aqueous extracts was assayed as reported by El-Guendouz et al. (14) with a little modification. Two hundred µL of different concentration of extract and (25 µL) $FeCl_2.4H_2O$ (2 mM) were added to the 50 µL of distilled water and (25 µL) ferrozine (5 mM) which initiated the reaction have mixed well, the absorbance at 562 nm was measured immediately. The results were calculated as reported previously. EDTA (Ethylenediaminetetraacetic acid) was used as standard (0.001 - 1 mg/mL).

2.8. Experimental Design

A three levels three factor full factorial design was used to evaluate the effect of the combinations of three independent variables *viz*. temperature, time, and plant-to-solvent ratio (PSR) on the extraction of phenolic compounds (TFC and TPC), antioxidant activity and α-glucosidase inhibition activity from *E. resinifera* and *E. officinarum* aqueous extracts. The experimental

design consists of 81 runs, including 27 experiments (Table 3.1) with three replicates for each. A second-order polynomial equation (Equation 1) was used to fit the experimental data. The general form of mathematical quadratic response equation was given as below.

$$Y = \beta 0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \varepsilon$$

Equation 1

where Y indicates the predicted response; β0, βi, βii, βij, are the regression coefficient for intercept, linear, quadratic, and interactive effect, respectively; and Xi, Xi2 and Xij are the coded independent variables; and k is equal to the number of the tested factors (k = 3 in this study).

2.9. Statistical analysis

All experiments were performed in three replicates. The results of analyses were reported as mean ± standard deviation (SD). The factorial design was designed and analyzed using the free version StatSoft, Inc. (2011), STATISTICA (data analysis software system), version 10. A total of 27 combinations were used (Table 3.1). Results were analyzed using ANOVA. Significant differences were determined by Tukey's test, with $p < 0.05$ as the significance criterion.

3. Results

3.1. Statistical analysis and model fitting

The identification of the main factors that affect the experimental response must be initiated by the determination of the optimal conditions in the extraction of phenol compounds. In this study, the three-level full factorial design was conducted to identify the main and interaction effects of process variables, namely, extraction temperature, extraction time, plant percent, and solvent-to-plant material ratio, PSR (expressed as the concentration of the plant material in the extraction solvent in mg/mL), on the extraction of phenolic compounds (total phenol content (TPC) and total flavonoid content (TFC), the anti-oxidant activity, and the α-glucosidase inhibition activity of the yielded aqueous extracts From *E. resinifera* and *E. officinarum*. Water was chosen as solvent extraction because the ethnobotanical studies reported the use of aqueous extracts and because it is generally safer for the potential applications in food or pharmaceutical fields. Analysis of variance (ANOVA) was performed with a confidence level of 95% (P = 0.05) to evaluate the robustness of the empirical model and verify the adequacy of the model generated by the factorial experiment. The probability value (p values) was used as a tool to verify the significance of each coefficient.

The test of reliability for predicting the equation had been carried out by Fisher's variance ratio test, known as the F-test. The fit of the model has also been expressed by the determination coefficient (R2) and the adjusted coefficients of determination (Adj-R^2), both indicating how well the polynomial equation predicts the data. The linear and quadratic effect of the independent variables as well as their interaction on the The determination coefficient value of the quadratic regression model coefficient (R^2), respectively, for TPC and TFC, was 0.97 and 0.94, indicating that only about 3% and 6% of the total variations were not explained by the model for the two responses, and can be explained by the residues. Meanwhile, the model explained 98% of the total variation in TPC and TFC values for *E. officinarum*. Furthermore, the adjusted (R^2) value was obtained from R^2 after the elimination of the unnecessary model terms. The adjusted (R^2 adj.) presented by the two species were 0.94 and 0.98 for TPC and 0.93 and 0.97 for TFC for *E. resinifera* and *E. officinarum,* respectively. Those values were very high and very close to the value of R^2 supporting the high correlation between the observed and predicted values for TPC and TFC. Concerning the DPPH antioxidant activity, the values of R^2 were, respectively, 0.79 and 0.93 for *E. resinifera* and *E. officinarum*. Closer values of 0.73 and 0.91 were presented, respectively, by adjusted R^2.

Regarding the α-glucosidase inhibitory activity, the values of R^2 were 0.92 and 0.98, respectively, for *E. resinifera* and *E. officinarum*, which imply that more than 92% and 98% of experimental data can be, respectively, explained by the model for the two responses. Additionally, the high correlation between the observed and the predicted values was also observed because R^2 and adjusted R^2 (R^2 adj.) were in rational agreement. Concurrently, the chelating power also presented high R^2 values of 0.89 and 0.91 for *E. resinifera* and *E. officinarum*, respectively, which were in their turn strongly correlated to the adjusted R^2.

Figure 3.2 (a, b) display the normal probability distribution of residuals of ANOVA for all response variables for *E. resinifera* (Figure 3.2a) and *E. officinarum* (Figure 3.2b). The plots prove the adequacy of the model as the former is approximately a line in which the values of residuals fit on a straight line to a major extent.response variables were analyzed, and the results are summarized in Table 3.2.

3.2. Effect of the process variables on the total phenolic content (TPC)

Data regarding the effect of extraction parameters on the amount of total phenolic contents are presented in Table 1. Among the 27 extracts obtained from *E. resinifera*, the amount of TPC extracted ranged from 1.25 to 7.02 mg/g of dry plant material, measured as gallic acid equivalent (GAE). The mean value of TPC extraction was 4.64 mg GAE/g dw depending on the extraction conditions. Run 19 (T 50°C; time 60 min and PSR (10 mg/mL)) exhibited the highest TPC, whereas run 16 (T 37°C; time 480 min and PSR (10 mg/mL)) showed the lowest TPC. Likewise,

the amount of TPC in *E. officinarum* ranged from 2.80 to 8.65 mg GAE/g dw, found in run 12 (T 37°C; time 60 min and PSR (50 mg/mL)) and run 4 (T 24°C; time 120 min and PSR (10 mg/mL)), respectively.

The results depicted in Table 2 from ANOVA analysis indicate that time (L) and PSR (L and Q) were the most significant factors in determining the optimum TPC recovery for *E. resinifera*, with a P value of 0.000000, followed by the interaction temperature–PSR, temperature linear, and the interaction temperature–time with P values of 0.0004, 0.02, and 0.03, respectively, Whereas temperature (Q) and time (Q) were without significant effects ($p < 0.05$). Meanwhile, the factors having the greatest impact in the case of *E. officinarum* were temperature (Q), PSR (L and Q), and the interaction temperature–PSR ($p = 0.000000$), unlike time (L) and its interaction with PSR, which were without significant effects.

Multiple regression analysis was used to determine the correlation of the three process variables and TPC levels in the extracts. The second-order polynomial equation yielded after elimination of the non-significant coefficients is given by the following equations:

TPC (mg GAE/g DW) *Euphorbia resinifera* $= 7.2 + 5.2*10^{-2}(ET°) + 7.2*10^{-2}\ (ET) - 1.6*10^{-4}\ (ET)^2 - 4.8*10^{-1}\ (PSR) - 4.4\ *10^{-3}\ (ET°)\ (ET) + 7.2*10^{-6}\ (ET°)\ (ET)^2 + 5.5*10^{-5}\ (ET°)^2(ET) - 9.03*10^{-8}\ (ET°)^2(ET)^2$ (Equation 2)

TPC (mg GAE/g DW) *Euphorbia officinarum* $= 29.420 - 1.086(ET°) + 1.34*10^{-2}(ET°)^2 - 1.077\ (PSR) + 1.35*10^{-2}\ (PSR)^2 + 3.81*10^{-3}\ (ET°)\ (ET) - 5.66*10^{-5}\ (ET°)^2(ET) + 9.17*10^{-8}\ (ET°)^2(ET)^2 + 3.60*10^{-2}\ (ET°)\ (PSR) - 4.52*10\text{-}^4\ (ET°)\ (PSR)^2$ (Equation 3)

Where; (ET°): Extraction temperature; (ET): Extraction time (min); (PSR): plant/solvent ratio (mg/mL).

All the presented factors in the equations are significant ($p < 0.05$). The negative sign in the equation represents an antagonistic effect of the variables while the positive sign represents a synergistic effect.

Table 3.1. Experimental design

	Temperature (°C)	Time (min)	PSR (mg/mL)	TPC		TFC		DPPH		Inhibition of α-Glucosidase		Chelating Power	
	Run			*Euphorbia resinifera*	*Euphorbia officinarum*	*Euphorbia resinifera*	*Euphorbia officinarum*	*Euphorbia resinifera*	*Euphorbia officinarum*	*Euphorbia resinifera*	*Euphorbia officinarum*	*Euphorbia resinifera*	*Euphorbia officinarum*
1	24	60	10	6.90±0.38	8.05±0.57	1.28±0.02	0.54±0.01	0.69±0.04	0.57±0.02	71.83±1.34	83.23±0.41	6.47±0.10	0.73±0.00
2	24	60	20	5.42±0.39	4.85±0.24	1.59±0.06	0.28±0.01	1.22±0.04	1.02±0.01	66.54±0.82	39.46±1.00	11.15±0.45	5.86±0.00
3	24	60	50	3.86±0.12	3.04±0.11	1.19±0.01	0.19±0.01	0.89±0.07	1.88±0.02	30.93±0.46	17.52±1.02	15.49±0.21	26.82±0.40
4	24	120	10	6.74±0.45	8.65±0.32	1.46±0.09	0.62±0.01	0.59±0.01	0.49±0.07	66.02±1.13	92.09±0.99	6.27±0.13	1.02±0.01
5	24	120	20	5.73±0.22	4.88±0.25	1.38±0.03	0.35±0.02	0.66±0.03	0.92±0.05	50.66±0.37	45.90±0.86	9.01±0.27	0.93±0.01
6	24	120	50	4.05±0.09	3.11±0.11	1.35±0.03	0.18±0.00	0.86±0.03	1.42±0.03	29.69±0.30	22.25±0.35	0.44±0.01	9.67±0.43
7	24	480	10	1.80±0.01	8.07±0.33	0.39±0.03	0.44±0.02	0.98±0.07	0.58±0.01	75.50±0.75	75.01±1.85	ND	0.89±0.01
8	24	480	20	4.31±0.28	5.09±0.21	0.78±0.00	0.24±0.02	0.92±0.06	1.21±0.00	42.33±0.83	45.45±0.48	ND	3.73±0.01
9	24	480	50	3.58±0.09	3.21±0.07	0.97±0.03	0.16±0.00	0.99±0.03	1.59±0.07	25.60±0.09	18.58±0.36	1.28±0.02	11.28±0.04
10	37	60	10	6.92±0.29	6.05±0.27	1.64±0.10	0.49±0.03	0.74±0.04	0.62±0.02	68.80±1.20	94.99±0.23	ND	0.94±0.00
11	37	60	20	6.12±0.10	4.55±0.22	1.83±0.04	0.29±0.00	0.87±0.03	1.05±0.07	56.51±0.53	39.84±0.35	ND	4.56±0.02
12	37	60	50	4.09±0.15	2.80±0.06	1.74±0.04	0.21±0.01	0.98±0.08	1.56±0.02	27.93±0.19	19.45±0.16	23.78±1.66	11.73±0.26
13	37	120	10	6.26±0.46	7.04±0.41	1.47±0.07	0.60±0.01	0.63±0.02	0.62±0.01	65.25±1.36	77.42±0.83	ND	1.30±0.01
14	37	120	20	5.76±0.35	4.68±0.21	1.76±0.03	0.22±0.02	0.69±0.04	1.02±0.02	48.38±0.34	45.33±0.93	ND	0.67±0.01
15	37	120	50	3.87±0.38	2.95±0.11	1.47±0.04	0.20±0.01	1.03±0.07	1.48±0.02	29.83±0.41	21.27±0.04	15.95±2.45	8.54±0.01
16	37	480	10	1.25±0.01	7.19±0.03	0.39±0.03	0.38±0.03	1.31±0.04	0.79±0.03	37.06±0.76	69.43±0.75	ND	0.85±0.01
17	37	480	20	3.25±0.29	4.37±0.32	0.81±0.05	0.16±0.01	1.06±0.03	1.59±0.11	45.98±0.53	35.95±0.74	ND	3.64±0.00
18	37	480	50	4.09±0.08	2.89±0.15	1.42±0.05	0.11±0.00	0.74±0.04	1.54±0.05	32.58±0.23	13.80±0.15	3.89±0.03	6.28±0.16
19	50	60	10	7.02±0.31	7.52±0.10	1.72±0.05	0.43±0.01	0.54±0.03	0.69±0.02	97.50±1.75	61.26±1.57	ND	1.54±0.00
20	50	60	20	5.76±0.23	5.31±0.04	1.63±0.04	0.32±0.02	0.64±0.01	1.11±0.01	50.67±0.96	40.73±0.35	ND	1.37±0.08
21	50	60	50	3.94±0.08	3.12±0.04	1.28±0.05	0.19±0.00	0.82±0.03	1.79±0.05	31.94±0.21	29.06±0.27	6.00±0.58	2.15±0.12
22	50	120	10	6.69±0.45	7.10±0.15	1.67±0.03	0.45±0.04	0.53±0.03	0.71±0.01	82.02±1.11	73.22±0.70	ND	2.36±0.06
23	50	120	20	5.62±0.16	5.22±0.08	1.06±0.05	0.33±0.01	0.61±0.00	0.93±0.04	47.24±1.97	44.18±0.65	8.77±0.03	1.20±0.00
24	50	120	50	3.63±0.06	3.06±0.04	1.30±0.02	0.19±0.00	0.95±0.07	1.57±0.03	28.88±0.21	22.23±0.25	6.92±0.15	7.96±0.05
25	50	480	10	2.23±0.06	6.65±0.25	0.61±0.01	0.25±0.00	0.88±0.02	1.08±0.03	58.92±1.25	57.17±1.92	ND	2.49±0.01
26	50	480	20	3.71±0.50	5.00±0.24	0.80±0.02	0.30±0.01	1.17±0.03	1.26±0.02	48.66±0.59	43.08±0.97	9.28±0.09	1.69±0.01
27	50	480	50	2.66±0.16	2.95±0.08	0.59±0.01	0.11±0.01	1.52±0.10	2.93±0.11	23.04±0.29	15.35±0.38	10.04±0.16	1.27±0.05
All Runs				4.64±1.65	5.09±1.86	1.24±0.43	0.30±0.14	0.87±0.25	1.18±0.53	49.86±19.9	83.23±0.41	6.47±0.10	4.49±5.55

Table 3.2. Analysis of variance for the response surface quadratic model

	Euphorbia resinifera		***Euphorbia officinarum***	
	F	***p***	**F**	***p***
TPC				
(1) Temperature(L)	5.76	0.019328	14.92	0.00027
Temperature(Q)	0.00	0.96522	36.93	0.00000
(2) Time (min)(L)	516.17	0.000000	0.01	0.92193
Time (min)(Q)	2.73	0.103503	2.97	0.08955
(3) PSR (mg/mL) (L)	63.87	0.000000	2985.93	0.00000
PSR (mg/mL) (Q)	39.86	0.000000	386.37	0.00000
1*2	2.80	0.033211	5.40	0.00085
1*3	5.96	0.000391	20.14	0.00000
2*3	5.76	0.019328	1.28	0.28600
	R-sqr: 0.97**; R-Adj:**0.95		**R-sqr**=0.98**; R^2-Adj:**0.97	
TFC				
(1) Temperature(L)	0.80	0.374588	32.18	0.000000
Temperature (Q)	70.68	0.000000	10.72	0.001740
(2) Time (min)(L)	513.65	0.000000	140.57	0.000000
Time (min)(Q)	0.03	0.863763	19.41	0.000043
(3) Plant %(mg/mL) (L)	21.33	0.000020	1686.63	0.000000
PSR (mg/mL) (Q)	10.47	0.001950	298.86	0.000000
1*2	3.77	0.008333	3.28	0.016738
1*3	19.39	0.000000	50.10	0.000000
2*3	0.80	0.374588	23.57	0.000000
	R-sqr:0.94**; R-Adj:**0.93		**R-sqr**=0.98**; Adj:**0.97	
DPPH				
(1) Temperature(L)	3.44	0.068435	56.1248	0.000000
Temperature(Q)	0.00	0.989553	6.0009	0.017137
(2) Time (min)(L)	40.10	0.000000	33.4127	0.000000
Time (min)(Q)	12.54	0.000763	18.7680	0.000055
(3) PSR (mg/mL) (L)	26.58	0.000003	552.8890	0.000000
PSR (mg/mL) (Q)	0.80	0.374274	19.3065	0.000044
1*2	9.44	0.000005	9.6806	0.000004
1*3	7.89	0.000033	10.6834	0.000001
2*3	6.79	0.000133	2.5006	0.051426
	R-sqr:0.79**; R-Adj**:0.73		**R-sqr**=0.93; **R-Adj**:0.91	
Chelating Power (IC_{50})				
(1) Temperature(L)	1.4829	0.227932	72.0713	0.000000
Temperature(Q)	44.8097	0.000000	0.3672	0.546761
(2) Time (min)(L)	0.5262	0.470956	37.0449	0.000000
Time (min)(Q)	9.6883	0.002804	23.4855	0.000009
(3) PSR (mg/mL) (L)	236.3545	0.000000	176.9106	0.000000
PSR (mg/mL) (Q)	1.0483	0.309872	13.8364	0.000000
1*2	17.8756	0.000000	29.8266	0.000000
1*3	16.8026	0.000000	11.8049	0.000000
2*3	5.6204	0.000632		
	R-sqr=0.89; Adj:.85911		**R-sqr**=0.91; **R-Adj**:0.88	
α - Glucosidase				
(1) Temperature(L)	0.04	0.848560	19.41	0.000043
Temperature(Q)	6.98	0.010444	2.23	0.140751
(2) Time (min)(L)	42.19	0.000000	26.66	0.000003
Time (min)(Q)	6.80	0.011390	8.10	0.006006
(3) PSR(mg/mL)(L)	455.04	0.000000	2886.67	0.000000
PSR(mg/mL)(Q)	24.68	0.000006	388.45	0.000000
1*2	2.12	0.088456	6.68	0.000155
1*3	13.08	0.000000	31.57	0.000000
2*3			9.69	0.000004
	R-sqr:0.92**; R-Adj:**0.90		**R-sqr=.98; Adj:.98**	

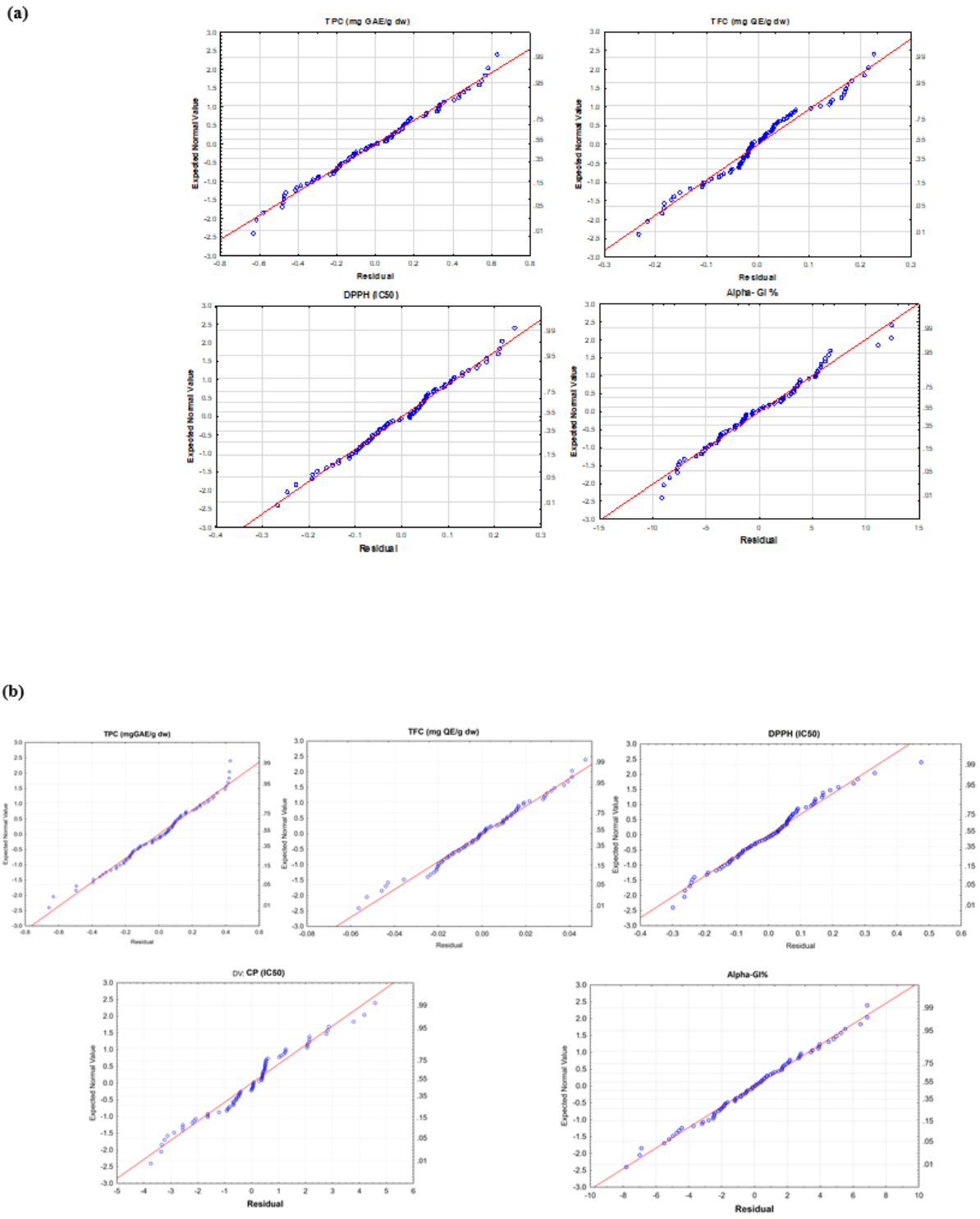


Figure 3.2. Normal probability plot of residuals (TPC, TFC, DPPH, alpha-GI%) for *Euphorbia resinifera* (a) and (TPC, TFC, DPPH, Chelating power CP, alpha-GI%) for *Euphorbia officinarum.*

According to Equation 2, the extraction temperature and time had a linear positive effect on TPC recovery, in contrast to the linear effect of the PSR, which was negative, indicating that more plant material in the extraction medium results in less TPC extraction efficiency. In terms of interactions, we noticed that the process variable interactions had a negative effect on TPC extraction, except the interaction (ET°) $(ET)^2$. With regard to *E. officinarum* (Equation 3), temperature (L) and PSR (L) had a significant negative effect on TPC recovery unlike their quadratic terms, which had a positive effect along with the linear and quadratic interactions temperature–time and temperature–PSR (L), whereas the rest of the coefficients did not show any significant effect.

To investigate the linear, quadratic, and interactive effects of the process variables on the total phenolic compound's extraction from the two species, the three-dimensional surface plots (Figure 3.3: a, b, c, d, e, and f) were constructed according to Equations 2 and 3. The color changing from green to red indicates an increase in the response.

The plot exhibiting the function of time versus PSR effect on the extract TPCs at a fixed temperature is depicted in Figure 3.3(a). It was noticed that TPC levels decrease sharply with the increase of PSR as well as with the increase of extraction time. This effect is more pronounced when the two factors increase simultaneously. The best levels of TPC (>7 mg GAE/g dry plant) can be achieved using a lower PSR at the shortest extraction time, during a maximum period of 1 hour. At high PSR, the extraction time did not have a significant effect and vice versa. As for *E. officinarum*, the increase in PSR during extraction decreased highly TPC recovery, without the noteworthy effect of temperature, Figure 3d.

Regarding the time and temperature of the extraction effect on *E. resinifera*, as shown in Figure 3b, the TPC values decrease with increasing extraction time (from >7 mg/g dry plant at T = 0 min to <1.25 g of a dry plant at T = 500 min) without any remarkable effect of temperature.

The plot presenting the interaction effect between extraction T° and PSR for *E. resinifera* (Figure 3.3c) revealed that the maximum of phenol extracted (>5 mg GAE/g dw) was found using PSR between 30 to 35 mg/mL and a temperature between 22 and 28. C. Regardless of the extraction temperature, TPC recovery first increases with the rise of PSR and starts to decrease at PSR higher than 35 mg/100 mL. However, the TPC recovery for *E. officinarum* was found to increase along with the decrease in PSR values (Figure 3.3e).

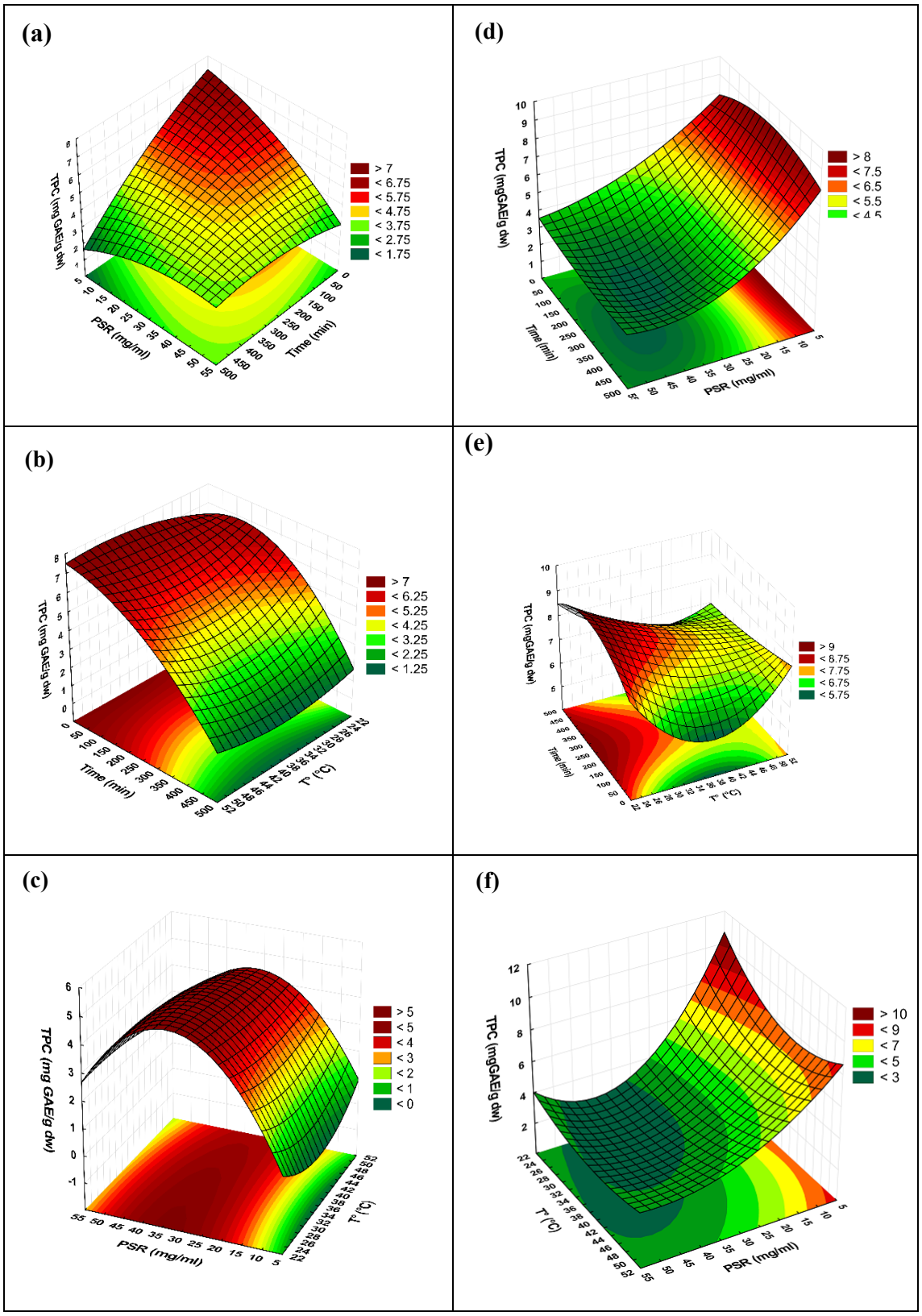


Figure 3.3. Three-Dimensional reponse surface plot showing the combined effects of time (min), temperature Tº (C), and plant solvent ratio (PSR) on total phenol content (TPC) in *Euphorbia resinifera* (a-c) and *Euphorbia officinarum* (d-f) extracts

3.3. Effect of the process variables on the total flavonoids content (TFC)

The TFCs of the aqueous extracts from the two species are listed in Table 1. The maximum TFC yield (1.83 ± 0.04 mg QE/g dw) was obtained in run n° 11, with the following extraction conditions: temperature 37°C, plant-to-solvent ratio 20 mg/mL, 60 min (Table 1). According to Table 3.2, the most significant factors presenting the lowest *P* values, influencing TFC extraction for *E. resinifera*, were temperature (Q), time (L), and the interaction of PSR with both temperature and time (P-value < 0.000001), followed by the linear and quadratic effects of PSR and the interaction time–temperature, whereas the linear effect of temperature and the quadratic effect of time in addition to the interaction time–PSR were not significant (P > 0.05). With respect to *E. officinarum*, we noticed that all factors and their interactions were significant, emphasizing that time (Q), temperature (Q), and PSR (L + Q), besides the interactions of PSR with temperature and time, were highly significant ($p < 0.000001$) (Table 3.2).

Applying RSM, the regression equation (Equation 4 and Equation5) for TFC, after elimination of the non-significant coefficients, is presented as

TFC (mg QE/g dw) *Euphorbia resinifera* $= -1.2 + 5.1\ 10^{-2}(ET°) + 4.7*10^{-2}\ (ET) - 8.6*10^{-5}\ (ET)^2 - 1.8*10^{-3}\ (ET°)\ (ET) + 3.0*10^{-6}\ (ET°)\ (ET)^2 + 2.1*10^{-5}\ (ET°)^2(ET) - 3.5*10^{-8}\ (ET°)^2(ET)^2 + 1.2*10^{-2}\ (ET°)\ (PSR) - 1.4*10^{-4}\ (ET°)\ (PSR)^2 - 2.0*10^{-4}\ (ET°)^2(PSR) + 2.3*10^{-6}\ (ET°)^2(PSR)^2 - 1.4*10^{-3}\ (ET)(PSR) + 2.24*10^{-5}\ (ET)(PSR)^2 + 2.64*10^{-6}\ (ET)^2(PSR) - 4.17*10^{-8}\ (ET)^2(PSR)^2$ (Equation 4)

TFC (mg QE/g dw) *Euphorbia officinarum* $= -0.975 + 0.104\ (ET°) - 1.59*10^{-3}\ (ET°)^2 + 0.009\ (ET) - 1.64*10^{-5}\ (ET)^2 + 0.105\ (PSR) - 1.80*10^{-3}\ (PSR)^2 - 8.49*10^{-3}\ (ET°)\ (PSR) + 1.37*10^{-4}\ (ET°)\ (PSR)^2 + 1.32*10^{-4}\ (ET°)^2(PSR) - 2.10*10^{-6}\ (ET°)^2(PSR)^2 - 3.15*10^{-4}\ (ET)(PSR) + 4.45*10^{-6}\ (ET)(PSR)^2 + 6.36*10^{-7}\ (ET)^2(PSR) - 9.00*10^{-9}\ (ET)^2(PSR)^2$ (Equation. 5)

Where; (ET°): Extraction temperature; (ET): Extraction time (min); (PSR): plant/solvent ratio (mg/mL).

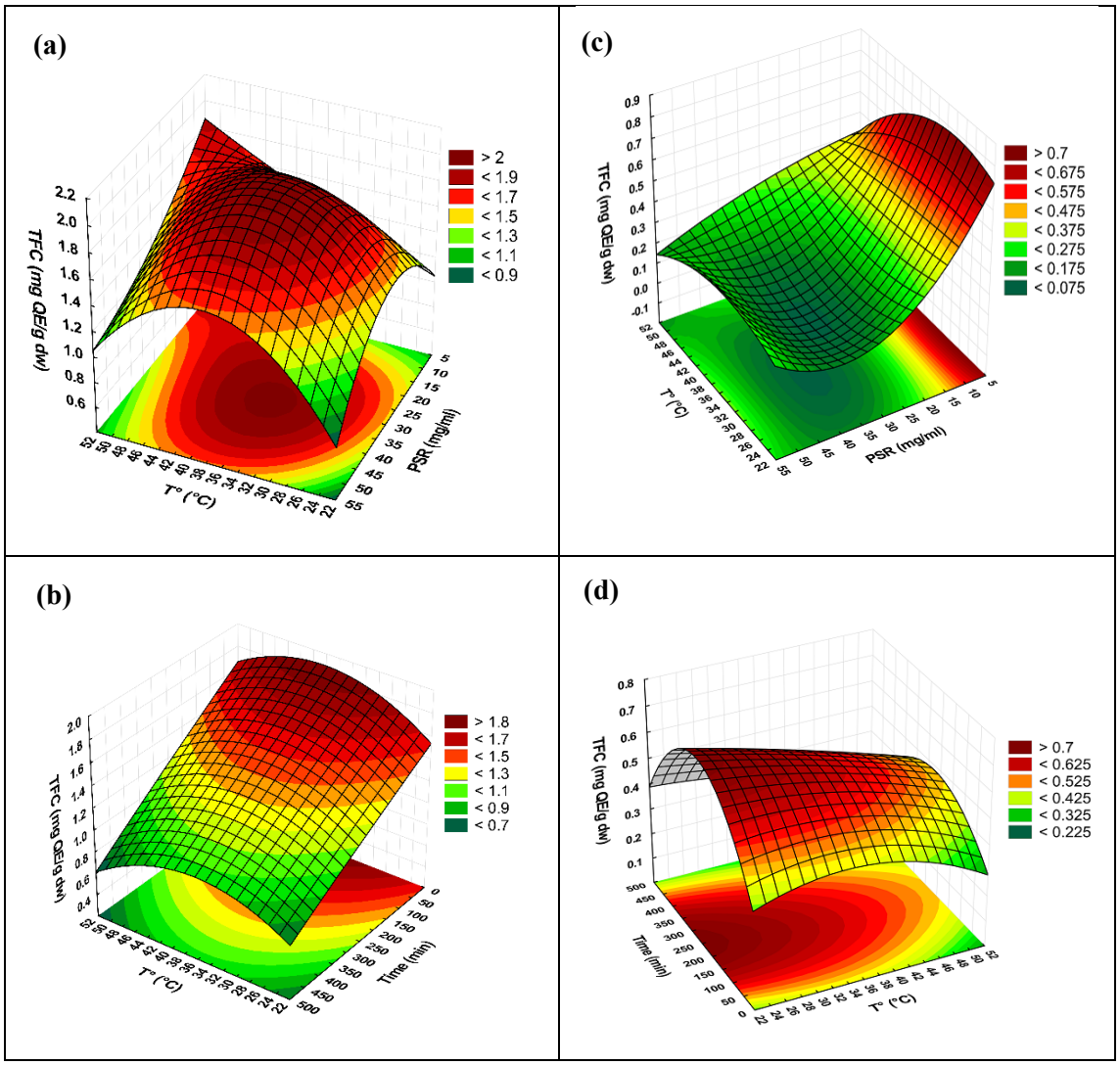


Figure 3.4. Three-Dimensional reponse surface plot showing the combined effects of time (min), temperature Tº (C), and plant solvent ratio (PSR) on total flavonoid concentration (TFC) in the *Euphorbia resinifera* (a and b) and *Euphorbia officinarum* (c and d) extracts.

According to Equation 4, the linear effect of temperature (ET°) and time (ET) of extraction in addition to the interactions $(ET°)^2$, (ET°) $(ET)^2$, $(ET°)^2(ET)$, (ET°) (PSR), $(ET°)^2(PSR)^2$, and $(ET)(PSR)^2$ had a significant positive effect on the yield of flavonoids from *E. resinifera*, indicating that their increase increases the TFC recovery. However, the rest of the interaction had an antagonistic effect. The linear effect of time was positive, whereas its quadratic effect was negative, indicating that the increase of extraction time leads to TFC increase to reach an optimum in the first hour of extraction, after which the recovery of TFC starts to decline.

Concerning *E. officinarum* (Equation 5), we noticed that the linear effect of the three extraction factors exhibited the highest positive influence on TFC extraction, whereas their quadratic effects

had a negative effect. Correspondingly, time was reported to have a negative quadratic effect because the TFC yield increased for the first 76 min and then decreased. The three-dimensional surface plots were designed to ascertain the effect of temperature and the percentage of the plant (PSR) on TFC recovery (Figure 4a and b). The results indicate that the content of flavonoids found in the aqueous extracts of *E. resinifera* increased slightly with the temperature rise from 0.9 mg QE/g in T = 24°C to >1.5 mg QE/g in T = 37°C; after that, the rate starts to decrease slightly until <1.1 5 mg QE/g for T = 50°C. On the other hand, we can see that PSR equal to 30 mg/mL of a plant used in the extraction records the greatest content on flavonoids (>1.5 mg QE/g), and thereafter, the rate begins to decrease with the increase in the PSR (<1 mg QE/g), Figure 3a. The combined effect of time and temperature of extraction revealed a significant negative effect of extending extraction time over 60 min, which results in a decrease of TFC level with the continuous increase of extraction time, regardless of the temperature used for extraction, and this was more pronounced at the low PSR. As shown by the same figures, the highest TFC levels are obtained at 38°C using a PSR ranging From 25 to 35 mg/mL, whereas their contents decreased at higher and lower temperatures. Likewise, increasing PSR from 10 to 30 mg/mL improved TFC recovery, but over 35 mg/mL, the recovery starts to diminish. As for *E. officinarum*, the yield of TFC increased with the extent of extraction time to reach its maximum after 3 hr; thereafter, it starts to decrease (Figure 3.4c). However, their yield decreased with the increase of extraction temperature and PSR (Figure 3.4d).

3.4. DPPH scavenging activity

The total phenolic content along with the antioxidant capacity is a useful tool in determining the potential of an extract for its application in functional foods, cosmetics, nutraceuticals or any other field. The free radical scavenging capacity from all *E. resinifera* and *E. officinarum* extracts increased in a concentration dependent manner (data not shown) and the extract concentration providing 50% scavenging (IC_{50}) are listed in Table 3.1. The results show that *E. resinifera* extracts exhibited good antioxidant activity (IC_{50} = < 0.3 mg/mL).

According to ANOVA analysis (Table 3.2), the linear effect of temperature, besides the quadratic effect of both temperature and PSR did not show any significant effect ($p > 0.05$), although the remaining of the parameter and their interactions had a significant effect, noting that time (L), was the most significant in terms of antioxidants recovery from *E. resinifera* ($P < 0.000001$). In the case of *E. officinarum*, all the coefficients were significant, drawing attention that the linear effects and the interaction time-PSR were the most significant ($p < 0.000001$), followed by the interaction temperature-time and the quadratic effects of the three extraction factors.

The second-order equations for DPPH scavenging activity after elimination the non-significant coefficients were shown in Equations 6 and 7.

DPPH scavenging activity (IC_{50}) *Euphorbia resinifera* $= -2.3*10^{-2}$ (ET°) $- 2.7*10^{-2}$ (ET) $+ 2.68*10^{-5}$ $(ET)^2 + 1.7*10^{-1}$ (PSR) $+ 1.6*10^{-3}$ (ET°) (ET) $- 3.0*10^{-6}$ (ET°) $(ET)^2 - 1.9*10^{-5}$ $(ET°)^2(ET) + 3.5*10^{-8}$ $(ET°)^2(ET)^2 - 9.3*10^{-4}$ (ET)(PSR) $+ 1.7*10^{-5}$ (ET)$(PSR)^2 + 1.5*10^{-6}$ $(ET)^2$(PSR) $- 2.8*10^{-8}$ $(ET)^2(PSR)^2$ (Equation 6).

DPPH scavenging activity (IC_{50}) *Euphorbia officinarum* $= 3.18*10^{-3}$ (ET°) $- 3.09*10^{-4}$ (ET°) $(PSR)^2 -$ $2.31*10^{-4}$ $(ET°)^2$(PSR) $+ 4.65*10^{-6}$ $(ET°)^2(PSR)^2$ (Equation 7).

Where; (ET°): Extraction temperature; (ET): Extraction time (min); (PSR): plant/solvent ratio (mg/mL).

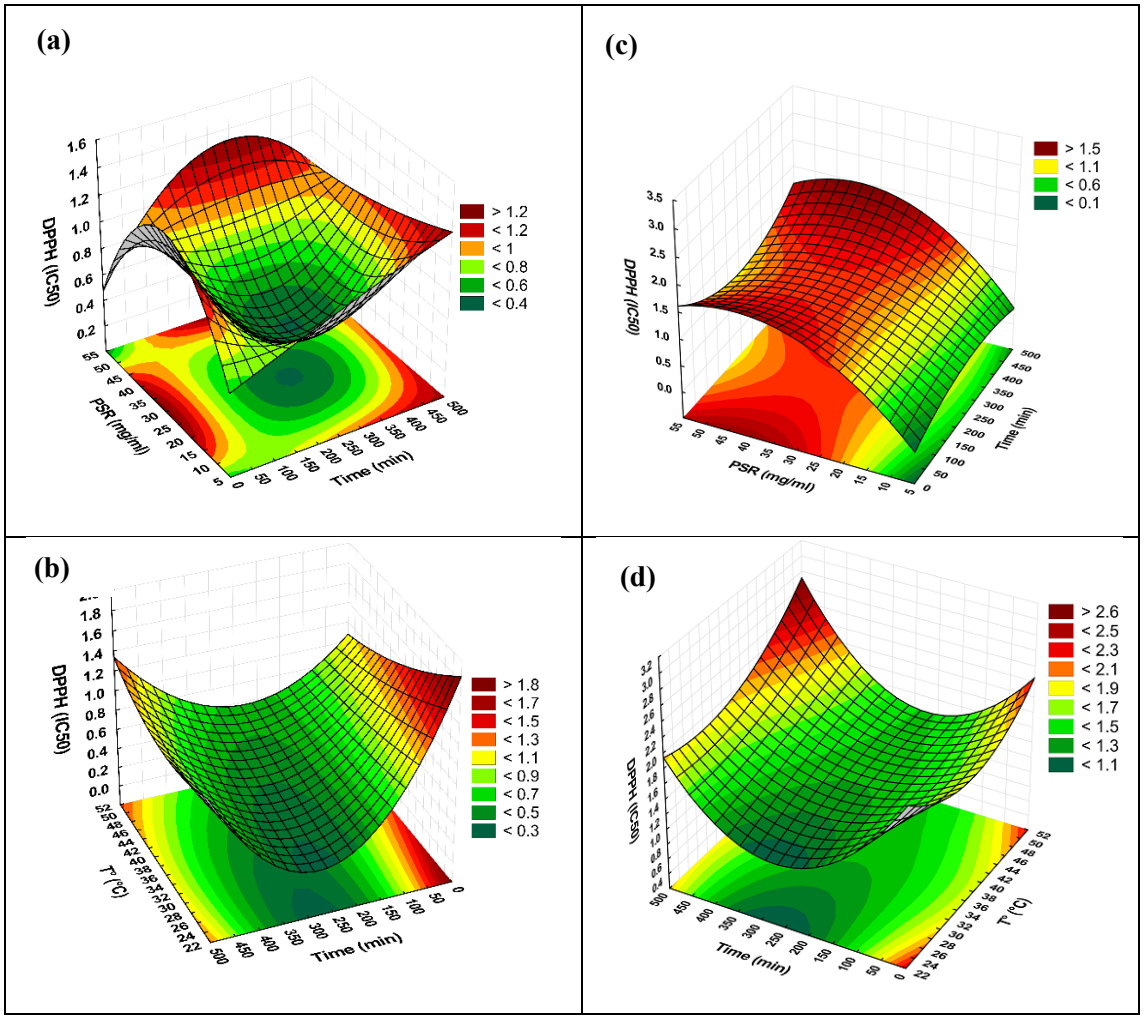


Figure 3.5. Three-Dimensional reponse surface plot showing the combined effects of time (min), temperature Tº (C), and plant solvent ratio (PSR) on the ability for scavenging DPPH free radicals mesured though IC_{50} values of the extracts of *Euphorbia resinifera*(a and b) and *Euphorbia officinarum* (c and d).

Related to the Equation 6, expressing the relationship between antioxidant activity, expressed as IC_{50} values, and the extraction conditions, we found that the IC_{50} values were negatively influenced by the linear and quadratic effects of extraction temperature, indicating that lower extraction temperatures resulted in decreased IC_{50} values thus higher antioxidant activity.

Concerning the extraction time, the linear effect was negative while the quadratic effect was positive, which implies that increasing extraction time till certain levels rises the antioxidant activity, but increasing it too much has an inverse effect, by lowering the recovery of molecules endowed antioxidant activity in the yielded extracts. Furthermore, PSR have a positive effect and increases the IC_{50} of the extracts indicating that lower PSR are suitable for extraction antioxidants from this plant. It is noteworthy to state that the most important effect on increasing antioxidant extraction was the linear effect of extraction time, whereas the linear effect of PSR, mostly decreased their extraction. As for *E. officinarum*, the quadratic effect of temperature and its interaction with the quadratic effect of PSR affected positively the antioxidant activity the yielded extracts, unlike the interactions T° (L) – PSR (Q) and T° (Q) – PSR (L) which had a negative impact, pointing out that the rest of the factors and their interactions did not have any significant effect. The surface plots (Figures 3.5a and b), indicate that both the extraction temperature and PSR along with extraction time, have an influence on the extraction of antioxidant compounds from *E. resinifera*. At lower levels of PSR (below 30 mg/mL) and extraction times (below 300 min), the ability to eliminate free radicals by *E. resinifera* aqueous extracts increases with the increase of the two factors, conversely, those factors start to have a negative impact on extracting antioxidants when they exceed those levels (Figure 3.5a), whereas extraction temperature had a negative effect regardless the other factors, because over time and with increasing temperature the antioxidant activity decreases (IC_{50} = >1.8 mg/mL) (Figure 3.5b). The best antioxidant activity of *E. resinifera* extract has been recorded for extraction times ranging from 200 to 350 min, using a PSR ranging from 25 to 35 mg/mL at low temperatures. According to previous reported results, the amount of the sample added in the solvent exerted the important effects on the extraction of antioxidants and beyond the optimal value of sample to solvent ratio of 28 mg/mL, the total the antioxidant activity of the extracts decreased progressively.

In relation to *E. officinarum*, the IC_{50} values increases with the increase of PSR (Figure 3.5c), indicating that high PSR result in low antioxidants yields. The extraction time exerted a positive effect during the first 3 hours of extraction, afterwards the antioxidant activity starts to diminish (Fig. 3.5d).

3.5. α-Glucosidase inhibition activity

The α-glucosidase inhibitory activity results of water extracts are depicted in Table 3.1. Run 19 (T° 50°C; time 60 min and plant ratio 10%) showed the greatest α-glucosidase inhibitory activity. The most efficient extracts to inhibit this enzyme were obtained using a PSR concentration below 15 mg/mL regardless of the temperature and the time of extraction. The second-order polynomial equation stating the relationship between extraction conditions and α-glucosidase inhibitor recovery is given by the Equations 8 and 9.

α-Glucosidase inhibition (%) *Euphorbia resinifera* $= 482.93 - 2.29\ (ET°) + 0.32\ (ET°)^2 - 23.18\ (PSR) - 0.30\ (PSR)^2 + 1.29\ (ET°)\ (PSR) - 1.79*10^{-2}\ (ET°)\ (PSR)^2 - 1.88*10^{-2}\ (ET°)^2(PSR) + 2.62*10^{-4}\ (ET°)^2(PSR)^2$

Equation 8

α-Glucosidase inhibition (%) *Euphorbia officinarum* $= 15.020\ (ET°) - 0.223(ET°)^2 + 1.466\ (ET) - 0.002\ (ET)^2 - 0.088\ (ET°)\ (ET) + 1.46*10^{-4}\ (ET°)(ET)^2 + 1.15*10^{-3}\ (ET°)^2(ET) - 1.89*10^{-6}\ (ET°)^2(ET)^2 - 0.623\ (ET°)(PSR) + 9.00*10^{-3}\ (ET°)(PSR)^2 + 1.01*10^{-2}\ (ET°)^2(PSR) - 1.44*10^{-4}\ (ET°)^2(PSR)^2$

Equation 9

Where; (ETº): Extraction temperature; (ET): Extraction time (min); (PSR): plant/solvent ratio (mg/mL).

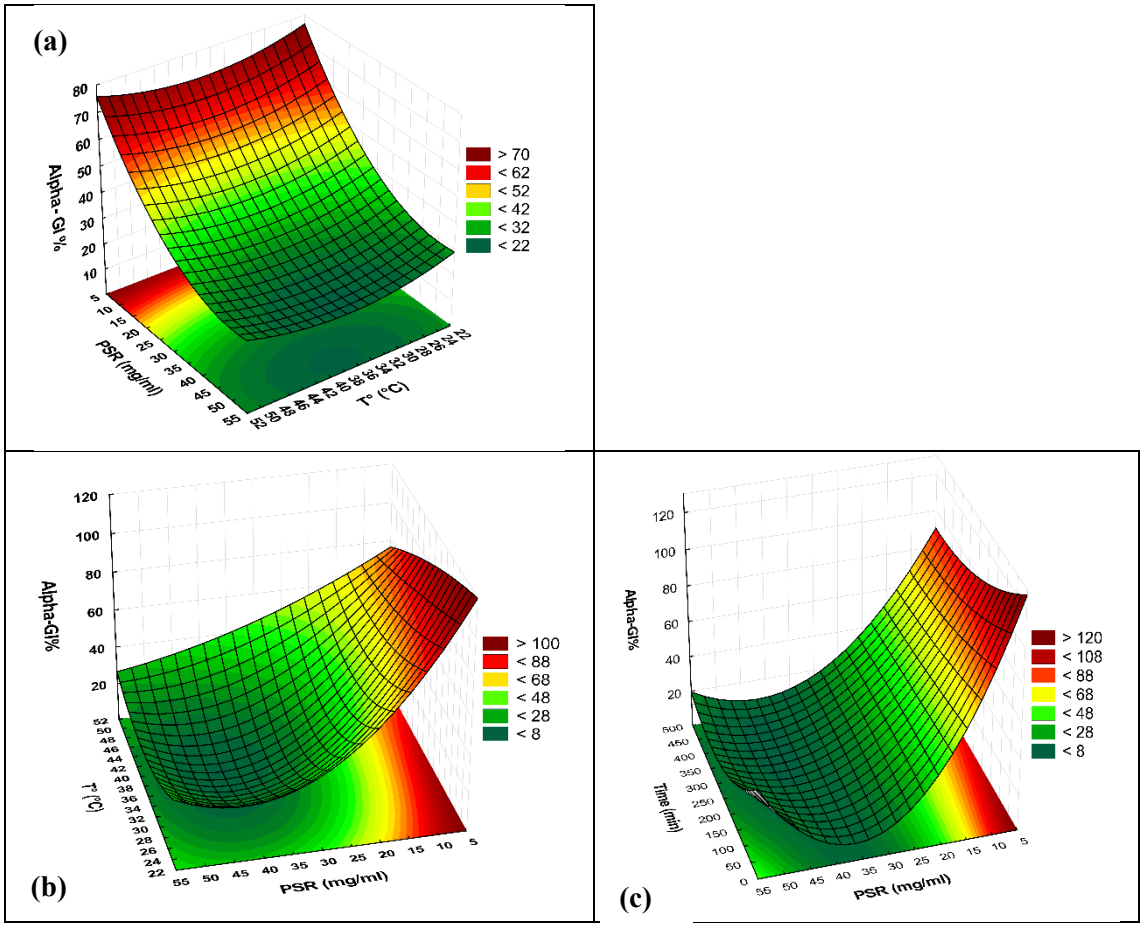


Figure 3.6. Three-Dimensional reponse surface plot showing the combined effects of time (min), temperature Tº (C), and plant solvent ratio (PSR) on alpha-Glucosidase inhibition percentage (alpha-Gl%) by the extracts of *Euphorbia resinifera* (a) and *Euphorbia officinarum* (b and c).

All the presented coefficients had a significant effect ($p < 0.05$) on extracting molecules with α-glucosidase inhibitory activity. Those molecules in the yielded extracts were negatively influenced by extraction temperature and PSR, whereas their linear and quadratic interaction resulted in a positive effect, indicating a synergetic effect between the two factors. The highest negative effect was expressed by PSR. Thus, increasing PSR resulted in a great decrease in extraction efficiency of molecules inhibiting the α-glucosidase enzyme (Figures 3.6a-c). However, the linear effect of temperature and time increased the yield of anti-diabetic molecules in the case of *E. officinarum* (Equation 9). The α-glucosidase inhibition capacity decreased

strongly with the enhancement of the PSR in *E. officinarum* extracts despite the time and temperature of extraction (Figure 3.6b and c).

With regard to the plot (Figure 3.6a), depicting the combined effect of PSR and extraction temperature, the anti-diabetic activity of the *E. resinifera* aqueous extracts strongly decreased from >80 (%) to <20 (%) as the PSR used during extraction increased from 10 to 50 mg/mL. The temperature of extraction had a weak significant effect, whereas the extraction time did not have any significant effect ($p < 0.05$).

3.6. Chelating power

This assay was performed to evaluate the chelating capacity of the yielded extracts from the two Euphorbia species, and the second-order polynomial equation stating the relationship between extraction conditions and α-glucosidase inhibitor recovery is given by Equations 10 and 11.

Chelating power (IC_{50}) *Euphorbia resinifera* $= +2.4\ (ET°)\ (PSR) - 4.5*10^{-2}\ (ET°)\ (PSR)^2 - 3.8*10^{-2}\ (ET°)^2\ (PSR) + 6.9*10^{-2}\ (ET°)^2\ (PSR)\ 2 - 0.15\ (ET)(PSR) + 2.3*10^{-3}\ (ET)(PSR)^2 + 2.5*10^{-4}\ (ET)^2\ (PSR) - 4.1\ 10^{-6}\ (ET)^2\ (PSR)^2$ (Equation 10)

Chelating power (IC_{50}) *Euphorbia officinarum* $= 1.41*10^{-2}\ (ET)(PSR) + 2.58*10^{-5}\ (ET)^2(PSR) - 3.47*10^{-7}\ (ET)^2(PSR)^2$ (Equation 11)

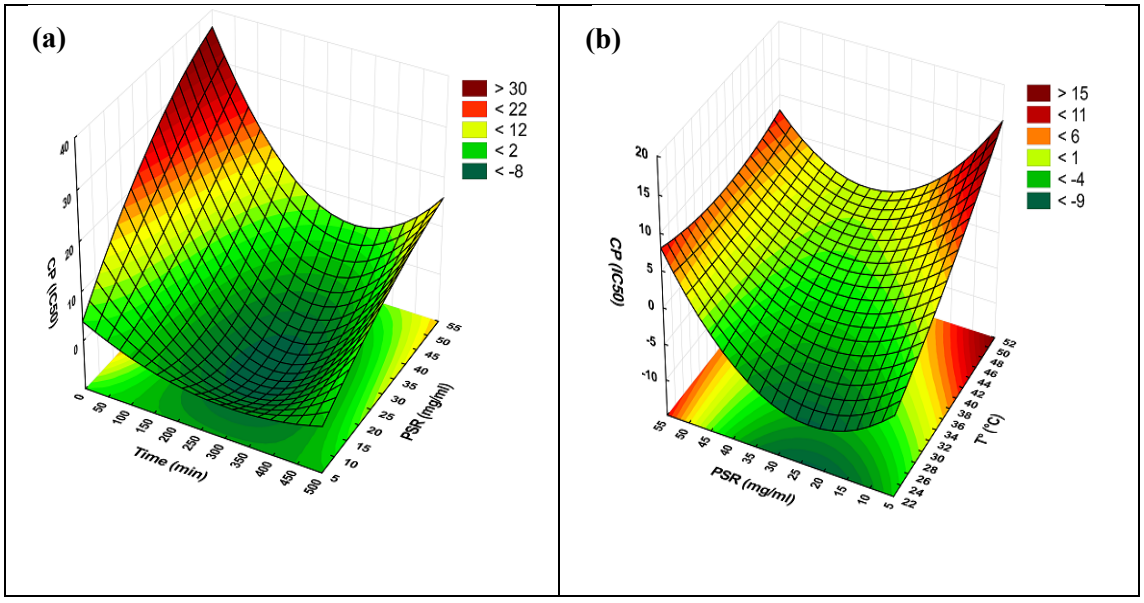
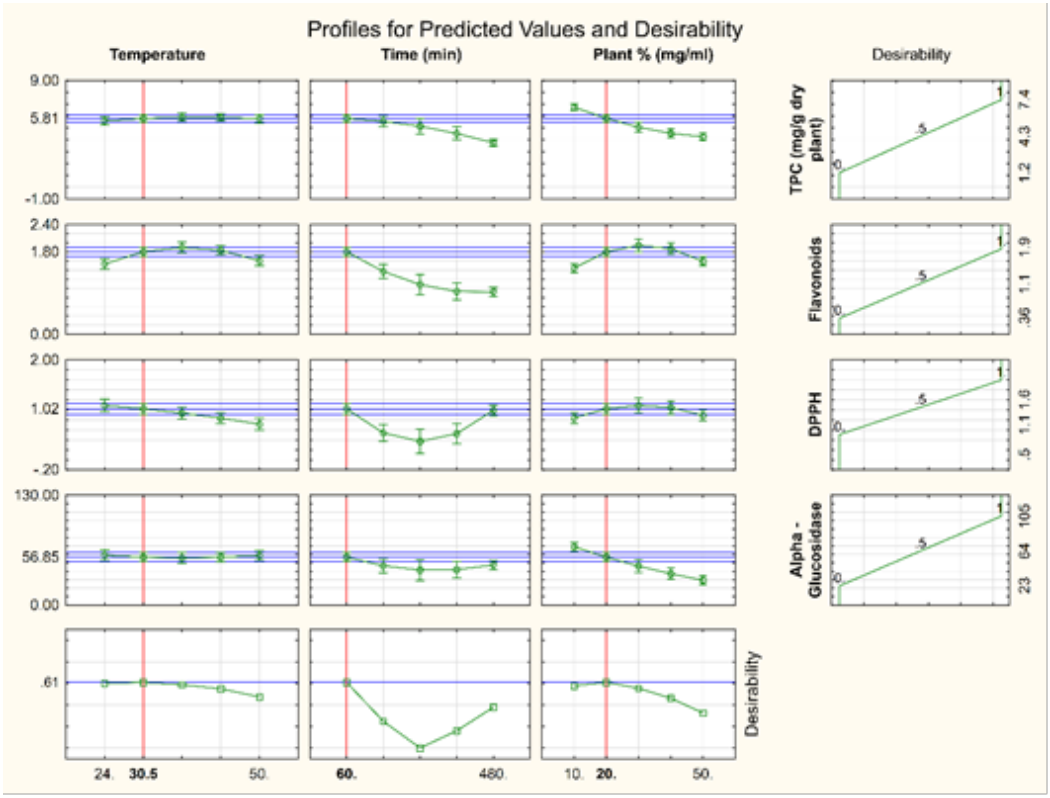


Figure 3.7. Three-dimensional response surface plots showing the effect of time (min) and plant-to-solvent ratio (PSR) on the chelating power activity for *Euphorbia officinarum* (a); Effect of temperature T° (°C) and PSR on the chelating power activity for *Euphorbia officinarum* (b).

According to Equation 10, the linear and the quadratic interactions between extraction time and PSR as well as the interactions (ET) $(PSR)^2$ and (ET)2 (PSR) had a significant positive consequence on the extraction of molecules endowed chelating power, whereas the rest of the interactions decreased their extraction from *E. resinifera*. With respect to *E. officinarum* (Equation 11), the linear and the quadratic interactions between extraction time and PSR decreased the chelating power of the yielded extract, whereas the interaction $(ET)^2(PSR)$ enhanced their chelating capacity. Figure 3.7a shows the surface plots with the chelating power from *E. resinifera* as a function of time and PSR. The chelating power of the extracts increased with extraction time to reach its maximum after 3 hr; after that, it begins to decrease. The same behavior has been observed with the PSR, in which an increase is observed when the concentration goes from 5 to 10 mg/mL; after that, it begins to decrease gradually. With respect to *E. officinarum* (Figure 3.7b), the increase in temperature extraction corresponds to a reduced chelating power of the extracts, whereas the best PSR concentration was found to be between 15 and 30 mg/mL.

3.7. Desirability

Optimization using the desirability function was performed in order to maximize the total phenolic compounds and also to maximize the antioxidant capacity (DPPH) and α-glucosidase inhibitory activity of the extracts. The results are presented in Figure 3.8 (a) and (b) for *E. resinifera* and *E. officinarum*, respectively. The result for the simultaneous optimization including all responses using the desirability function suggested that aqueous extraction of *E. resinifera* dried aerial part powder during 60 min at 30°C using a plant-to-solvent ratio of 2% extracts yielded extracts with the high TPC and TFC contents along with high antioxidant and α-glucosidase inhibitory activities. However, extraction during 270 min at 50°C using a PSR concentration of 10 mg/mL (1%) was the best condition to yield the extract with the optimum values for all the studied responses from *E. officinarum*.



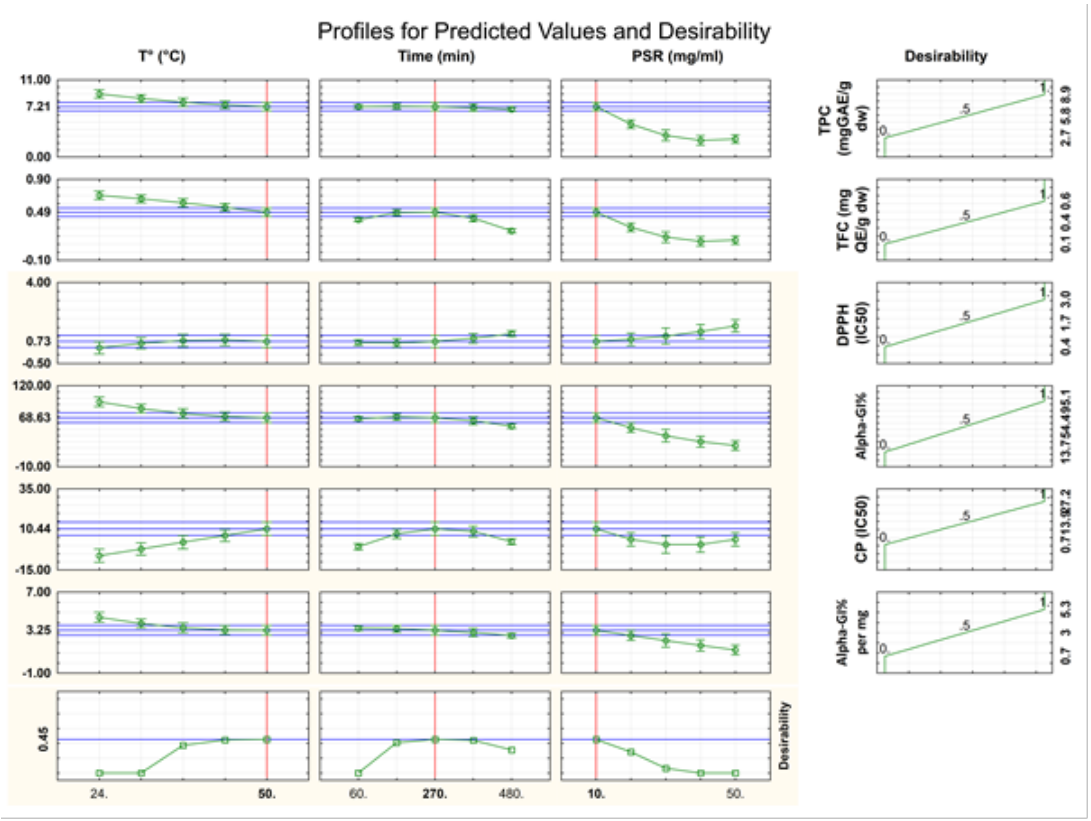


Figure 3.8. Best experimental parameters (Temperature, time, and plant (%)) using the desirability function that maximize phenol and flavonoid contents and anti-oxidant and alpha-glucosidase inhibitory activities of (a) *Euphorbia resinifera* and (b) *Euphorbia officinarum*

3.8. Principal component analysis (PCA)

Plotting PCA scores in two or three dimensions provides an effortless way to observe the data distribution. The first three PCs explained 88.07 % of the variance in the data for *E. resinifera* and 90.4 for *E. officinarum*, which was high enough to represent all the variables. In the PC1-PC2 plot (Figure 3.9a), the first two PCs contain about 73.75 % (PC1: 49.78 %, PC2: 23.97 %) information of the raw data, whereas the third component (PC3) explained 14.32 % of the total variance, while the PC1-PC2 plot (Figure 3.9c), of *E. officinarum* explained 77.6 % of the total variance.

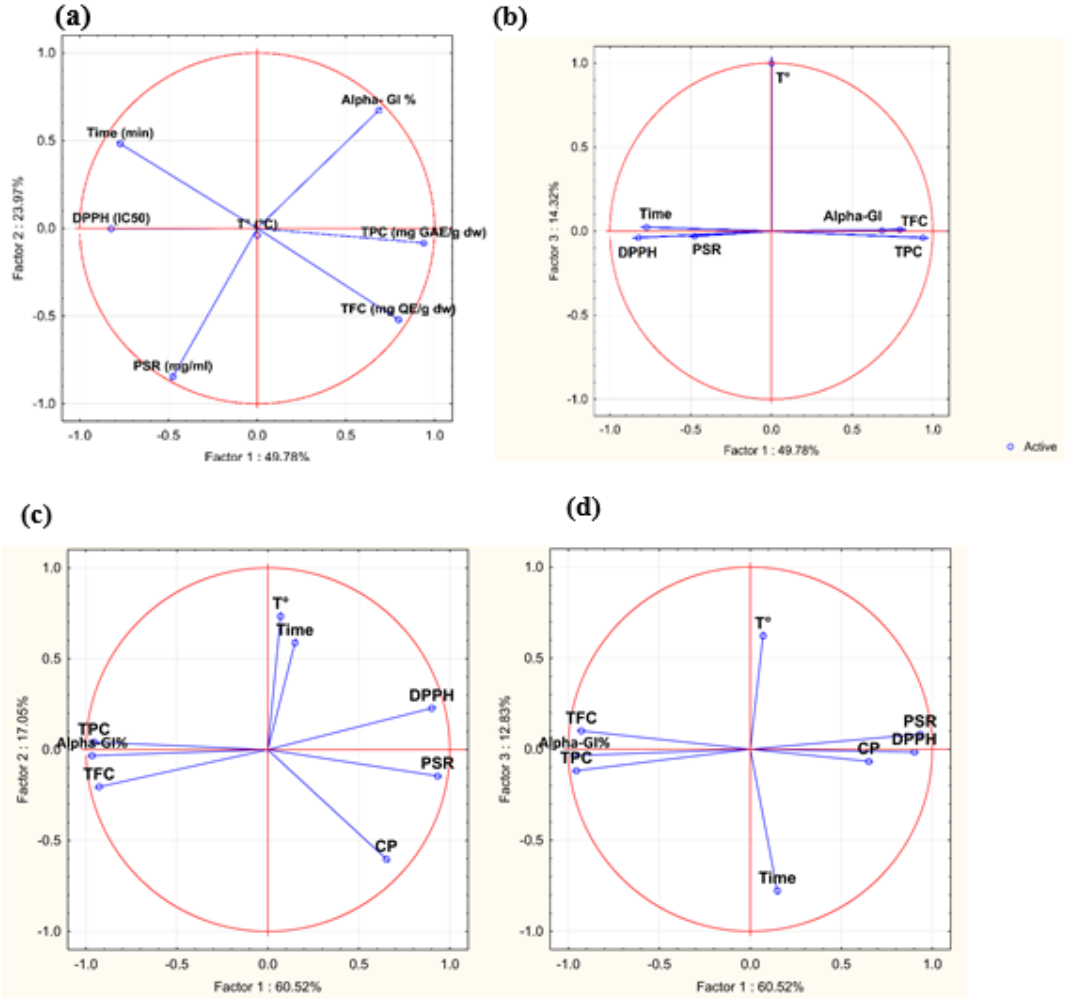


Figure 3.9. Principal component analysis: (a and b) represent *Euphorbia resinifera*; (c and d) represent *Euphorbia officinarum*. (a) and (c) Plot using the first and second components; (b) and (d) Plot using the first and third components.

Related to Figures 3.9 (a) and (b) corresponding to *E. resinifera*, in PC1, TPC has a heavy positive loading, while DPPH has negative loading, whereas Time, PSR, TFC and α-glucosidase have a load in both axes. Meanwhile, extraction temperature did not present any load for the two axes. This plot clearly shows a strong correlation between DPPH and TPC, indicating their high contribution to the antioxidant activity of the extracts. TFC recovery was negatively affected by extraction time, while α-glucosidase inhibition activity was negatively affected by PSR. According to the Figure 3.9b, showing the PC1-PC3 plot, all the factors were strongly loaded on the PC1 except extraction temperature which was highly loaded on PC3. The antioxidant and α-glucosidase inhibitions activities along with TPC and TFC recovery were negatively influenced by both time and PSR. As for *E. officinarum* (Figures 3.7 b and c), TPC, TFC and α-glucosidase inhibition and DPPH scavenging activity were positively correlated and simultaneously were negatively correlated to PSR.

4. Discussion

Secondary metabolites with antioxidant activity can also act as anti-inflammatory agents because the inflammatory response is an oxidative burst that can occur in monocytes, neutrophils, eosinophils, and macrophages (15). For this reason, the search of these bioactive natural compounds can be considered so important. Excess metal ions can contribute to oxidative damage in some neuro-degenerative disorders such as Alzheimer's and Parkinson's diseases. Moreover, the formation of ROS can be triggered by the presence of metal ions; thereby, the reduction of their formation can be achieved by using adequate chelating agents (16). The development of the α-glucosidase inhibitors can be a new approachin the handling of diabetes (17). Inhibition of intestinal α-glucosidase delays the digestion of starch and sucrose, therefore reducing the post-prandial blood glucose, and consequently mimics the effects of dieting on hyperglycemia (18). *E. resinifera* leaf stem decoction (3) or one drop of latex in a glass of water once a day (4) has been used orally to treat diabetes in Morocco. However, this species is among the medicinal plants from Morocco that have not been explored experimentally for anti-diabetic activity (7).

The determination coefficient (R^2), which was defined as the ratio of the explained variation to the total variation, was a measure of the degree of fit (19). The empirical model fits the actual data in a better way when the R^2 value is closer to unity. This coefficient was used to check the robustness of the fit of the model. Normal probability plots of the residuals were used for checking the adequacy of the model, and the results are presented in Table 3.2. ANOVA must statistically satisfy the fundamental assumption of the experience, in which the legitimacy of the model was diagnosed using residual plots. Thus, the adequacy of the model was also evaluated with the help of the residuals. The straight line in the normal plot of the residuals means a normal distribution of the errors and adequacy of the constructed model (20).

According to Equation 2, the extraction temperature and time had a linear positive effect on TPC recovery, in contrast to the linear effect of the PSR, which was negative, indicating that more plant material in the extraction medium results in less TPC extraction efficiency. Likewise, Pinelo et al. (21) obtained the highest phenolic concentration and anti-radical activity by increasing the solvent-to-solid ratio, that is, a lower PSR. A higher PSR leads to a decrease of the phenol extraction as well as of the biological activities, which is expected because it is consistent with mass-transfer principles. According to those authors, the concentration gradient between the solid and the bulk of the liquid is the driving forcé during mass tranfer, which is greater when a higher solvent-to-solid ratio is used. Similarly, Cheok et al. (22) noticed an increase in the TPC yield as the solid-to-solvent ratio decreases.

The significant influence of extraction time on total polyphenol content was reported previously for black tea (23). Upadhya et al. (24) reported that 5 min was found as being the most adequate for TPC and antioxidant activities in continuous shaking extraction from different parts of *Achyranthes aspera*, whereas 1 hour of extraction time was sufficient for the extraction of phenolic compounds from mangosteen hull poder (22). Divergent results related to the temperature effect during extraction have been reported, even though the increase of extraction temperature tends to improve extraction (21). In fact, the temperature has a positive effect on the extraction of phenols and, consequently, on the antioxidant activity. According to the results obtained, temperature and time are two factors essential to have better phenol yields and antioxidant activity through scavenging the DPPH free radicals or to inhibit the α-glucosidase activity. The temperature improved the extraction at short periods, but for relatively long periods, the effect was the opposite. Similar results were reported by Yim et al. (25) in the aqueous extracts of *Schizophyllum commune*. According to these authors, better diffusion coefficients of polyphenols were observed with increased temperatures allowing higher extraction yields; nevertheless, above a limit, the inverse occurs, which was explained by the decomposition of thermo-sensistive compounds. This decomposition is also responsible for the loss of antioxidant activity for higher-temperature extraction, which is accentuated when the time of extraction is prolonged.

Flavonoids' extraction has been reported to be influenced by many factors including time, temperature, solid–liquid ratio, and extraction cycle (26). Concerning *E. officinarum*, we noticed that the linear effect of the three extraction factors exhibited the highest positive influence on TFC extraction, whereas their quadratic effects had a negative effect. Time was reported to have a negative quadratic effect because the TFC yield increased for the first 76 min and then decreased. A possible explanation can include a decomposition phenomenon with a relatively extended extraction time (27) and is already reported for TPC. Because total phenols including flavonoids

have an important role in the antioxidant activity and inhibition of α-glucosidase, the parameters that influence the extraction also have repercussions on the biological activities found.

5. Conclusion

The present study established that RSM was effective tool for optimizing the extraction conditions of *E. resinifera* and *E. officinarum* aerial parts and allows a better understanding of the relationship between independent variables and response variables. Results reported a decrease in extraction efficiency with the increase of the plant ratio. The best extraction temperature was between 30 and 35°C. Extraction using PSR of 20 mg/mL during one hour at 30°C, yielded extracts with optimal phenolic content and optimal values of the studied activities for *E. resinifera*. Whereas, extraction during 270 min, at 50°C, using PSR of 10 mg/mL, where the best extraction conditions to yield extracts with optimum values of the studied responses for *E. officinarum*.

Acknowledgements

The authors acknowledge Prof. Manuela David, curator of ALGU Herbarium, in supervising tasks involving the identification, preparationand deposit of the voucher specimens. The authors also acknowledge Mr.Jawad Elamrani, beekeeper that provided plant samples for this study.

Financial support and sponsorship

The authors would like also to acknowledge the support granted from FCT under the project UIDB/05183/2020.

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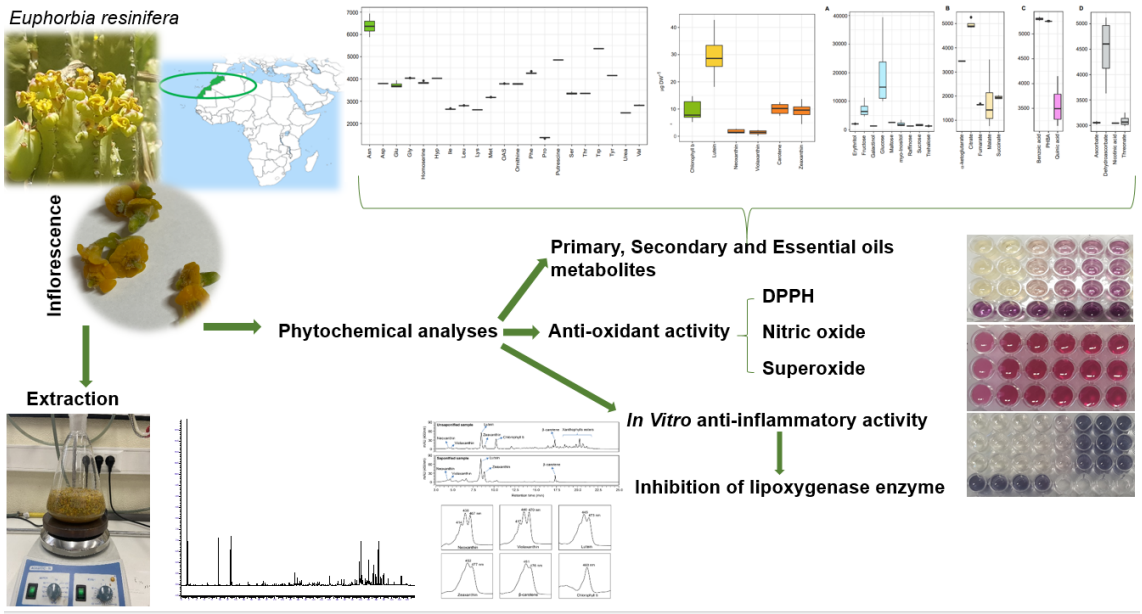
Chapter 4

The biochemical characterization of *Euphorbia resinifera* floral cyathia provides insights into the physiological role of flowers in integrating honey bee nutrition and medicinal properties of plant extracts



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Chapter IV: The biochemical characterization of Euphorbia resinifera floral cyathia provides insights into the physiological role of flowers in integrating honey bee nutrition and medicinal properties of plant extracts*

Résumé

Euphorbia resinifera O. Berg est une plante endémique des régions du Nord et du Centre du Maroc, utilisée depuis l'Antiquité romaine et grecque pour ses propriétés médicinales. La plante est principalement connue pour sécréter un latex toxique contenant de la resiniferatoxin. Cependant, les pseudo-inflorescences d'*E. Resinifera* appelées cyathes sont dépourvues de laticifères et ne sécrètent donc pas de latex. Au lieu de cela, ils exsudent le nectar que les abeilles locales collectent et transforment en miel, aujourd'hui labellisé par l'Indication Géographique Protégée du Maroc pour ses qualités. Les extraits de miel et d'eau de cyathia floral trouvent un large éventail d'applications dans la médecine traditionnelle d'Afrique du Nord sous forme de pommades et de décoctions d'eau. Compte tenu de la pertinence des produits dérivés des fleurs d'*E. Resinifera* pour la nutrition des abeilles et la production de miel et des bienfaits pour la santé des décoctions d'eau et des onguents, cette étude visait à fournir un dépistage phytochimique complet de ses cyathes. Nos analyses ont révélé que *E. resinifera* cyathia produit une pléthore de différentes classes de métabolites spécialisés, notamment des caroténoïdes, des flavonoïdes et des polyamines qui confèrent des propriétés antioxydante aux décoctions d'eau, comme nous l'avons mesuré avec des dosages antioxydants *in vitro*. Nous avons également mesuré la teneur abondante en hexoses, acides aminés et vitamines que les abeilles peuvent collecter à partir du nectar et transformer en miel. De plus, nous avons détecté des niveaux élevés de benzaldéhyde et de nonanal, qui sont des composés organiques volatils connus émis par les fleurs des espèces pollinisées par les abeilles et les bourdons. Finalement, nous pouvons émettre que, nos analyses ont révélé que *E. resinifera* cyathia est une excellente source de molécules antioxydante et une bonne source de nourriture pour les abeilles butineuses locales, révélant ainsi le rôle central des fleurs dans la médiation des interactions avec les pollinisateurs locaux et la transmission de propriétés médicinales aux plantes extraits.

Mots clés : épicéa africain, pseudo-inflorescence de cyathium, métabolites primaires, caroténoïdes, flavonoïdes, volatils

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* Oumaima Boutoub, Sagar Jadhav, Xiongjie Zheng, Lahsen El Ghadraoui, Salim Al Babili, Alisdair R. Fernie, Ana Cristina Figueiredo, Maria Graça Miguel, Monica Borghi. The biochemical characterization of *Euphorbia resinifera* floral cyathia provides insights into the physiological role of flowers in integrating honey bee nutrition and medicinal properties of plant extracts. Soumis, **2023**.

4

The biochemical characterization of *Euphorbia resinifera* floral cyathia provides insights into the physiological role of flowers in integrating honey bee nutrition and medicinal properties of plant extracts*

Abstract

Euphorbia resinifera O. Berg is a plant endemic to the Northern and Central regions of Morocco, which has been used since the ancient Roman and Greek times for its medicinal properties. The plant is mainly known for secreting a poisonous latex containing resiniferatoxin. However, *E. resinifera* pseudo-inflorescences called cyathia are devoid of laticifers, therefore, do not secrete latex. Instead, they exudate nectar that local honeybees collect and craft into honey, nowadays labeled with the Protected Geographic Indication of Morocco for its qualities. Honey and water extracts of floral cyathia find a broad range of applications in the traditional medicine of Northern Africa as ointments and water decoctions. Given the relevance of *E. resinifera* floral-derived products for bee nutrition and honey production and the health benefits of water decoctions and ointments, this study aimed to provide a comprehensive phytochemical screening of its cyathia. Our analyses revealed that *E. resinifera* cyathia produce a plethora of different classes of specialized metabolites, including carotenoids, flavonoids, and polyamines which confer antioxidant properties to water decoctions as we measured with in vitro antioxidant assays. We also measured abundant content of hexoses, amino acids and vitamins that honeybees may collect from nectar and craft into honey. Moreover, we detected high levels of Benzaldehyde and nonanal, which are known volatile organic compounds emitted by flowers of species pollinated by bees and bumblebees. In conclusion, our analyses revealed that *E. resinifera* cyathia are a great source of antioxidant molecules and a good food source for the local foraging honeybees, so revealing the central role of flowers in mediating interactions with local pollinators and the conferral of medicinal properties to plant extracts.

Keywords: African spruce, cyathium pseudo-inflorescence, primary metabolites, carotenoids, flavonoids, volatiles

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* Oumaima Boutoub, Sagar Jadhav, Xiongjie Zheng, Lahsen El Ghadraoui, Salim Al Babili, Alisdair R. Fernie, Ana Cristina Figueiredo, Maria Graça Miguel, Monica Borghi. The biochemical characterization of *Euphorbia resinifera* floral cyathia provides insights into the physiological role of flowers in integrating honey bee nutrition and medicinal properties of plant extracts. Submitted, **2023**.

1. Introduction

Euphorbia resinifera O. Berg, commonly known to as “resin spurge” or “African spurge”, is a plant endemic to the Northern and Central regions of Morocco, where it is frequently found along

the slopes of the Atlas Mountains (Figures 4.1A-B). Like other species of the Euphorbiaceae family, also *E. resinifera* evolved adaptations to dry climates, such as a succulent habitus with thick stems and spines for which the plant is often mistaken for a member of the Cactaceae family (Figures 4.1C-D) (1). At maturity, *Euphorbia* species develop pseudo-inflorescences arising from a peculiar ontogenesis of the reproductive structures, in which a spiral of male elements develops to enclose a central dome of cells that elongate to form a protruding gynoecium (2). As the boundaries between individual floral organs never develop to completion, the resulting overlapping rearrangement of male and female organs gives rise to a cup-like pseudanthial inflorescence called cyathium. In *E. resinifera*, cyathia gather in small groups at the top of thick succulent stems forming clusters of pseudo-inflorescences of a deep egg-yolk yellow color (Figures 4.1E-G).

Attracted by a combination of color and scent, local Moroccan honeybee species, *Apis mellifera intermissa* and *Apis mellifera major*, visit the cyathia to collect the nectar (Figures 4.1F), which is secreted by a rime of nectary glands encircling the androecia (3,4). Apiculture in Morocco is a widespread business of socio-economic relevance with a long historical tradition and regional specialization characterized by the authentic use of medicinal plants for honey production (4). The cohort of the principal Moroccan melliferous plants counts approximately fifteen species, among which *E. resinifera* dominates in the region of Tadla-Azilal. In this region, *E. resinifera* blooms from May till the end of summer with seasonal variation depending on rainfall and altitude (4,5). The monofloral honey that local honeybees craft on the nectar collected from *E. resinifera* cyathia is recognized for its outstanding quality and labeled with the appellation of Protected Geographical Indication (PGI). A palatable taste and high scores in selected parameters, including color, crystallization potential, and low counts of exogenous pollen grains are just a few among the many requirements needed to receive the certification (Ministry of Agriculture of Morocco, 2019) (6). The PGI has contributed to the promotion of this product on international markets, but in the local tradition of Morocco and Northern Africa, *E. resinifera* monofloral honey has been utilized for centuries for its medicinal properties. In particular, honey topical applications in the form of ointments and bandages have largely been employed to treat wounds in animals and humans (7). Similarly, *E. resinifera* cyathia have for a long time been utilized in the form of water decoctions for the treatment of inflammations of the respiratory tract and the throat (8). Contrary to other *E. resinifera* plant organs, flowers are devoid of laticifers secreting resiniferatoxin, a potent capsaicin analog listed as the hottest chemical compound existing in nature (1,000 times hotter than capsaicin) known to cause severe irritation upon contact (5,8,9). Nonetheless, cyathia may synthetize other beneficial chemical compounds that are secreted into the nectar and ultimately found in honey and water decoctions. These metabolites may add to those of the central pathway, which honeybees also retrieve from nectar as food resources for their brood. Given the

relevance of products derived from *E. resinifera* inflorescences, in this study we performed a thorough analysis of primary and specialized metabolites and volatile organic compounds (VOCs) from floral cyathia. Moreover, we determined the antioxidant potential of water-based cyathium extracts which, in the traditional medicine of Morocco and Northern Africa, are utilized as water decoctions. Our analyses revealed that *E. resinifera* cyathia accumulate a wide variety of metabolites with strong antioxidant properties, which render flowers and honey a great source of free radical scavengers and vitamins. Additionally, cyanthia contain very high levels of sugar hexoses and amino acids, which represent great food sources for honeybee feeding.

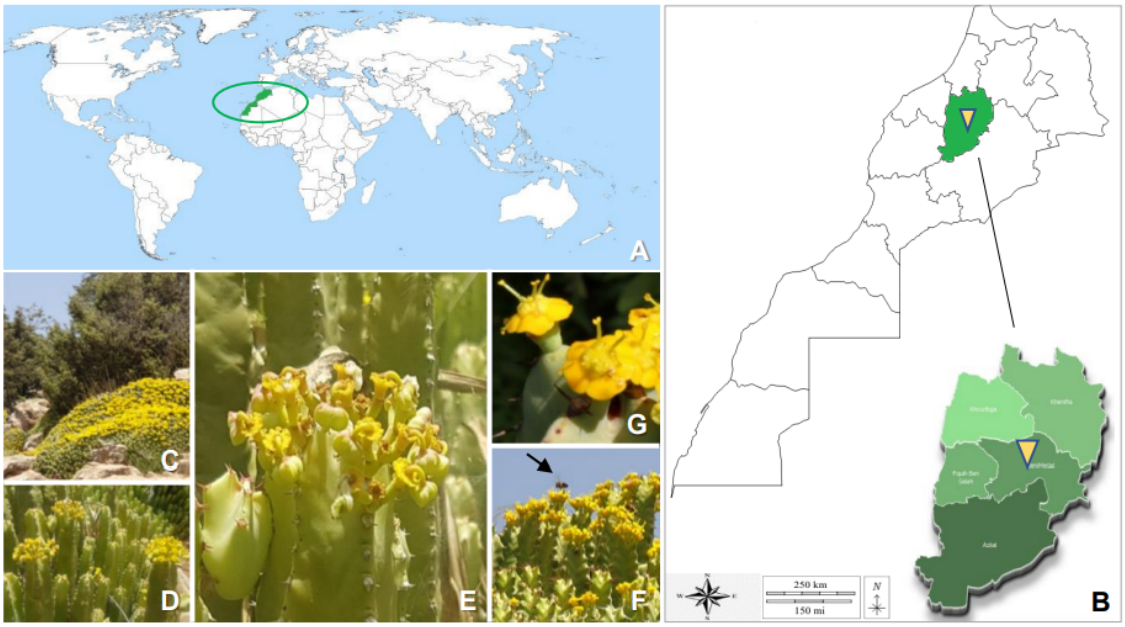

Figure 4.1. Location and photos of *Euphorbia resinifera* plants used in this study. **A** Location of Morocco in Northwest Africa and **B** Beni Mellal-Khénifra region where samples were collected. **C** and **D** Images of *Euphorbia resinifera* plants in full bloom. **E** Cluster of cyathia on top of a succulent stem. **F** Close-up view of blooming cyathia. **G** Overview of blooming cyathia and a foraging honeybee (black arrow).

2. Experimental

2.1. Plant material

Euphorbia resinifera cyathia were collected during July 2021 in the city of Beni Mellal located in the Beni Mellal-Khénifra region of Morocco (geographic coordinates: 32°20′22″ N, 6°21′39″W) and kept at -80°C until further analysis.

2.2. Analysis of primary and specialized metabolites

Cyathia were reduced to fine powder using a ball mill (Tesch, Haan, Germany) at liquid N_2 temperature. Primary and specialized metabolites were extracted from 50 mg of powder with 0.7 mL of methanol containing ribitol (2 mg/L) and isovitexin (5 mg/L) as internal standards. A phase separation with chloroform was used to remove chlorophylls before dividing each sample into two aliquots for the analysis of primary and specialized metabolites, respectively. Primary metabolites were derivatized with *N*-Trimethylsilyl-N-methyl trifluoroacetamide (10) and measured via GC-MS following the procedure described by (11) and (12). Curves of primary metabolite standards purchased at Sigma were utilized for metabolite quantification. Specialized metabolites were separated and measured via liquid chromatography mass spectrometry as previously described by (13) and (14). Mass features were obtained using the XCMS online platform with inbuilt METLIN metabolomics database (15). Thereafter, metabolites were annotated following the analysis of mass spectra and comparisons with fragmentation patterns of previously reported compounds.

2.3. UHPLC analysis of carotenoids and chlorophylls

Carotenoids were extracted as previously described (16,17). Briefly, 20 mg of lyophilized powder of *E. resinifera* cyathia were extracted with 250 µL of methanol and 500 µL of chloroform. The samples were mixed thoroughly and incubated on ice for 20 min before adding 250 µL of water. Phase separation was obtained with centrifugation at 11000 rpm in an Eppendorf 5424 centrifuge for 5 min, after which the aqueous phase was re-extracted with 500 µL of chloroform. The chloroform phases were pooled together, dried in a vacuum centrifuge, and resuspended in ethyl acetate prior to perform UHPLC analysis in a Dionex Ultimate 3000 UHPLC-DAD system (Thermo Fisher Scientific). Carotenoids were separated in a C30 column (150 × 3.0 mm, 3 µm, YMC) kept at 35 °C with mobile phase A (MeOH-MTBE (50:50)) and phase B (MeOH-H2O-MTBE (6:3:1)) at a flow rate of 0.8 mL.min 1. The elution gradient was set as it follows: 0-19 min, 30-100 % A and 70-0 % B; 19-24 min, 100% A; 24-25 min, 100% A; 25-26 min, 100-30% A and 0-70% B; 26-30 min, 30% A and 70% B. Identification of carotenoids and chlorophylls was obtained by comparison with retention times and UV–visible spectra of authentic carotenoid standards (CaroteNature, Switzerland) and the literature (16,18).

2.4. Volatile organic compound extraction, analysis, and annotation

The cellular pool of volatile organic compounds (VOCs) was isolated by hydrodistillation in a Clevenger-type apparatus for 3 h at a distillation rate of 3 mL/min according to the European Pharmacopoeia protocol (Council of Europe, 2010) (19). At the end of the distillation procedure, the apparatus was cooled down for approximately 10 to 15 min and the VOCs recovered from the graduated tube by rinsing it with n-pentane distilled in the laboratory. This was accomplished by introducing the distilled *n*-pentane in the filling funnel after flowing out part of the hydrolate from the connecting tube until just below the filling funnel. The residual heat of the distillation flask evaporated the distilled n-pentane, which then condensed and dissolved the volatiles above the aqueous phase in the graduated tube. The mixture of distilled *n*-pentane and volatiles was transferred to a clean glass vial and concentrated to approximately 10 µL using a blow-down evaporator system under a flux of nitrogen at room temperature. The concentrated samples were stored at -20 °C in the dark until further analysis. VOCs were quantified in a Clarus 400 Gas Chromatograph (PerkinElmer, Waltham, MA, USA) equipped with two flame ionization detectors (GC-FID). Two columns of different polarities were inserted into the injector port: a DB-1 fused-silica column (100% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Folsom, CA, USA) and a DB-17HT fused-silica column ((50 % phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific). The oven temperature was programmed to rise from 45 to 175 °C at a rate of 3 °C/min, then to 300 °C at a rate of 15 °C/min and finally held isothermal for 10 min, for a total run time of 61.67 min. The split injector ratio was 1:40 and the injector and detector temperatures were 280 and 290 °C, respectively; the carrier gas was hydrogen, adjusted to a linear velocity of 30 cm/s. The percentage composition of the volatiles was computed by the normalization method from the GC peak areas, without the use of correction factors, calculated as mean values of two injections from each sample, in accordance with ISO 7609 (ISO 7609:1985) (20). For the identification of compounds, the samples were analyzed by GC-MS in a Perkin Elmer Clarus 690 gas chromatograph equipped with a DB-1 fused-silica column (100% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific) connected to a Perkin-Elmer SQ-8-T Mass Spectrometer (software version 6.1, PerkinElmer, Shelton, CT, USA). Injector and oven temperatures were set as previously described above with the following additional settings: transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40 300 *m/z*; scan time, 1 s. Compounds were annotated after the calculation of their retention index (RI) relative to a C7–C31 *n*-alkane series (Sigma) in accordance with the ISO 7609 protocol and followed by the comparison of spectra from a laboratory made library of essential oils, laboratory-synthesized compounds, and commercially available standards.

2.5. Total flavonoid content

Total flavonoid content (TFC) was determined with the method described by (21). Briefly, 150 µL of cyathium extract were mixed with 100 µL of 20% $AlCl_3$ and incubated for 1 h at room temperature. The absorbance of the extracts was measured in a spectrophotometer at 420 nm and TFC expressed as mg of Quercetin Equivalents (QE) after integration of calibration curves obtained with commercial quercetin standards ranging in concentration from 0.002 to 1 mg/mL.

2.6. Extraction for in-vitro antioxidant activity

Two grams of *E. resinifera* cyathia were extracted by maceration with 100 mL of distilled water on an orbital shaker under mild agitation (250 rpm) for 24 h. Thereafter, the supernatant was retrieved by centrifugation at 5,000 rpm for 20 min and kept at -20 ºC until further analyses.

2.6.1. DPPH radical scavenging assay

The antioxidant activity of cyathium extracts was determined as described by (22) and performed with 25 µL of liquid extract in the presence of 275 µL of 63.4 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) for 30 min. The antioxidant activity was calculated after measuring the absorbance in a spectrophotometer at 517 nm and expressed as percentage of inhibition accordingly to the formula Inhibition = $((A_0-A_1)/A_0) \times 100)$, where A_0 represents the absorbance of a water control and A_1 represents the absorbance of the samples. The half maximal inhibitory concentration (IC_{50}) is obtained by plotting the inhibition percentage as a function of sample concentration. Butylated hydroxytoluene (BHT) at the concentration from 0.03 to 1 mg/mL was used as a positive control.

2.6.2. Superoxide radical scavenging assay

The scavenging activity toward superoxide radical was assessed accordingly to (23) using 25 µL of cyathium extract in the presence of 50 µL of 514 µM nitroblue tetrazolium (NBT) and 50 µL of 1989 µM nicotinamide adenine dinucleotide solution (NADH). Thereafter, 100 µL of distillated water and 75 µL of 2.7 µM phenazine methosulfate (PMS) were added to the mix immediately before reading the absorbance in a spectrophotometer at 560 nm. IC_{50} was calculated as previously described using ascorbic acid from 0.01 to 1 mg/mL as positive control.

2.6.3. Nitric oxide scavenging assay

The scavenging activity toward nitric oxide (NO) was measured as reported by (24) using 150 µL of cyathium extract mixed with 150 µL of 10 mM sodium nitroprusside prepared in phosphate-buffered saline (PBS) at pH 7.4 for 1 h. Then, 20 µL of a solution constituted by 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in water and 1% sulfanilic acid in phosphoric acid (1:1) was added to the initial mix, followed by the addition of 130 µL of water. The scavenging activity toward NO was calculated after measuring the absorbance in a spectrophotometer at 540 nm and expressed as IC50 as previously described. Curcumin ranging in concentration from 0.022 to 1 mg/mL was used as a positive control.

2.6.4. Lipoxygenase assay

The cyathium extract (150 µL)was mixed with 50 µL of 0.001 M linoleic acid and 777 µL of 0.1 M boric acid pH = 9.0 to which tween 0.005% was added as surfactant (24). 15 µL of lipoxygenase enzyme at the concentration of 0.018 g/mL were added to the mixture before reading the absorbance in a spectrophotometer at 234 nm. The IC50 was calculated as previously described using nordihydroguaiaretic acid (NDGA) as positive control at a concentration from 0.003 to 1 mg/mL.

3. Results and discussion

While *E. resinifera* has reached a peak of notoriety for the secretion of its poisonous latex (8,25), the local medicine of Morocco and Northern Africa has, for centuries if not millennia, utilized products derived from its inflorescences, which lack laticifers, for their healing properties. Therefore, the objective of this study was to provide a comprehensive phytochemical screening of *E. resinifera* cyathia to unravel the full chemical potential of the inflorescences for bee nutrition, honey production, and to better understand the health benefits of water decoctions. For this, we collected fully mature cyathia from *E. resinifera* plants growing in the Beni Mellal-Khénifra region of Morocco (Figure 4.1), and utilized gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) to fully characterize the chemical products that they synthetize and accumulate. Finally, we investigated the antioxidant potential of water extracts with *in vitro* enzymatic assays.

3.1. Metabolites of the central pathway

It is accepted that flowers, despite behaving for the most part of their lifespan as heterotrophic organs, synthetize, accumulate, and secrete a plethora of primary and specialized metabolites with a wide array of physiological functions. These functions include protection towards abiotic and biotic stressors (26), pollinator attraction (27), and finally promotion of fruit and seed set (28). Metabolites of the central pathway fulfill most of these purposes as they not only sustain key steps in the developmental transition from pre-anthesis to post-anthesis (29), but also represent important food sources that animal pollinators retrieve from nectar. To gather information about the composition of primary metabolites of *E. resinifera* cyathia, methanol extracts were characterized via GC-MS after derivatization with N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA). Thereafter, absolute metabolite composition was obtained following the integration of calibration curves with the corresponding metabolite commercial standards. Finally, to facilitate data interpretation, metabolite amounts were grouped by compound class and association with known metabolic pathways, and represented as bar plots of metabolite abundance per fresh weight (Figure 4.2).

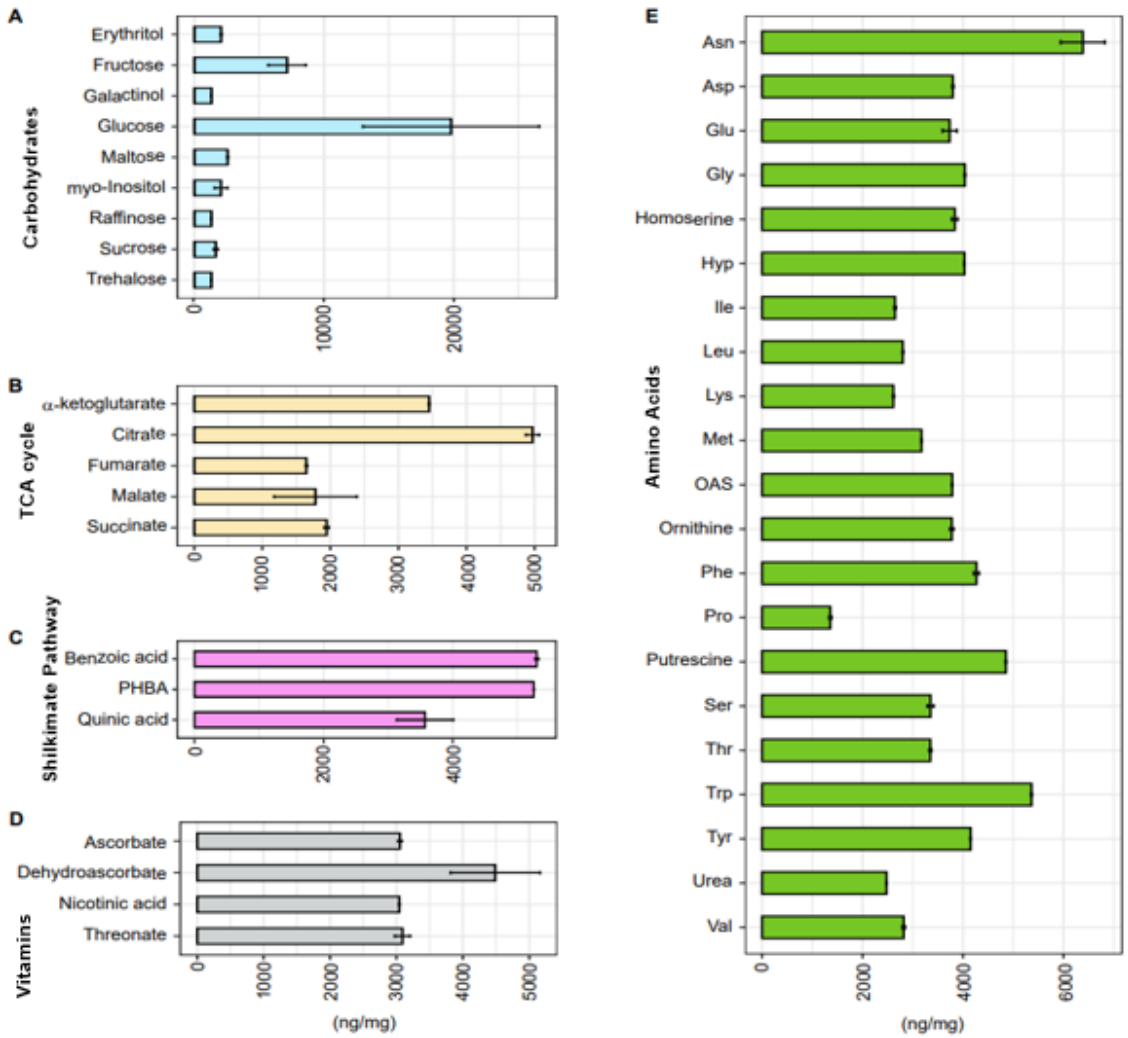

Figure 4.2. Primary metabolites. Absolute amounts of metabolites associated with the central pathway measured in mature cyathia. The bar plots show the average of four independent measurements and the error bars the standard deviation. The color of the bar plots indicates the following metabolite-pathway associations: A blue, carbohydrates; B yellow, TCA cycle (tricarboxylic acid); C pink, shilkimate pathway; D grey, vitamins and others; E green, amino acids. Hyp, hydroxyproline; OAS, O-acetylserine; PHBA, *p*-hydroxybenzoic acid.

Five groups of primary metabolite classes were targeted for this analysis, including carbohydrates, amino and organic acids, vitamins, and intermediates of the shikimate pathway. In C3 plants with actinomorphic (regular) and perfect (with male and female organs) flowers, sucrose is the main sugar transported via the phloem from source tissues to sink organs, where it is hydrolyzed into glucose and fructose, and ultimately glucose transported into the flowers. Thereafter, cycles of sucrose resynthesis and break-down support the transport of carbohydrates across the cell walls of floral organs, as these chemical compounds cross the cellular barriers via high- and low-affinity transporters (27). During the ontogenesis of *E. resinifera* cyathia the boundaries between individual floral organs never develop to completion. Therefore, carbohydrates could be mobilized via plasmodesmata connections between the symplast of adjacent cells. Indeed, the predominant sugar that we measured in *E. resinifera* cyathia was glucose followed by fructose, while the level of sucrose appeared secondary and similar to that of sugars usually present in lower abundance, such as raffinose and trehalose (Figure 4.2A). Abundant glucose measured in cyathia mirrors high glucose content also measured in honey (24), which aligns very well with its medicinal properties. Indeed, when honey is used as a topical ointment, the burst of H_2O_2 that follows the reduction of glucose by glucose-oxidase catalysis is a trait mostly sought for during wound healing as it helps to keep the wounded tissues sanitized (7,30).

Maltose follows as the third most abundant sugar in floral cyathia. However, it is challenging to infer a possible physiological explanation for this happening. Crassulacean acid metabolism (CAM) is of frequent occurrence among the Euphorbiaceae (31) and *E. resinifera* plants display a succulent habitus. Therefore, high levels of maltose could be attributed to a CAM type of photosynthesis presents in *E. resinifera* (32). Nonetheless, high maltose could also derive from the degradation of starch, which plausibly accumulates in the nectary glands surrounding the androecia. Giving the little knowledge currently available about the biology of *E. resinifera* plants and their cyathia, a deductive conclusion cannot be made at this time. Organic acids were also elevated in floral cyathia, with citrate and α-ketoglutarate being the most abundant (Figure 4.2B). High level of citrate may indicate that cellular respiration through the tricarboxylic acid (TCA) cycle is very active, although fast rates of glycolysis could also support the cellular requirements for energy of floral tissues, since carbohydrates were also high. Whether citrate is also secreted into nectar is a possibility (33), and if this is the case, it may account for the low acidity of *E. resinifera* honey (pH=4.0; (15). Elevated level of ketoglutarate may indicate an active ammonium metabolism, and, in fact, amino acids were also present in abundance in cyathia, with asparagine (Asn) being the most concentrated (Figure. 4.2E). The presence of the amide group in the Asn molecule is of great relevance for the N metabolism of flowers, as the elevated N:C ratio makes it suitable for the storage of N into fruits and seeds, as well as in support of N nutritional needs of animal pollinators (34). Moreover, alkaline soils typical of desertic areas are usually poor in N

content, for which strengthening N assimilation could be an adaptive response typical of this species. Proline (Pro), the metabolic marker of plant responses to drought and heat stress was surprisingly present at low level (Figure 4.2E). As we explained further below, *E. resinifera* synthetizes a plethora of specialized metabolites for the conferral of resilience to abiotic stressors, for which enhancing Pro biosynthesis and storage in cyathia could be redundant. All aromatic amino acids were well represented in flowers and similarly were the intermediates of the shikimate pathway and, in particular, benzoic acid and p-hydroxybenzoic acid (PHBA; Figure 4.2C). Finally, cyathia reveled to be great sources of vitamins including nicotinic acid (also known to as niacin or vitamin B3), threonate (a metabolite of vitamin C), and antioxidants such as ascorbate and dehydroascorbate. In conclusion, *E. resinifera* cyathia revealed abundant content of primary metabolites that may satisfy the nutritional requirements of animal pollinators and also mirror the medicinal properties of nectar.

3.2. Non-polar pigments

Except for the Caryophyllales which accumulate betalains, all other plant species obtain yellow flowers through synthesis and accumulation of carotenoids or phenylpropanoid-acetate derivatives such as flavones, flavonols, and flavanones (35). Therefore, the resulting yellow hue of flowers ultimately depends on the relative ratio of each class of pigments and their combination. Carotenoids when present alone give rise to flowers of a deep-yellow or orange color, while flavones and flavonols are pale-yellow, and aurones contribute a bright yellow hue (36). *E. resinifera* cyathia are of a deep egg-yolk yellow, for which we hypothesized that they accumulate carotenoids in large abundance. To test our hypothesis chloroform extracts of non-polar metabolites were analyzed via ultra-high-performance liquid chromatography (UHPLC), and retention times and UV spectra of the corresponding chromatographic peaks compared with those of authentic carotenoid standards. This analysis confirmed our initial hypothesis and revealed that *E. resinifera* accumulates a few classes of major carotenoid compounds (Figure 3). Among all, lutein was by far the most abundant as it reached a concentration of approximately 30 µg/g of cyathium dry tissue. Carotene and zeaxanthin followed at a concentration of approximately 10 µg/g, while neoxanthin and violaxanthin were present at much lower level. Carotenoids greatly contribute to physical quenching of singlet molecular oxygen (1O2), one of the most potent reactive oxygen species (ROS), by dissipating energy in the surrounding cellular environment after physically interacting with it (37). *E. resinifera* plants that colonize the desertic slopes of the Atlas Mountains in Northern Africa are exposed to numerous abiotic stressors including high temperatures, UV radiance, and drought, which all lead to cytotoxic concentrations of ROS (38,39). Moreover, floral cyathia gather atop of tall succulent stems where they are exposed for their entire lifespan to direct solar radiation and the associated UV-B high energy photons. While protecting from photo-quenching and the damaging effects of high ROS,

carotenoids may also contribute to preserve androeacia and gynoeacia from abiotic stressors, and consequently contribute to the reproductive success of this species and its biological fitness.

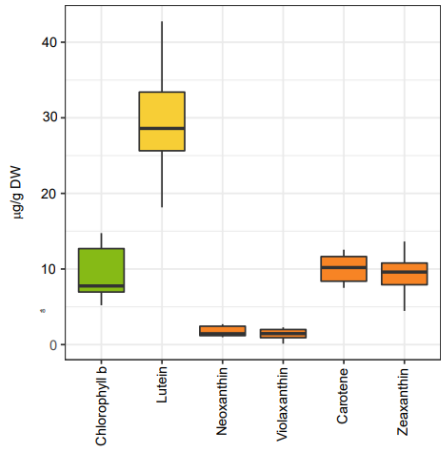

Figure 4.3. Non-polar pigments. Absolute amount of clorophill b and carotenoids in full blooming cyathia obtained from six independent samples. The boxplots show the median (central bar), the interquartile range (box), and minimum and maximum values (vertical bars) and represent µg/g of sample dry weight (DW)

Among the non-polar metabolites, relatively high levels of chlorophyll b (10µg/g) were also measured in *E. resinifera* cyathia. As the color of cyathia intensifies from pale-yellow and green to dark-yellow during development and maturation, chlorophylls could still be present in the deeper tissues while inflorescences start accumulating carotenoids. Indeed, scanning electron microscopy (SEM) images of cyathia from *Euphorbia* species showed the presence of numerous stomata (2) for which active photosynthesis presumably occur in these reproductive organs.

3.3. Flavonoids and polyamines

The total content of flavonoid compounds in *E. resinifera* cyathium water extracts was determined by reading the absorbance in a spectrophotometer at 420 nm. This measurement revealed relatively high content of flavonoid compounds corresponding to an average of 17.62 ± 0.17 mg quercetin equivalent (QE)/g floral tissue. Thereafter, to gain a comprehensive overview of the different classes of compounds present in the inflorescences, cyathium methanol extracts were analyzed via LC-MS (14, 13) and compounds annotated following comparisons with fragmentation patterns of previously identified metabolites (40). This analysis revealed the presence of numerous flavonol glycosides consisting of quercetin (Q), kaempferol (K), and isorhamnetin (Is) aglycones in combination with glucose (G) and rhamnose (R) sugars (Table 4.1). The spectrum of flavonol glycosides was dominated by quercetin derivatives, with quercetin-3-*O*-glucoside-7-*O*-rhamnoside (Q3G7R), quercetin-3-*O*-rhamoside-7-*O*-rhamnoside

(Q3R7R), quercetin-3-*O*-glucoside-2"-*O*-glucoside (Q3GG), and quercetin-3-*O*-(2"-*O*-rhamnosyl)glucoside-7-*O*-rhamnoside (Q3GR7R) being the most abundant compounds. The second largest class of flavonoid compounds was represented by isorhamnetin glucosides with isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside (Is3G7R) and isorhamnetin-3-*O*-glucoside (Is3G) being among the most abundant. As the synthesis of isorhamnetin follows the methylation of quercetin (41), there is the possibility that the flavonoid biosynthetic pathway in *E. resinifera* cyathia is diverted towards the biosynthesis of quercetin rather than kaempferol. Nonetheless, a few kaempferol derivatives, including kaempferol-3-*O*-glucoside-7-*O*-rhamnoside (K3G7R), kaempferol-3-*O*-glucoside-2"-*O*-glucoside (K3GG) and keampferol-3-*O*-rhamnoside-7-*O*-rhamnoside (K3R7R) were also identified in cyathia. A recent literature survey of flavonoid molecules from Euphorbia species listed 80 different compounds among which glycosylated flavonoids were the most common (42). However, this survey did not include annotations of flavonoid compounds from *E. resinifera*. In fact, many publications report the total content of phenolic compounds as gallic acid equivalents (GAE) or QE, without providing details concerning the chemical structure of flavonoid compounds (43).

Floral cyathia also displayed elevated content of polyamines (PAs). In particular, we annotated spermidine derivatives covalently conjugated with phenolic compounds, among which tri-*p*-coumaroyl spermidine (PA 3) was the most abundant. As the levels of ornithine and putrescine were also elevated in cyathium extracts (Figure 4.2), the pathway of PA biosynthesis may be very active in these tissues. As PAs regulate flower development and also confer protection towards high temperature stress (44), they could exert a dual biological function also in *E. resinifera* cyathia.

In conclusion, our analysis showed that the metabolism of specialized compounds, and in particular the synthesis of Q, Is and PAs is intense in *E. resinifera* cyathia as elevated levels of different classes of compounds accumulate in these tissues.

Table 4.1. Flavonoids and polyamine compounds isolated from *Euphorbia resinifera* cyathia

No.	Abbreviation	Metabolite name	Chemical class	Molecular Formula	Knapsack ID	RT	Ionization mode	(M-H)- m/z	Average	Standard Deviation	References
1	Q3GR7R	Quercetin-*3-O*-(*2″-O*-rhamnosyl) glucoside-*7-O*-rhamnoside	Flavonol	$C_{33}H_{40}O_{20}$	C00005475	5.48	negative	755.22	4.09E+04	2.65E+04	(45)
2	K3GR7R	Kaempferol-*3-O*-(*2″-O*-rhamnosyl) glucoside-*7-O*-rhamnoside	Flavonol	$C_{33}H_{40}O_{19}$	-	5.91	negative	739.22	7.66E+02	8.22E+02	(13,45)
3	Q3GG	Quercetin-*3-O*-glucoside-*2″-O*-glucoside	Flavonol	$C_{27}H_{30}O_{17}$	-	6.05	negative	625.16	1.45E+05	1.37E+05	(46)
4	Q3G7R	Quercetin-*3-O*-glucoside-*7-O*-rhamnoside	Flavonol	$C_{27}H_{30}O_{16}$	C00005428	6.70	negative	609.16	3.41E+05	1.13E+05	(47,48)
5	Q3A7R	Quercetin-*3-O*-arabinoside-*7-O*-rhamnoside	Flavonol	$C_{26}H_{28}O_{15}$	-	6.95	negative	579.19	2.07E+03	1.28E+03	(49)
6	K3GG	Kaempferol-*3-O*-glucoside-*2″-O*-glucoside	Flavonol	$C_{27}H_{30}O_{16}$	C00005165	7.16	negative	609.20	3.76E+03	4.39E+03	(49)
7	K3G7R	Kaempferol-*3-O*-glucoside-*7-O*-rhamnoside	Flavonol	$C_{27}H_{30}O_{15}$	-	7.29	negative	593.15	3.54E+04	2.04E+04	(47,48)
8	Q3R7R	Quercetin-*3-O*-rhamoside-*7-O*-rhamnoside	Flavonol	$C_{27}H_{30}O_{15}$	C00005432	7.32	negative	593.17	1.89E+05	9.74E+04	(47,48)
9	Is3G7R	Isorhamnetin-*3-O*-glucoside-*7-O*-rhamnoside	Flavonol	$C_{28}H_{32}O_{16}$	C00005557	7.49	negative	623.18	6.31E+04	4.57E+04	(50)
10	Is3R7R	Isorhamnetin-*3-O*-rhamnoside-*7-O*-rhamnoside	Flavonol	$C_{28}H_{32}O_{15}$	-	7.95	negative	607.09	3.06E+03	1.06E+03	(50)

11	K3R7R	Keampferol-*3*-*O*-rhamnoside-*7*-*O*-rhamnoside	Flavonol	$C_{27}H_{30}O_{14}$	C00005189	8.02	negative	577.10	3.32E+03	6.72E+02	(50)
12	Is3G	Isorhamnetin-*3*-*O*-glucoside	Flavonol	$C_{22}H_{22}O_{12}$	C00005525	8.31	negative	477.19	4.25E+04	1.29E+04	(40)
13	K3G	Kaempferol-*3*-glucuronide	Flavonol	$C_{21}H_{18}O_{12}$	C00005139	8.67	negative	461.22	1.14E+03	2.15E+02	(52)
14	PA 1	*N1,N5*-di(hydroxyferuloyl)-*N10*-sinapoy-spermidine	Polyamine	$C_{38}H_{45}O_{12}N_3$	-	8.96	positive	736.10	6.74E+02	2.29E+02	(40,53)
15	PA 2	mono(hydroxyferuloyl)-diferuloyl-spermidine	Polyamine	$C_{37}H_{43}O_{10}N_3$	-	9.57	positive	690.17	9.18E+03	3.66E+03	(40,53)
16	PA 3	Tri-p-coumaroyl-spermidine	Polyamine	$C_{34}H_{37}O_6N_3$	-	10.13	positive	438.24	3.55E+05	1.37E+05	(40,53)
17	PA 4	*N1,N10*-di(feruloyl)-*N5*-coumaroyl-spermidine	Polyamine	$C_{36}H_{41}O_8N_3$	-	10.26	positive	498.20	7.36E+02	1.55E+02	(40,53)
18	PA 5	*N1,N5-di*(coumaroyl)-*N10*-feruloylspermidine	Polyamine	$C_{35}H_{39}O_7N_3$	-	10.30	positive	614.29	4.71E+04	1.70E+04	(40,53)
19	PA 6	Tri-*p*-feruloyl-spermidine	Polyamine	$C_{37}H_{43}O_9N_3$	-	10.76	positive	674.48	4.57E+03	1.13E+03	(40,53)

3.4. Volatile organic compounds

The phenotypical expression of a species genotype includes, among others, volatile organic compounds (VOCs) that each species synthetizes and emits from their tissues (54). The rate of VOC biosynthesis and emission are also modulated by agents of environmental disturbance. In particular, VOC synthesis is highly affected by high temperature and drought stress, and exposure to atmospheric ozone, which all contribute to reducing the availability of metabolic precursors and the rate of catalysis of biosynthetic enzymes. Moreover, these environmental stressors alter VOC partitioning between the air and the cellular space and consequently the emission of volatiles (52,55). Here, we utilized hydrodistillation to isolate essential oils (EOs) from *E. resinifera* cyathia, followed by gas chromatography coupled to flame ionization detector (GC-FID) and GC-MS measurements to quantify and annotate these metabolites (Table 4.2). Our analysis identified 76 compounds belonging to the chemical classes of alkanes, fatty acids, and oxygen-containing terpenes and terpene hydrocarbons. Other classes of compounds including aldehydes, benzoic acid derivatives, and C6 compounds often referred to as green leaf volatiles (GLVs) were also present in variable amount. The spectrum of cyathium VOCs was dominated by different classes of aldehydes either present in large abundance or trace amount (<0.05%). In this class of compounds, *n*-nonanal (pelargonaldehyde; **17**) and phenylacetaldehyde (**10**) were the most abundant, but altogether hexanal (**1**), heptanal (**5**), and octanal (**9**), which were present in trace amounts, and decanal (**22**) and dodecanal (**36**), which were low in abundance, reached rather elevated levels. Some of these compounds are constitutively emitted by plants, while for others emission increases upon exposure to stress. *n*-Nonanal is an example of this kind (56), although we speculated that high *n*-nonanal in *E. resinifera* cyathia could be constitutive. Indeed, *n*-nonanal is ubiquitously present in the floral bouquet of honeybee-pollinated species, in addition to being secreted into their nectar, and probably persisting also in the honey (57). The second most abundant aldehyde was (phenylacetaldehyde (**10**), a common VOC emitted by a vast array of flowers including petunias and roses. In petunia, phenylacetaldehyde and its (phenyl ethyl alcohol derivative (**16**) are synthetized from phenylalanine (Phe)) via a combined decarboxylation-amine oxidation reaction catalyzed by phenylacetaldehyde synthase (58). Conversely, in roses, two enzymes catalyze deamination and decarboxylation reaction, respectively (59). As we have already mentioned, current knowledge about *E. resinifera* biology and biochemistry is insufficient to determine how the biosynthesis of phenylacetaldehyde occurs in this species. In the same phenylpropanoid/benzenoid group of compounds, benzaldehyde (**6**), a VOC that seemingly evolved across different plant lineages to mediate interactions with pollinators (60) was also detected in the amount of 0.5%. Cyathia also accumulated oxygen-containing terpenes and terpene hydrocarbons in large amounts. Abundant sesquiterpene hydrocarbons included germacrene D (**39**), bicyclogermacrene (**40**), δ cadinene (**44**), *trans,trans*-α-farnesene (**42**), α-

muurolene (**41**), α-copaene (**33**), β-bourbonene (**34**), β-elemene (**35**) and δ-elemene (**30**). In addition, we annotated and quantified oxygenated sesquiterpenes such as α-cadinol (**52**) and β-eudesmol (**51**). While sesquiterpenes dominated the spectrum of terpene compounds, we also annotated monoterpenes and monoterpenoids. For example, geranyl acetone (**38**) was present at 0.6% and nerol oxide (**21**) and geraniol (**23**) at 0.2%. Trace amounts of monoterpenes like *p*-cymene (**11**), limonene (**14**), and linalool (**18**) were also detected. Finally, we identified abundant saturated fatty acids such as palmitic acid (**62**), myristic acid (**57**) and lauric acid (**45**), as well as 1.9% of unsaturated oleic acid (**66**).

A survey of the recent literature concerning VOCs identified from various tissues and latex of thirty *Euphorbia* species (Table 4.3) revealed a widespread production of sesquiterpenes, among which caryophyllene and caryophyllene oxide were the most common. Alkanes were also frequently found, as well as monoterpenes although less abundant. When restricted to floral cyathia, the comparison revealed the presence of heptacosane, pentacosane, tricosane and 9-tricosene which are part of the female sex pheromone of the bee *Andrena* (Melandrena) nigroaenea with a natural habitat all around the Mediterranean basin (61). Indeed, many *Euphorbia* species are pollinated by bees and a wide variety of insects of the orders of Diptera and Himenoptera (62,63). Ants also visit the cyathia to collect droplets of nectar and ultimately interfere with seed dispersal (64,65). Interestingly, phenylacetaldehyde, one of the preferred scent used by bees and bumblebees to locate rewarding flowers (66), was only detected in *E. resinifera* (Table 4.2). Nonanal, a typical VOC of honeybee-pollinated species, was detected in *E. resinefera* and *E. characias* (also known to as Mediterranean spruce), the latter pollinated by bees and butterfly.

In conclusion, our analyses indirectly revealed that in *E. resinefera* cyathia multiple metabolic pathways leading to the synthesis of diverse classes of VOCs are activated, ultimately supporting the productions of *E. resinifera* specific VOCs, as well as compounds shared among various *Euphorbia* species. Indeed, we annotated compounds of the phenylpropanoid/benzenoid pathway, as well as oxygen-containing terpenes and terpene hydrocarbons possibly synthetized via the mevalonate pathway in the cytosol (sesquiterpenes) and the methylerythritol 4-posphate (MEP) pathway in the plastid (monoterpenes). The pathway of fatty acid biosynthesis, which in plants also takes place in plastids, is also active in cyathia, as it supports the production of multiple unsaturated fatty acids. Despite our analysis suggesting high rates of VOC synthesis, whether these compounds are transported out into the atmosphere or stored in glandular trichomes and eventually secreted into nectar only measurements of VOC emission would clarify. SEM images of cyathia from Euphorbiaceae species revealed the presence of numerous trichomes on the surface of the perianth and along the anther filaments (2). However, if these trichomes have the potential to accumulate VOCs is not reported in the current literature.

Table 4.2. Essential oils isolated by hydro-distillation from *Euphorbia resinifera* cyathia.

N.	GCA	Compound name	RI	%
1	O	Hexanal	739	t
2	O	2-Furfural	800	0.1
3	A	*n*-Octane	800	t
4	O	Hexanol	883	t
5	O	Heptanal	897	t
6	O	Benzaldehyde	927	0.5
7	O	6-Methyl 5-hepten-2-one	960	t
8	O	2-Pentyl furan	973	0.1
9	O	*n*-Octanal	973	t
10	O	Benzene acetaldehyde (=Phenylacetaldehyde)	1002	7.8
11	MO	*p*-Cymene	1003	t
12	O	2-Ethyl-1-hexanol	1004	t
13	OCM	1,8-Cineole	1005	t
14	MO	Limonene	1009	t
15	O	*n*-Octanol	1045	0.1
16	O	Phenyl ethyl alcohol	1067	t
17	O	*n*-Nonanal (= Pelargonaldehyde)	1073	8.8
18	OCM	Linalool	1074	t
19	O	Benzyl cyanide (= Benzyl nitrile)	1077	0.6
20	O	2-*trans*-Nonenal	1124	0.1
21	OCM	Nerol oxide	1127	0.2
22	O	*n*-Decanal	1180	0.6
23	OCM	Geraniol	1236	0.2
24	O	2-*trans*-Decenal	1236	0.4
25	FA	Nonanoic acid	1263	0.6
26	O	2-Undecanone	1275	0.4
27	O	*cis*-Theaspirane	1286	0.6
28	O	*n*-Undecanal	1288	0.2
29	O	*trans*-Theaspirane	1300	0.2
30	SH	δ-Elemene	1332	0.1
31	O	1,2-Dihro-1,1,6-trimethyl-naphthalene *	1332	t
32	O	*trans*-2-Undecenal	1334	0.3
33	SH	α-Copaene	1375	0.3
34	SH	β-Bourbonene	1379	0.2
35	SH	β-Elemene	1388	0.2
36	O	*n*-Dodecanal	1397	0.7
37	A	*n*-Tetradecane	1400	0.5
38	OCM	Geranyl acetone	1434	0.6
39	SH	Germacrene D	1474	2.7
40	SH	Bicyclogermacrene	1487	1.6
41	SH	α-Muurolene	1494	0.4
42	SH	*trans,trans*-α-Farnesene	1500	0.8
43	A	*n*-Pentadecane	1500	0.5
44	SH	δ-Cadinene	1505	1.1
45	FA	Dodecanoic acid (= Lauric acid)	1550	0.9
46	OCS	Spathulenol	1551	t
47	O	Ethyl laureate	1580	0.8

N.	GCA	Compound name	RI	%
48	O	*n*-Tetradecanal	1596	0.3
49	OCS	T-Cadinol	1616	t
50	OCS	δ-Cadinol	1621	t
51	OCS	β-Eudesmol	1622	0.3
52	OCS	α-Cadinol	1630	0.8
53	SH	Cadalene	1640	t
54	O	*n*-Pentadecanal	1688	1.9
55	A	*n*-Heptadecane	1700	0.5
56	O	Methyl tetradecanoate	1706	0.3
57	FA	Tetradecanoic acid (= Myristic acid)	1723	2.1
58	O	Ethyl tetradecanoate	1774	1.1
59	O	*n*-Hexadecanal	1776	t
60	A	*n*-Nonadecane	1900	1.0
61	O	Methyl hexadecanoate (= Methyl palmitate)	1904	0.5
62	FA	Hexadecanoic acid (= Palmitic acid)	1908	8.2
63	O	Ethyl hexadecanoate (= Ethyl palmitate)	1936	4.9
64	O	*n*-Octadecanal	2008	1.0
65	O	*n*-Octadecanol	2095	1.7
66	FA	Oleic acid	2108	1.9
67	O	Ethyl linoleate	2119	5.5
68	O	Ethyl oleate	2151	t
69	O	Ethyl stearate (= Ethyl octadecanoate)	2163	0.4
70	A	*n*-Tricosane	2300	2.0
71	A	*n*-Tetracosane	2400	0.4
72	A	*n*-Pentacosane	2500	2.4
73	A	*n*-Hexacosane	2600	0.2
74	A	*n*-Heptacosane	2700	1.1
75	A	*n*-Nonacosane	2900	0.6
76	A	*n*-Hentriacontane	3100	0.6
		% Identification		72.9
		Grouped compounds		
	MO	Monoterpene hydrocarbons		t
	OCM	Oxygen-containing monoterpenes		1.0
	SH	Sesquiterpene hydrocarbons		7.4
	OCS	Oxygen-containing sesquiterpenes		1.1
	FA	Fatty acids		13.7
	A	Alkanes		9.8
	O	Others		39.9

RI: Retention Index relative to C7-C31 *n*-alkanes on a DB-1column. GCA: Grouped components abbreviation. * Identification based on mass spectra only**. t: trace amount (< 0.05%).**

Table 4.3. VOCs identified in *Euphorbia* species and reported in the current literature

Species	Country of plant origin	Plant organ or exudate	Extraction/ collection	Time (h)	Analytical method	Compounds (≥ 5%)	Reference
E. acanthothamnos	Greece	F	H	3	GC-MS	Phytol (28), Phytol acetate (9), β-Caryophyllene (8), Tricosane (5)	(67)
E. apios	Greece	F	H	3	GC-MS	Germacrene D (30), Heptacosane (13), β-Caryophyllene (10), Tirocsane (7), Pentacosane (6)	(67)
E. caracasana	Venezuela	L	H	4	GC-FID GC-MS	β-Caryophyllene (39), Germacrene-D (22), α-Copaene (9), α-Humulene (5)	(68)
E. characias	Greece	F	H	3	GC-MS	Nonanal (23), Phytol (14), Pentacosane (9), Heptacosane (7), Hexadecanoic acid (6), Nonacosane (6)	(67)
E. convolvuloides	Ivory Coast	AP_{bloom}	H	6	GC-FID GC-MS	Phytol (21), Caryophyllene oxide (13), Dimethoxyageratochromone (12), Hexahydrofarnesyl acetone (12), β-Caryophyllene (6), *n*-Hexadecanoic acid (5), Spathulenol (5)	(69)
E. cotinifolia	Venezuela	L	H	4	GC-FID GC-MS	β-Caryophyllene (34), α-Humulene (19), Aromadendrene (8), α-Selinene (8), Vianol (7), Caryophyllene oxide (6), Junipene (5), Phytol (5)	(68)
E. dendroides	Greece	F	H	3	GC-MS	Unknown m/z 272 (13), Heptacosane (11), 4-Terpineol (6), Pentacosane (6), Tricosane (5)	(67)
E. densa	Syria	AP_{veg}	H	3	GC-MS	1,8-Cineole (19), Linalool (14), Carvacrol (13), (E)-Caryophyllene (10), α-Pinene (5)	(70)
E. fischeriana	China	R	H	2.5	GC-FID GC-MS	*β*- Eudesmol (18), Caryophyllene oxide (9), *p*-Menth-8-en-2-ol (9), β-Selinenol (7)	(71)
E. gaillardotii	Turkey	AP_{veg}	H	3	GC-FID GC-MS	Arachidic acid (32), Hexatriacontane (9), Hexadecanoic acid (8), Mint furanone (8), Octadecane (6), Tetratetracontane (6), α-Selinene (5)	(72)

E. hebecarpa	Iran	AP_{bloom}	H	3	GC-FID GC-MS	α-Bisabolol (31), *cis*-Cadin-4-en-7-ol (20), *trans*-Piperitol (9), *cis-p*-Menth-2-en-1-ol (6), *trans-p*-Menth-2-en-1-ol (6)	(73)
E. helioscopia	Greece	F	H	3	GC-MS	Phytol (21), β-Caryophyllene (10), Docosanoic acid, methyl ester (8), Tetracosanoic acid methyl ester (6), Heptacosane (5), 9-Tricosene (5)	(67)
E. helioscopia	UK	Latex	SPME (80 °C)	0.5	GC-MS	β-Caryophyllene (45-59), (+)-Epi-bicyclosesquiphellandrene (12-41) *	(74)
E. helioscopia	China	AP_{veg}	H	6	GC-FID GC-MS	1,6-Dihydrocarveol (31), Carvone (17), Menthol (8), *trans*-Dihydrocarvone (6)	(75)
E. helioscopia	Egypt	F	H	3	GC-MS	Thymol (48), Caryophyllene (24), Caffeic acid (7), Carvacrol (7), Caryophyllene oxide (6)	(76)
E. heterophylla	Egypt	AP_{veg}	H	3	GC-MS	1,8-Cineole (32), Camphor (17), β-Elemene (6), *endo*-Borneol (5)	(77)
E. heterophylla	Ivory Coast	AP_{bloom}	H	6	GC-FID GC-MS	Caryophyllene oxide (15), *n*-Hexadecanoic acid (13), Hexahydrofarnesyl acetone (8), Dimethoxyageratochromone (7), Phytol (5)	(69)
E. heterophylla	Nigeria	L	H	3.5	GC-MS	3,7,12,15-Tetramethyl-2-hexadecen-1-ol (12), Octadecanoic acid (11), Oleic acid (10), *cis-cis*-Linoleic acid (9), 1, 2-Epoxy-cyclododecane (8), 13-Tetradece-11-yn-1-ol (8), *cis-cis*-7,10-hexadecadienal (8), 1,2-Benzene dicarboxylic acid diisooctyl (7), 1,2,15,16-Diepoxyhexadecane (6), Phytol (6), Palmitin or 2-monopalmitic acid (5), Aminoethoxyethanediylester or ethynediyl (5), Methyl ester (5), Phathalic acid, Butyl tetradecyl ester (5), Hexadecanal (5)	(78)
E. heterophylla	Nigeria	S	H	3.5	GC-MS	Octadecanoic acid (11), Oleic acid (10), *cis-cis*-Linoleic acid (9), 1, 2-Epoxy-Cyclododecane (8), 13-Tetradece-11-yn-1-ol (8), *cis-cis*-7 ,10-	(78)

						Hexadecadienal (7), 1, 2, 15, 16-Diepoxyhexadecane (6), Phytol (6), Palmitin or 2-Monopalmitic acid (6), 2-Aminoethoxyethynediyl methyleste (6), Methyl ester (5), Phathalic acid, Butyl tetradecyl ester (5)	
E. hirta	Ivory Coast	AP_{bloom}	H	6	GC-FID GC-MS	Dimethoxyageratochromone (31), *n*-Hexadecanoic acid (18), Caryophyllene oxide (10), Hexahydrofarnesyl acetone (6), Selin-11-en-4 α-ol (6), β-Caryophyllene (5)	(69)
E. intisy	Italy	S	H	3	GC-MS	Heptacosane (16), Phytol (8), Unknown m/z 161 (5)	(79)
E. macroclada	Turkey	AP_{veg}	H	3	GC-FID GC-MS	Tetratetracontane (43), Hexatriacontane (12), α-Selinene (7), Mint furanone (6)	(72)
E. macrorrhiza	China	AP_{veg}	H	4	GC-MS	Acorenone B (17), (+)-Cycloisosativene (15), 3a-Hydroxy-5b-androstane (11), β-Cedrene (8), Copaene (7), Palmitinic acid (6)	(80)
E. macrorrhiza	China	R	H	4	GC-MS	Acorenone B (26), (+)-Cycloisosativene (12),β-Cedrene (8), Copaene (6), 3a-Hydroxy-5b-androstane (6), 1-Calamenene (5),	(80)
E. mauritanica	Egypt	WP	H	3	GC-MS	(3*E*)-Cembrene A (19), Verticiol (17), Limonene (8), Eucalyptol (7), α-Pinene (6)	(81)
E. peplus	United Kingdom	Latex	SPME (80 °C)	0.5	GC-MS	α-Copaene (3-15), β-Caryophyllene (18-72), α-Humulene (1-6), (+)-Epi-bicyclosesquiphellandrene (nd-17) *	(74)
E. pilosa	India	AP_{bloom}	H	6	GC-FID GC-MS	Phytol (6), n-Pentadecanal (5)	(82)

E. rigida	Greece	F	H	3	GC-MS	Heneicosane (14), Heptacosane (13), β-Caryophyllene (9), Linalool (7), Pentacosane (7), Germacrene D (5)	(67)
E. sanctae-catharinae	Egypt	AP_{veg}	H	3	GC-MS	Valencene (16), (+) Spathulenol (15), (-)-Caryophyllene oxide (11), Limonene (8)	(83)
E. teheranica	Iran	AP_{bloom}	H	3	GC-MS	Elemol (58), β-Caryophyllene (8), Caryophyllene (8)	(67)

A Pbloom: aerial parts during plant blooming period; A Pveg: aerial parts during plant vegetative growth; F: Floral cyathia; L: leaves; S: stems; R: roots; WP: whole plant. Compound concentrations are reported between squared brackets. The presence of an asterisk indicates that the concentration of identified compounds varied depending upon place and duration of VOC collection. Only compounds with a concentration ≥ 5% are reported in the table.

3.5. Antioxidant activity

In the traditional medicine of Morocco, *E. resinifera* cyathium water decoctions have for a long time been utilized to reduce inflammations of the respiratory tract and the throat. Moreover, we have recently shown that *E. resinifera* honey has good antioxidant properties, possibly conferred by compounds that flowers secrete into nectar, which bees craft into honey (24). Therefore, by mimicking the traditional procedure utilized to prepare water decoctions, we obtained cyathium water extracts and assessed their antioxidant potential and inhibition of lipoxygenase enzyme activity (Figure 4.4).

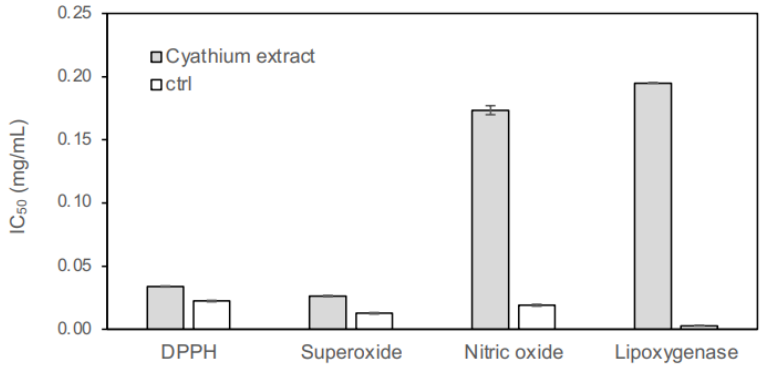

Figure 4.4. Antioxidant and anti-lipoxygenase potential of cyathium water extracts. Concentrations of water cyathium extract quenching 50% of DPPH, superoxide and nitric oxide radicals and inhibiting the activity of the lipoxygenase enzyme (grey bars). The corresponding concentrations obtained with BHT, ascorbic acid, Curcumin, and NDGA pure standards are represented with white bars. Bar plots and error bars represent the average of three replicates and the standard deviation, respectively.

The general radical scavenging potential of water cyathium extracts was assessed with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay using butylated hydroxytoluene (BHT) as a positive control, which reveled anti-DPPH activity with an IC_{50} of 0.0338 ± 0.0004 mg/mL. Anti-superoxide radical activity was instead determined using ascorbic acid as a positive control, which revealed an IC_{50} of 0.0263 ± 0.0004 mg/mL. These data showed that the overall quenching potential of cyathium extracts is very high, as it corresponds to two third and half the quenching capacity of BHT and ascorbic acid pure compounds, respectively. IC_{50} for anti-nitric oxide radical and anti-lipoxygenase activity were 0.1734 ± 0.0036 mg/mL and 0.1955 ± 0.0000 mg/mL, respectively. When IC_{50} of cyathium extracts were compared to the corresponding values measured in *E. resinifera* honey (24), it became evident that cyathia have remarkable antioxidant and lipoxygenase inhibition potential ranging hundred times higher than the honey. The antioxidant potential of cyathium extracts possibly resides in the abundant specialized compounds

synthetized and stored in floral cyanthia including carotenoids (Figure 4.3), vitamins (Figure 4.2D) and flavonoids (Table 4.1). Moreover, the presence of terpenes in floral extracts (Table 4.2), may contribute to alleviate mild infections given the antimicrobial properties of these compounds (38).

4. Conclusions

In this study we performed a comprehensive phytochemical screening of *E. resinifera* cyathia harvested in mid-July 2021 from the Beni Mellal-Khénifra region of Morocco. Our analyses revealed that these inflorescences produce a plethora of different classes of specialized metabolites including carotenoids, flavonoids, and PAs. Many of these compounds have antioxidant properties and exert direct protection toward abiotic stress, in particular high solar radiance and heat stress, as well as indirect protection towards the damaging effect of free radicals, of which the content increases when plants are exposed to agents of environmental disturbance. The presence of these compounds in floral cyathia mirrors the antioxidant properties of cyathium water extracts, which in the traditional medicine of Morocco have a centennial tradition of usage as water decoctions. Cyathia also contain elevated content of primary metabolites and vitamins which could also be secreted into nectar. Finally, the spectrum of VOCs includes compounds known to attract honeybee pollinators to flowers, such as benzaldehyde and nonanal. In conclusion, our analyses reveled that *E. resinifera* cyathia are a great source of antioxidant molecules and also represent a good source of food for the local foraging honeybees, so revelling the central role of flowers in mediating interactions with local pollinators and the conferral of medicinal properties to plant extracts.

Funding

This research was supported by funding from the Fundação para a Ciência e a Tecnologia (FCT/MCTES) Grant No. MED UIDB/05183/2020 to MGM and Grant No. CESAM UIDP/50017/2020, UIDB/50017/2020, and LA/P/0094/2020 to ACF.

Author contributions

MB conceived the research with inputs from MGM and ACF. OB, SJ, XZ, ACF and MB extracted and analyzed metabolites. OB performed the antioxidant assays. SAB, ARF, ACF, MGM, and MB provided analytical support and supervision. MB wrote the manuscript with inputs from OB, MGM and ACF. All authors edited and approved the final version of the manuscript.

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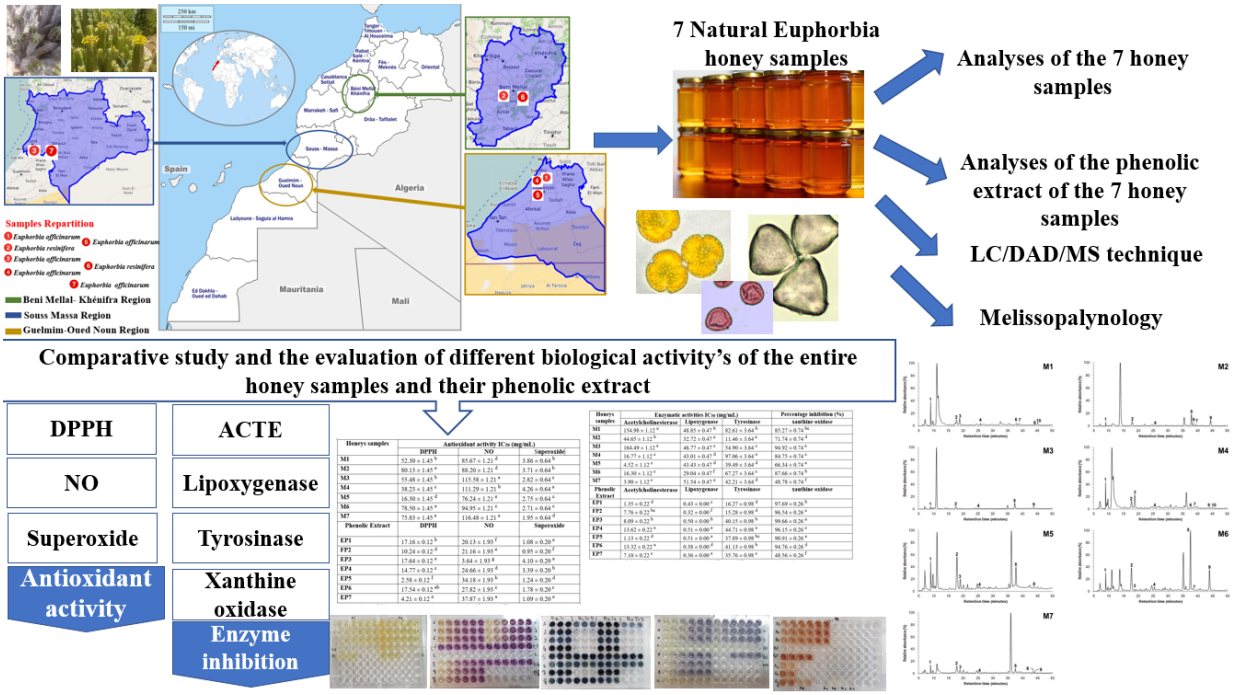
Chapter 5

Comparative study of the antioxidant and enzyme inhibitory activities of two types of Moroccan *Euphorbia* entire honey and their phenolic extracts



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Chapter V: Comparative study of the antioxidant and enzyme inhibitory activities of two types of Moroccan Euphorbia entire honey and their phenolic extracts*

Résumé

Le miel est un produit alimentaire naturel très réputé pour ses bienfaits pour la santé car il est une source importante de composés antioxydants et phénoliques. Des miels d'*Euphorbia* provenant de différentes régions du Maroc ont été évalués pour leur capacité à inhiber les enzymes acétylcholinestérase, lipoxygenase, tyrosinase et xanthine oxydase. Leurs propriétés antioxydantes ont été évaluées à l'aide de la capacité de piégeage des radicaux 2,2-diphényl-1-picrylhydrazyle (DPPH), de l'activité de piégeage de l'oxyde nitrique (NO) et de la capacité de piégeage du radical anion Superoxyde. Ensuite, les extraits phénoliques des mêmes échantillons de miels entiers ont été évalués par chromatographie liquide couplée à la détection par barrette de diodes et à la spectrométrie de masse et testés pour les activités biologiques préalablement évaluées sur les miels entiers, afin de réaliser une étude comparative étude entre les deux (miel et extraits phénoliques). Les profils chromatographiques des extraits de miel d'*Euphorbia* étudiés étaient différents. Des composés phénoliques tels que l'acide gallique, l'acide 4-hydroxybenzoïque et l'acide p-coumarique ont été détectés dans tous les échantillons, alors que le kampferol n'était présent que dans deux échantillons. Les paramètres physico-chimiques et la teneur phénolique totale ont également été déterminés. Le miel entier qui a enregistré le taux le plus élevé de phénols était l'échantillon M6 (E. resinifera) = 69,25 mg GAE/100g. En revanche, les meilleures activités antioxydante étaient : pour le DPPH : IC50 de l'échantillon PE5 (extrait phénolique de miel d'E. officinarum) = 2,58 mg/mL ; pour NO : IC50 de PE3 (extrait phénolique de miel d'E. officinarum) = 3,64 mg/mL ; pour la capacité de piégeage du Superoxyde : IC50 de PE2 = 0,95 mg/mL pour le miel d'Euphorbia resinifera. Concernant les activités enzymatiques, anti-acétylcholinestérase IC50 PE5 = 1,13 mg/mL E. officinarum ; inhibition de la lipoxygenase IC50 PE2 = 0,32 mg/mL E. resinifera ; anti-tyrosinase IC50 PE2 = 15,28 mg/mL E. resinifera ; et anti-xanthine oxydase IC50 PE3 = 99,66 E. officinarum, ont été enregistrées pour les extraits méthanoliques. En conclusion, les miels d'Euphorbia étudiés peuvent avoir un grand potentiel en tant que sources antioxydante pour des applications pharmaceutiques et cosmétiques.

Mots clés : Miel, composés phénoliques, activité antioxydante, *Euphorbia resinifera*, *Euphorbia officinarum*, profil phénolique

* Adapté de Oumaima Boutoub, Soukaina El-Guendouz, Ana Manhita, Cristiana Barrocas Dias, Letícia M. Estevinho, Vanessa B. Paula, Jorge Carlier, Maria C. Costa, Brígida Rodrigues, Sara Raposo, Smail Aazza, Lahsen El Ghadraoui, and Maria G. Miguel. Comparative study of the antioxidant and enzyme inhibitory activities of two types of Moroccan Euphorbia entire honey and their phenolic extracts. *Foods*. **2021**, DOI: 10.3390/foods10081909.

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Comparative study of the antioxidant and enzyme inhibitory activities of two types of Moroccan *Euphorbia* entire honey and their phenolic extracts*

Abstract

Honey is a natural food product very famous for its health benefits for being an important source of antioxidant and phenolic compounds. *Euphorbia* honeys obtained from different re-gions of Morocco were evaluated for their ability to inhibit acetylcholinesterase, lipoxygenase, tyrosinase and xanthine oxidase enzymes. Their antioxidant properties were evaluated using the: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity, nitric oxide scavenging activity (NO) and scavenging ability of superoxide anion radical. Then, the phenolic extracts of the same entire honey samples were evaluated by liquid chromatography coupled to diode array detection and mass spectrometry (LC-DAD-MS) and tested for the biological activities previously evaluated on the entire honeys, in order to conduct a comparative study between both (honey and phenolic extracts). The chromatographic profiles for the studied *Euphorbia* honey extracts were different. Phenolic compounds gallic acid, 4-hydroxybenzoic acid and *p*-coumaric acid were detected in all samples, whereas kampferol was only present in two samples. Physico-chemical parameters and total phenolic content were also determined. Entire honey that has recorded the highest rate of phenols was sample M6 (*E. resinifera*) = 69.25 mg GAE/100 g. On the other hand, the best antioxidant activities were: for DPPH: IC_{50} of sample PE5 (phenolic extract of *E. officinarum* honey) = 2.58 mg/mL; for NO: IC_{50} of PE3 (phenolic extract of honey) = 3.64 mg/mL; for superoxide scavenging ability: IC_{50} of PE2 = 0.95 mg/mL for *E. resinifera* honey. Regarding the enzymatic activities, anti-acetylcholinesterase IC_{50} PE5 = 1.13 mg/mL *E. officinarum*; lipoxygenase inhibition IC_{50} PE2 = 0.32 mg/mL *E. resinifera*; anti-tyrosinase IC_{50} PE2 = 15.28 mg/mL *E. resinifera*; and anti-xanthine oxidase IC_{50} PE3 = 99.66 *E. officinarum*, have been recorded for methanolic extracts. In conclusion, the studied *Euphorbia* honeys may have a great potential as antioxidant sources for pharmaceutical and cosmetic applications.

Keywords: Honey, phenolic compounds, antioxidant activity, *Euphorbia resinifera*, Euphorbia officinarum, phenolic profile

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1. Introduction

The Green Morocco Plan has permitted a sustainable development of the agricultural sector of this country contributing to increase its added value. The country has established programs in order to leave the subsistence farming, focusing on the promotion of the specific products of the territories, identifying hundreds of local products and, consequently, labeling them under Geographical Indications, Designations of Origin or Agricultural Labels. These labeled products include fresh and dried fruits, medicinal and aromatic plants, olive and argan oils and animal origin products such as honey (1). Three labeled honeys were registered, being two of them of *Euphorbia* monofloral origin: geographical indication "honey of desert *Euphorbia*" and protected geographical indication "*Euphorbia* honey of Tadla-Azilal" (2). The organoleptic characteristics for the former are the color dark amber, dry herbal, wax and spicy taste, and a permanent, intense and prickly aftertaste; and for the last one the color is dark golden, bitter and peppery at the throat taste (2). In both cases, the plant species is not provided although three monofloral *Euphorbia* honeys can be found in Morocco: *Euphorbia officinarum* subsp. *echinus*, *E. regis-jubae*, and *E. resinifera*. *E. resinifera* is an endemic species of Morocco mainly distributed in Azilal and Beni Mellal regions (Middle Atlas), whereas *E. officinarum* subsp. *echinus* and *E. regis-jubae* can be found in the south-western region (3).

Beyond the nutritive aspect of honeys, *Euphorbia* honey has been target of study due to its economic importance in Morocco, thereby investigation has increased on the melissopalynological and physico-chemical characterization (4–8) and biological properties with potential application on human health (9–11). Anti-inflammatory, analgesic and antimicrobial properties have been attributed to *Euphorbia* honeys along with the ability to promote wound healing, nevertheless, the majority of works has been focused on the anti-microbial activity (9–11), and much less, antioxidant activity (12). More recently, our team compared the antioxidant activity and some enzyme inhibitory activities of *E. officinarum*, *E. resinifera* plants with those of the respective two monofloral honeys (13). This work permitted detected relative high amounts of Al, Cu and Fe in *E. officinarum* honey, suggesting environmental pollution and/or inadequate storage of honey.

In continuation of our studies, the present work aims to evaluate the antioxidant, anti-inflammatory, anti-acetylcholinesterase, anti-tyrosinase and anti-xanthine oxidase activities of seven entire *E. resinifera* and *E. officinarum* honeys from Morocco as well as their phenolic extracts for better understanding if the activities are due to the entire honey and/or to their secondary metabolites.

2. Experimental

2.1. Honey samples

Seven (n = 7) unifloral honey samples of *Euphorbia* sp. were acquired directly from the beekeepers between June and July 2018. These samples were stored in dark at room temperature until time of testing, no more than two months after collection. The Figure 5.1 represents the regions of harvest and the palynological classification of the samples.

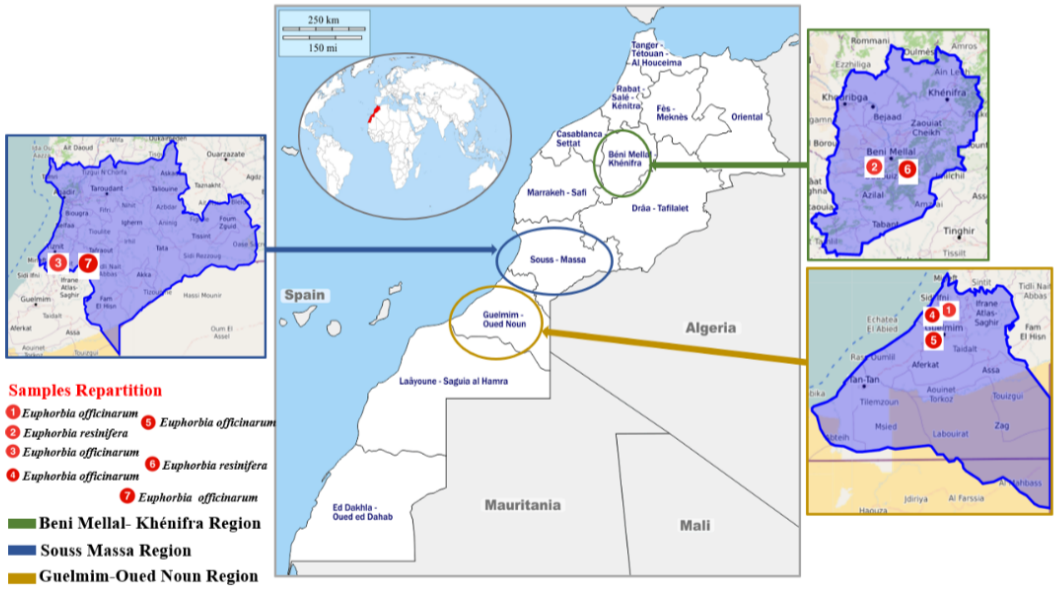

Figure 5.1. Map showing the location of the apiaries in which samples were collected.

2.2. Melissopalynological analysis

Honey samples pollen qualitative and quantitative analysis was performed according to the International Commission for Bee Botany (ICBB) (14).

2.3. Physico-chemical parameters of honey

Free acidity, lactonic acidity, total acidity, pH, ash content, electrical conductivity, moisture, proline content, diastase activity, HMF content, reducing sugars have been determined according to the methods used by Bogdanov (15).

2.3.1. Free acidity

The sample solutions were neutralized with a standard solution of sodium hydroxide (0.1 M) to pH 8.30 using a potentiometer combined with glass electrode (Thermo Electron Corporation, Orion 3 STAR; Beverly, MA, USA).

2.3.2. Lactonic acidity

The lactonic acidity was determined by adding the excess of sodium hydroxide (0.05 M) to the honey solution and back titrated with sulfuric acid (0.025 M), using a potentiometer combined with glass electrode (Thermo Electron Corporation, Orion 3 STAR; Beverly, MA, USA).

2.3.3. Total acidity

Total acidity is obtained by making the sum of free acidity plus lactonic acidity.

2.3.4. pH

The pH was measured using a pH-meter with glass electrode (Thermo Electron Corporation, Orion 3 STAR; Beverly, MA, USA) from the honey solution prepared in CO_2-free distilled water.

2.3.5. Ash content

Five grams of the honey sample were put in a porcelain dish and burned in a temperature between 350–400 ◦C in electric furnace for at least 2 h, after cooling the porcelain dish was placed in desiccator and weighted.

2.3.6. Electric conductivity

This parameter was obtained by using a conductivity meter (Thermo Electron Corporation, Orion 3 STAR; USA) in the aqueous honey solution.

2.3.7. Water content (moisture)

Water content (moisture %) was determined with a Abbe refractometer (HANNA HI96801, HANNA Instruments, Nus, falău, Romania) at 20 ◦C and using the Wedmore's Table.

2.3.8. Proline content

For proline content, 5 g of honey was dissolved in 100 mL of distilled water in a volumetric flask. To carry out this test it is necessary to have three test tubes for each sample. The first tube (blank test) contains 500 µL of distilled water mixed with 1 mL of formic acid (H.COOH) (98%) and 1 mL ninhydrin solution (3%) in ethylene glycol monomethyl ether. The second tube (sample test), 500 µL of honey solution was mixed with 1 mL of formic acid (98%) and 1 mL of ninhydrin solution (3%). The last test tube contains 500 µL of proline solution standard (0.8 mg/25 mL) mixed with 1 mL of formic acid (98%) and 1 mL of ninhydrin solution (3%). The three tubes were shaked for 15 min and put in a water bath for 15 min at 70 ◦C, by the end 5 mL of 2-propanol 50% were added and leave it to cool for 45 min, the absorbance was measured at λ = 510 nm, using a spectrophotometer Shimadzu 160-UV (Shimadzu Europe GmbH, Duisburg, Germany).

2.3.9. Diastase activity

Diastase activity (Shade units/g) was determined by weighing 10 g of honey dissolved in 15 mL and 5 mL of acetate buffer (pH 5.3), the mixture was transferred to 50 mL volumetric flask containing 3 mL of sodium chloride solution (2.9 g/100) and adjust the volume to the mark with water. Ten mL of this solution was measured and introduced into a 50 mL of flask and left at 40 ºC in a water bath along with a second flask with the same volume of starch solution. After 15 min, 5 mL of starch solution is pipetted into the honey solution. At periodic intervals, 0.5 mL of the mixture is pipetted for other tube and 5 mL of diluted iodine solution, and a volume water previously determined and the absorbance read at $\lambda = 660$ nm.

2.3.10. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural was determined by clarifying honey samples with Carrez solution (I and II) with a sodium bisulphite solution (0.20 g/100 g) and the absorbance was measured at $\lambda = 284$ nm.

2.3.11. Reducing sugars percentage

Reducing sugars percentage in each honey sample were determined with the titration in 2-time assay (preliminary assay and definitive assay) using the solutions: 5 mL of A and 5 mL of B. Fehling solution A: 69.28 g of copper sulphate pentahydrate ($CuSO_4$ $5H_2O$) in 1000 mL distilled water; Fehling solution B: 346 g of sodium potassium tartrate ($C_4H_4NaO_5$,$4H_2O$) and 100 g of sodium hydroxide (NaOH) in 1000 mL distilled water. Five mL of each solution were measured into an Erlenmeyer flask and from a burette, the honey solution was left drop until the upper phase remains colorless.

2.4. Estimation of honey colour

The colour of honey was evaluated as reported by Aazza et al. (16). A solution of 1 g in 2 mL of distilled water was prepared and the absorbance measured at $\lambda = 635$ nm (A_{635}). The colour was calculated using the equation: mm Pfund = $-38.7 + 371.39 \times A635$

2.5. Determination of mineral elements of honey samples

The mineral elements quantified were Fe, Zn, Mn, Cu, Al, Ca, K, Mg, Na. Five grams of honey were calcined at 550 ◦C and after cooling 5 mL of nitric acid 0.1 M were added, shaked and heated until completely dry on a hot plate. Ten mL of nitric acid 0.1 M was added and make up to 25 mL with distilled water (17). For the Ca, Mg, Mn, Zn, Cu, and Fe, the measurement was done through flame atomic absorption spectroscopy air-acetylene using a novAA 350 (Analytik Jena, Jena, Germany), while for the analysis of Na, K and Al, microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent, Santa Clara, CA, USA) was used. The concentrations were expressed as mg/ kg honey.

2.6. Carbohydrate content of honey samples by high performance liquid chromatography (HPLC)

The sugar quantification was done using a chromatograph Hitachi LaChrom Elite HPLC, Japan, equipped with a refractive index detector (Hitachi L-2490, Tokyo, Japan). The column used was a Purospher STAR NH2 (5 µm particle size) (Merck, Darmstadt, Germany). The separation of carbohydrates was achieved with an isocratic elution having as mobile phase acetonitrile and water (85:15, v/v), at room temperature. The preparation of honey samples and the quantification of carbohydrates was done according to the methodology previously reported (3).

2.7. Samples extraction for the determination of phenolic compounds

This study relates to the comparison between the seven *Euphorbia* entire honeys and their phenolic extracts. Accordingly, 5 g of each entire honey sample was diluted in 10 mL of distillated water in the day of the assay in order to study the antioxidant and enzymatic inhibitory activities. On the other hand, the extraction of phenolic compounds for the seven honey samples was performed according to procedures previously described (18) with slight modifications: 10 g of each honey sample was dissolved in 50 mL of distilled water and mixed well for 5 min using a vortex. In each flask solution, 50 mL of ethyl acetate were added and were placed for 45 min in a shaker (1700 rpm) (Edmund Bühler, TH 15, Bodelshausen, Germany). The mixture was then transferred to a separating funnel for separation of the phases. The liquid-liquid extraction was repeated three more times. Then, the combined ethyl acetate extracts were evaporated using a vacuum rotary evaporator (Heidolph, 94200, BIOBLOCK SCIENTIFIC, Schwabach, Germany) at 36 ◦C, after that the residue was collected in methanol (5 mL). The phenol content and their identification as well as the antioxidant and enzymatic activities were subsequently determined for each phenolic extract.

2.8. Total phenol content

The total polyphenol content in honey as well as in the methanolic extract were determined as stated by Boutoub et al. (13). The total polyphenol content was expressed as mg gallic acid equivalents per 100 g (mg GAE/100 g).

2.9. Liquid Chromatography Coupled to Diode Array Detection and Mass Spectrometry (LC/DAD/MS) for the identification of phenolic compounds

Five µL of the methanolic extracts M1–M7 prepared in step 2.7. (‘Samples extraction for the determination of phenolic compounds’) were analysed by LC/DAD/MS technique. The extracts were previously filtered using a 0.45 µm PTFE syringe filter. A mass spectrometer (LCQ Fleet, Thermo Finnigan, San Francisco, CA, USA), equipped with an electrospray ionization (ESI)

source and an ion trap mass analyser was used. MS conditions for the analysis were the following: 300 ◦C for capillary temperature, 5.0 kV for source voltage, 100.0 µA for source current and 3.0 V for capillary voltage, in negative ion mode. Samples were analysed both in full MS mode (m/z 50–800), and in Selective Reaction Monitoring (SRM) mode (normalized collision energy of 30%). Selected reactions are presented in the Section 3.6 and were optimized using standards available in the laboratory. The MS equipment was coupled to an LC system (Surveyor, Thermo Finnigan, San Francisco, CA, USA) with an autosamplerand a diode array detector (DAD). A reversed-phase analytical column was used (Zorbax Eclipse XDB C18, Rapid Resolution, particle size 3.5 µm, 150 mm 4.6 mm, Agilent Technologies, Santa Clara, CA, USA). Tray temperature was set at 24 ◦C and column temperature was set at 30 ◦C. Chromatographic separation was performed at a flow rate of 0.2 mL/min. The mobile phase was composed by 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) with the following elu- tion programme: 0.0% B (0–5 min), 0.0–10.0% B (5–20 min) and 10.0–50.0% B (20–60 min). DAD detector was programmed to acquire data from 200 to 800 nm.

2.10. Antioxidant activity

2.10.1. (2,2-Diphenyl-1-picrylhydrazy) DPPH scavenging activity

Free radical scavenging activity determination by DPPH scavenging activity was performed as described by Boutoub et al. (13). The volumes used for phenolic extract and honey samples were 25 and 200 µL, respectively. The percentage of inhibition was determined using the formula: Percentage of inhibition = ((A_0 A_1)/A_0 x 100); with A_0 representing the absorbance of the control and A_1 the absorbance of the sample. The sample concentration providing 50% inhibition (IC_{50}) was achieved by plotting the inhibition percentage against extracts concentrations.

2.10.2. Nitric oxide scavenging activity

The nitric oxide (NO) scavenging activity was carried out according to Boutoub et al. (13). In both phenolic extract and honey sample the volume used was 150 µL. The IC_{50} values were obtained as aforementioned.

2.10.3. Scavenging ability of superoxide anion radical

Scavenging ability of superoxide anion radical was assayed as reported by Boutoub et al. (13). In both phenolic extract and honey sample the volume used was 25 µL. The IC_{50} values were obtained as aforementioned.

2.11. Enzymatic activities

2.11.1. Inhibition of acetylcholinesterase

The acetylcholinesterase inhibition was carried out with few modifications as reported by Boutoub et al. (13). For phenolic extract 25 µL were used while for honey sample the volume was 300 µL. The percentage of inhibition of acetylcholinesterase activity was determined and the (IC_{50}) value was calculated.

2.11.2. Inhibition of lipoxygenase

The lipoxygenase assay is used as an indicator of anti-inflammatory and antioxidant activity (19). The inhibition action of honey solution and plant extract was determined as reported by Boutoub et al. (13) with some modifications. In short, 25 µL of phenolic extract were used while for honey solution, the volume was 150 µL. The results were expressed as IC_{50} value.

2.11.3. Inhibition of tyrosinase

The tyrosinase activity was determined based on the protocol reported by El-Guendouz et al. (20) with slight modification. The total assay mixture consisting on 50 µL of honey solution and 25 µL of phenolic extract was used for this activity. The results were expressed as IC_{50} value.

2.11.4. Inhibition of xanthine oxidase

The inhibitory activity of seven phenolic extracts and their honey solution was determined as described by El-Guendouz et al. (19), but using 25 µL of methanolic extract and 150 µL of honey solution. The results were expressed as IC_{50} value.

3. Results and Discussion

3.1. Pollen analysis

The microscopic analysis of the sediment (Table 5.1) showed that the predominant pollen grains of the two samples (M2 and M6) from Beni Mellal-Khénifra region was *E. resinifera*, with a percentage of 48% for M2 and 45% for M6, being both classified as monofloral. These findings are in accordance to those recently reported (7), in which lower than 50% of *E. resinifera* pollen could be found in more than 20 samples studied. The second most prevalent pollen types were *Caesalpinia pulcherrima* (21.80%) and *Genista hirsuta* (12.83%) in M2 and M6 honeys, respectively. Moreover, *C. pulcherrima* pollen was absent in sample M6. In contrast to M2, in M6 sample, pollen of *E. officinarum* was found (5.93%). These accompanying pollen species are not the same previously reported (7).

Table 5.1. Honey samples, place, and the most predominant pollen of seven *Euphorbia* honey samples from Morocco. The results of M2 and M3 samples were previously published (13). Reproduced with permission from Oumaima Boutoub et al., Antioxidant activity and enzyme inhibitory potential of *E. resinifera* and *E. officinarum* honeys from Morocco andplant aqueous extracts.

Honey	Honey type	Pollen species (%)	Region of production	Coordinates
M1	*Euphorbia officinarum*	*Euphorbia officinarum* 55.67 ± 1.78 *Quercus rotindifolia* 10.31 ± 0.35 *Genista hirsuta* 6.88 ± 079 *Thymus lotocephalus* 4.95 ± 0.2 *Cistus albidus* 4.08 ± 0.47 *Ilex aquifolium* 2.97 ± 0.10 *Malus sylvestris* 2.9 ± 0.15 *Malus domestica* 2.83 ± 0.75 *Eucalyptus cinereae* 2.32 ± 0.34 *Cistus crepis* 2.1 ± 0.35 *Campanula pimulifolia* 1.64 ± 0.11 *Lavandula viridis* 1.46 ± 0.13	Guelmim-Oued Noun	28° 27′ N, 10 ° 07 ′ W
M2	*Euphorbia resinifera*	*Euphorbia resinifera* 48.7 ± 1.1 *Caesalpinia pulcherrima* 21.8 ± 1.3 *Malvus domestica* 10.2± 0.4 *Cistus crepis* 7.9 ± 0.9 *Populus nigra* 4.0 ± 0.3 *Genista hirsuta* 2.9 ± 0.2 *Populus alba* 1.9 ± 0.2 *Ilex aquifolium* 2.6 ± 0.3	Beni Mellal-Khénifra	32 ° 22 ′ 06 ″ N, 6 ° 22 ′ 09 ″ W
M3	*Euphorbia officinarum*	*Euphorbia officinarum* 52.1 ± 1.6 *Caesalpinia pulcherrima* 11.8± 0.7 *Arbutus unedo* 6.1 ± 1.2 *Populus alba* 5.8 ± 0.8 *Pinus pinaster* 5.0 ± 0.2 *Eucalyptus globulus* 3.3 ± 0.6 *Malvus domestica* 3.0 ± 0.2 *Thymus lotocephalus* 2.4 ± 0.3 *Quercus suber* 2.0 ± 0.1 *Eucalyptus cinereae* 1.9 ± 0.2 *Populus nigra* 1.8 ± 0.3 *Caesalpinia spinosa* 1.7 ± 0.1 *Cistus albidus* 1.7 ± 0.2 *Trifolium arvense* 1.5 ± 0.3	Souss-Massa	30 ° 04 ′ 48 ″ N, 8 ° 28 ′ 48 ″ W
M4	*Euphorbia officinarum*	*Euphorbia officinarum* 51.78 ± 2.11 *Caesalpinia spinosa* 14.95 ± 1.84 *Arbustus unedo* 9.93 ± 0.61 *Cistus crepis* 9.89 ± 0.57 *Cistus populis* 5.62 ± 0.61 *Eucaliptus globulus* 5.01 ± 0.45 *Populus alva* 2.84 ± 0.39	Guelmim-Oued Noun	28° 27′ N, 10 ° 07 ′ W
M5	*Euphorbia officinarum*	*Euphorbia officinarum* 51.51 ± 2.53 *Quercus suber* 17.74 ± 2.01 *Quercus rotindifolia* 10.34 ± 0.11 *Caesalpinia spinosa* 6.91 ± 0.35 *Olea europaceae* 4.81 ± 0.27 *Trifolium arvenses* 4.32 ± 0.35 *Populus alva* 2.88 ± 0.21 *Malus domestica* 1.51 ± 0.06	Guelmim-Oued Noun	28° 27′ N, 10 ° 07 ′ W
M6	*Euphorbia resinifera*	*Euphorbia resinifera* 45.58 ± 1.98 *Genista hirsuta* 12.83 ± 0.38 *Salvia officinalis* 6.06 ± 0.72 *Euphorbia officinarum* 5.93 ± 0.15 *Cistus populis* 5.35 ± 0.68 *Malus domestica* 5.11 ± 0.33 *Cistus crepis* 3.22 ± 0.25 *Cistus albidus* 4.22 ± 0.33 *Quercus rotindifolia* 4.17 ± 0.39 *Campanula pimulifolia* 2.96 ± 0.07 *Populus alva* 2.40 ± 0.17 *Malus sylvestris* 2.17 ± 0.39	Beni Mellal-Khénifra	32 ° 22 ′ 06 ″ N, 6 ° 22 ′ 09 ″ W
M7	*Euphorbia officinarum*	*Euphorbia officinarum* 44.22 ± 4.57 *Pinus pinaster* 14.16 ± 1.82	Souss-Massa	30 ° 04 ′ 48 ″ N, 8 ° 28 ′ 48 ″ W

Caesalpinia pulcherrima 6.33 ± 0.35
Malus domestica 6.22 ± 0.35
Quercus suber 4.99 ± 0.24
Eucalyptus globulus 4.73 ± 0.73
Caesalpinia spinosa 4.35 ± 0.9
Cistus crepis 3.62 ± 0.16
Artenisa vulgaris 3.15 ± 0.72
Salvia officinalis 2.5 ± 0.15
Asparagus albus 2.05 ± 0.27
Populus nigra 1.90 ± 0.12
Lavandula viridis 1.81 ± 0.24

Regarding the other five samples (M1, M3, M4, M5, M7), the analysis showed the dominance of the pollen species of *E. officinarum*. The M7 sample from the Souss-Massa region (Table 5.1), more exactly Tiznit (Figure 5.1), recorded a value of 42.22% of the predominantly *E. officinarum* pollen species, on the other hand the M1 sample collected in the region of Guelmim-Oued Noun and closer to Ait Baamrane (Figure 5.1), recorded a value of 55.70% asthe highest percentage of *E. officinarum* pollen. The second pollen types comprised between10.3 and 17.74% were from *Quercus rotindifolia*, *Caesalpinia pulcherrima*, *C. spinosa*, *Quercus suber*, and *Pinus pinaster* (Table 5.1) sequentially regarding samples M1, M3, M4, M5and M7

3.2. Physicochemical parameters

The results obtained for the physicochemical parameters are summarized in Table 5.2. The pH values ranged from 3.74 to 4.08 with an average value of 3.86 to the seven samples. Our results are in agreement with those of Moujanni et al. (4), generally the pH value of *E. resinifera* honey is slightly higher than that of *E. officinarum* honey.

The acidity of honey is due to the presence of organic acids, produced from nectar during the maturation by glucose oxidase (4), and also to the organic acids such as gluconic acid and their lactones and esters (6). Concerning the free acidity values (Table 5.2.), they ranged from 10.08 to 19.04 meq/kg, all values below the Codex Alimentarius Commission (21) limit of tolerance (50 meq/kg). The results obtained were lower comparing with the results found by Bettar et al. (6) with values ranging from 16 to 80 meq/kg.

Table 5.2. Physicochemical parameters of Moroccan *Euphorbia* honeys. *Euphorbia officinarum* (samples M1, M3, M4, M5, M7) and *E. resinifera* (samples M2, M6).

	pH	Moisture (%)	Diastase (Shade units/g)	Proline (mg/kg)	Conductivity (µS/cm)	Ash (%)	HMF (mg/kg)	Colour (mm)	Free acidity (meq/kg)	Lactonic acidity (meq/kg)	Total acidity (meq/kg)	Reducing sugar (%)
M1	3.88±0.09^{bc}	19.63±0.00^{b}	11.93±1.23^{e}	584.96±42.05^{d}	553.67±0.57^{a}	0.17±0.00^{a}	51.74±0.01^{b}	136.84±0.48^{f} **Dark Amber**	15.68±1.75^{b}	7.84±2.92^{c}	23.52±2.88^{bc}	61.67±0.00^{c}
M2	4.08±0.04^{a}	18.62±0.00^{e}	37.36±1.46^{d}	953.94±36.50^{bc}	379.33±0.57^{f}	0.14±0.00^{c}	2.29±0.00^{d}	407.59±0.84^{b} **Dark Amber**	10.08±1.83^{d}	7.68±1.25^{c}	17.76±1.44^{d}	66.67±0.00^{b}
M3	4.06±0.02^{a}	19.00±0.00^{cd}	13.19±1.33^{e}	729.56±43.27^{cd}	342.33±1.52^{g}	0.14±0.00^{bc}	80.42±0.13^{a}	294.68±1.28^{c} **Dark Amber**	10.64±2.04^{cd}	10.96±0.91^{c}	21.60±2.19^{cd}	61.67±0.01^{c}
M4	3.40±0.02^{ab}	19.06±0.00^{c}	11.43±1.20^{e}	692.48±26.69^{d}	449.00±1.00^{d}	0.17±0.00^{ab}	41.02±0.14^{c}	191.56±0.84^{d} **Dark Amber**	12.60±0.37^{c}	10.44±1.61^{c}	23.04±0.83^{bc}	61.67±0.00^{c}
M5	3.84±0.01^{cd}	20.00±0.00^{a}	49.61±0.75^{c}	1169.45±18.9^{b}	514.00±1.00^{b}	0.19±0.00^{a}	7.04±0.14^{d}	144.90±0.84^{e} **Dark Amber**	19.04±0.77^{a}	15.60±0.43^{b}	34.08±0.83^{a}	61.00±0.01^{c}
M6	4.01±0.01^{a}	18.73±0.00^{de}	115.89±1.77^{a}	1485.71±23.6^{a}	455.33±0.57^{c}	0.18±0.00^{a}	2.40±0.00^{d}	510.96±0.48^{a} **Dark Amber**	17.36±0.37^{ab}	20.56±1.51^{a}	37.92±1.44^{a}	70.67±0.00^{a}
M7	3.75±0.01^{d}	19.13±0.00^{c}	103.23±2.42^{b}	962.80±44.43^{bc}	403.67±0.57^{e}	0.13±0.00^{c}	5.89±0.00^{d}	103.67±0.96^{g} **Amber**	19.04±1.54^{a}	8.32±0.69^{c}	27.36±1.66^{b}	61.00±0.01^{c}

The values in the same column followed by the same letter are not significantly different (P< 0.05) by Tukey's multiple range test. a: represent the higher value; b: represent the low value.

The moisture content in the honey samples was between 18.62% and 20.00% (Table 5.2), being within the limit (≤ 20%) recommended by the international quality regulations (21).The samples of *E. resinifera* had lower percentages of moisture (M2 = 18.62% and M6 = 18.73%) than the five remaining samples of *E. officinarum*. The values found in *E. resinifera* honey are within the mean values referred by Chakir et al. (8) (17.06%). Regarding the results of *E. officinarum*, there was also an agreement with the values found by Bettar et al. (6) (19.60–21.70%). These differences in moisture percentages can be attributed to climatic conditions (22) where honey samples were collected.

The diastase activity is an indicator of the freshness and the detection of heat induced defects and the improper storage of honey (22). Diastase activity shows values between 11.43 (M4) and 115.89 (M6) Shade units/g. The results were within the values found for the totality of *Euphorbia* honeys samples tested by Chakir et al. (8).

The hydroxymethylfurfural (HMF) is an important criterion to evaluate storage timeand the heat damage (6). In general, fresh honey does not contain or contain very low or trace amounts of HMF. In this study, all *Euphorbia* honey samples showed HMF values ranging between 2.29 to 80.48 mg/kg, being almost all samples within the limit established by the Codex Alimentarius Commission (80 mg/kg) (21).

Electrical conductivity of honey is related to the concentration of mineral and organic acids and dependent on the floral origin (8). In our results, the values found for the electrical conductivity were between 342.30 and 553.67 µS/cm, where *E. officinarum* samples recorded the higher values (M1 = 553.67 µS/cm). Our results are similar to those reported by other authors (561.18 µS/cm in *E. officinarum*) (8). Concerning the *E. resinifera* honey the values were: M2 = 379.33 µS/cm and M6 = 455.33 µS/cm. Previous results (8) obtained a value of 410.62 µS/cm for *E. resinifera*, quite similar to our results. In this study, honey colour was between 103.67 mm Pfund (M7) (dark) and 510.96 mm Pfund (M6), corresponding to dark-amber colour.

In our study, the ash content values varied between 0.13% and 0.19%. According to the European legislation (23) the value of ash content must not exceed 0.6% which means that our samples respect the proposed standards. In this study we did not find a positive correlation between the values of ash percentage and electrical conductivity, as it had been else where reported (17).

Honey contains several amino acids, being proline the major one (24). This parameter indicates honey maturity. In our study, the higher values were for *E. resinifera* honey M6 = 1485.71 mg/kg and the lowest one was for *E. officinarum* honey M1 = 584.96 mg/kg (Table 5.2), and both of them contained more than the minimum acceptable proline concentration 200 mg/kg for honey samples (25).

Glucose and fructose are the main sugars present in honey samples. The reducing sugars were significantly higher in *E. resinifera* samples (66.67% and 70.67%) than in the monofloral *E. officinarum* honeys (Table 5.2). In both cases, they are higher than the minimum required levels of 60% by the Codex Alimentarius Commission. Our results suggest that the reducing sugars' percentage may be related to the floral origin of honeys.

With very few exceptions, there were not significant differences in the physicochemical parameters between the two monofloral honeys. Similar results were already reported (6) but between *E. officinarum* and *E. regia-jubae*.

3.3. Sugar profile and quantification

Carbohydrates are the main constituents of honey representing at least 95-99% of the dry matter, being mainly constituted by fructose and glucose (21). Glucose and fructose were the dominant saccharides, with the values of 31.53 and 39.48 g/100 g, respectively, being fructose present in higher quantities. Concerning to glucose, the amounts ranged from 27.77 to 34.17 g/100 g, for the different samples. The values for sucrose ranged from 3.45 to 5.04 g/100 g) (Table 5.3). Other sugars in lower quantities were found, as turanose, maltose and trehalose. These results are concordant with previous works (9,13), for the different sugars presented for *E. resinifera* and *E. officinarum* honeys.

Table 5.3. Sugar content (g/100 g) of *E. resinifera* and *E. officinarum* monofloral honey samples from Morocco

	Fructose	Glucose	Sucrose	Turanose	Maltose	Trehalose
M1	36.40±3.10^{a}	32.31±2.70^{a}	5.37±0.13^{a}	5.40±0.59^{a}	3.21±0.40^{ab}	4.58± 0.50^{a}
M2	37.02±2.11^{a}	34.17±1.78^{a}	4.09±0.33^{a}	2.11±0.50^{b}	2.26±0.42^{bc}	2.80±0.52^{bc}
M3	34.95±1.08^{a}	30.23±1. 80^{a}	4.27±0.90^{a}	2.83±0.55^{b}	3.79±0.47^{a}	3.98±0.53^{ab}
M4	33.04±1.18^{a}	29.15±1.33^{a}	3.45±0.91^{a}	1.98±0.49^{b}	1.89±0.44^{bc}	1.70±0.50^{cd}
M5	39.48±1.11^{a}	31.53±2.30^{a}	5.04±0.91^{a}	2.57±0.41^{b}	1.75±0.48^{c}	1.04±0.51^{d}
M6	31.53±1.33^{a}	27.77±2.11^{a}	4.23±0.94^{a}	1.28±0.49^{b}	1.58±0.47^{c}	0.62±0.51^{c}
M7	33.79±3.02^{a}	30.85±2.83^{a}	4.36±0.90^{a}	2.36±0.50^{b}	2.49±0.49^{abc}	1.63±0.49^{cd}

3.4. Elemental mineral analysis

Contents of each mineral element found in the seven honeys expressed in mg/kg (fresh weight) are shown in Table 5.4. The potassium (K) was the most important mineral element with an average content of 409 mg/kg. *E. officinarum* honeys had the highest K content (M5 = 533 mg/kg) but also the lowest one (M7 = 324 mg/kg). Concerning the *E. resinifera* honeys, M6 had the highest value of potassium 503 mg/kg and M2 = 345 mg/kg shows the lowest content. Concerning the second most important mineral element for both species, calcium (Ca), *E. officinarum* samples, M1 records the highest value (153 mg/kg) and M3 shows the

smallest quantity (70.93 mg/kg). For *E. resinifera* M2 presented the highest Ca value (115 mg/Kg), followed by M6 with 103 mg/kg. These results agree with those already reported by Elamine et al. (17), the two highest minerals found were the K in the first range followed by the Ca, which agrees with our results, nevertheless there was not possible to detect differences between the two monofloral honeys (Table 5.4). The third most important mineral element was sodium (Na), nevertheless the values differed significantly, ranging from 34.96 to 125.05 mg/kg (Table 5.4). Such variability was also found by Bettar et al. (6) for *E. officinarum* honey and by Moujanni et al. (4) for *E. resinifera* honey.

Table 5.4. Mineral content (mg/kg) in Moroccan *Euphorbia* honeys. *Euphorbia officinarum* (samples M1, M3, M4, M5, M7) and *E. resinifera* (samples M2, M6).

	Fe	Zn	Mn	Cu	Al	Ca	K	Mg	Na
M1	5.79±0.01	3.30±0.24^{a}	0.81±0.00^{d}	< LOD	9.35±0.41	152.72±2.51^{a}	352.48±5.74^{c}	55.80±0.07^{a}	125.05±1.90^{a}
M2	10.27±0.21^{b}	6.29±0.21^{a}	1.08±0.00^{c}	< LOD	11.39±1.34^{c}	114.82±1.55^{b}	344.82±13.25^{c}	39.63±1.02^{b}	41.92±0.10^{c}
M3	332.47±14.46^{a}	1.77±0.16^{a}	1.46±0.26^{b}	109.68± 3,67^{a}	64.25±9.54^{a}	70.93±1.48^{e}	410.22±0.17^{b}	32.72±3.04^{c}	35.20±3.64^{c}
M4	6.68±0.02^{b}	2.14±0.04^{a}	1.09±0.01^{c}	< LOD	12.45±3.95^{bc}	119.83±3.42^{b}	396.80±8.13^{b}	32.88±1.23^{c}	87.30±2.11^{b}
M5	6.19±0.57^{b}	6.21±0.94^{a}	0.91±0.01^{cd}	< LOD	9.23±1.80^{c}	94.00±1.62^{d}	533.15±7.28^{a}	22.60±0.49^{d}	34.96±4.38^{c}
M6	12.92±3.56	2.5±0.17^{a}	1.71±0.03^{a}	< LOD	19.90±3.39^{b}	102.57±1.34^{cd}	502.48±3.85^{a}	31.93±0.81^{c}	44.43±1.52^{c}
M7	9.20±0.28^{b}	1.97±0.17^{a}	0.75±0.02^{d}	< LOD	6.32±0.03^{c}	105.75±1.06^{c}	323.95±2.13^{c}	31.20±0.49^{c}	42.25±1.13^{c}

The values in the same column followed by the same letter are not significantly different (P< 0.05) by Tukey's multiple range test.^{a}: represent the higher value; ^{b}: represent the low value, LOD (limit of detection) = 0.786 mg/ mL

3.5. Total phenolic content

Total phenolic content of the entire honey samples and their phenolic extracts are depicted in Table 5.5. Polyphenols are present in small amount and derived from the pollen of the plant foraging by the honeybee (27). The highest total phenolic levels were detected in the entire honeys and the lowest in their phenolic extracts (Table 5.5). These results are in opposite to that previously reported (28), but similar to that found by Ferreira et al. (29) for Portuguese honeys. In addition, these authors also reported a correlation between the total phenol content in the entire honeys and their colour. In our case, the darker entire honey sample (M6) had the higher total phenolic content contrariwise the amber honey sample (M7) that had the lower total phenolic content.

Another study (30), reported that the entire honey contains some non-phenolic reducing compounds contributing to increase the absorbance values (interferences) in the total phenol assay, which gives erroneous values on the rate of phenol present in the sample. In fact, honey contains reductive sugar or organic acids, and these compounds interfere and may cause the values found of total phenols, determined by Folin-Ciocalteu's method, in entire honey samples (30).

Table 5.5. Total phenols' content of Euphorbia honey samples (M1, M2, M3, M4, M5, M6, M7) and their phenolic extracts (PE1, PE2, PE3, PE4, PE5, PE6, PE7).

Total phenols' content of honey samples (mg GAE/100 g)		Total phenols' content of phenolic extract (mg GAE/100 g)	
M1	64.78 ± 0.02^{ab}	**PE1**	4.74 ± 0.00 ^{d}
M2	54.55 ± 0.02 ^{c}	**PE2**	5.93 ± 0.00 ^{d}
M3	61.82 ± 0.03 ^{b}	**PE3**	10.24 ± 0.01 ^{c}
M4	53.38 ± 0.02 ^{c}	**PE4**	13.88 ± 0.00 ^{b}
M5	64.94 ± 0.05 ^{ab}	**PE5**	30.74 ± 0.00 ^{a}
M6	69.25 ± 0.01 ^{a}	**PE6**	13.55 ± 0.01 ^{b}
M7	46.14 ± 0.03 ^{d}	**PE7**	12.52 ± 0.00 ^{b}

3.6. Phenolic profile of honey extracts

M1-M7 honey extracts chromatographic profiles are depicted in Figure 5.2 and Table 5.6. Nineteen phenolic compounds' standards were tested for SRM analysis, but some of them were not detected in honey samples, and therefore were not included in the results' table. In a general way, the chromatographic profiles for the studied *Euphorbia* honey extracts were quite different. Phenolic compounds gallic acid, 4-hydroxybenzoic acid and *p*-coumaric acid were detected in all samples, although in different ratios. Naringenin was identified in all but sample M5. Abscisic acid was detected in 5 of the 7 studied honey samples, being the major compound detected in honey M6. As a plant hormone, abscisic acid is responsible for regulating plant development, growth, and response to stress. It is a common compound in honey and has been proposed as a marker for checking adulterated honey and for quality control, in *Acacia* and *Erica* honey samples (31,32), although this type of pollen had not been detected (Table 5.1).

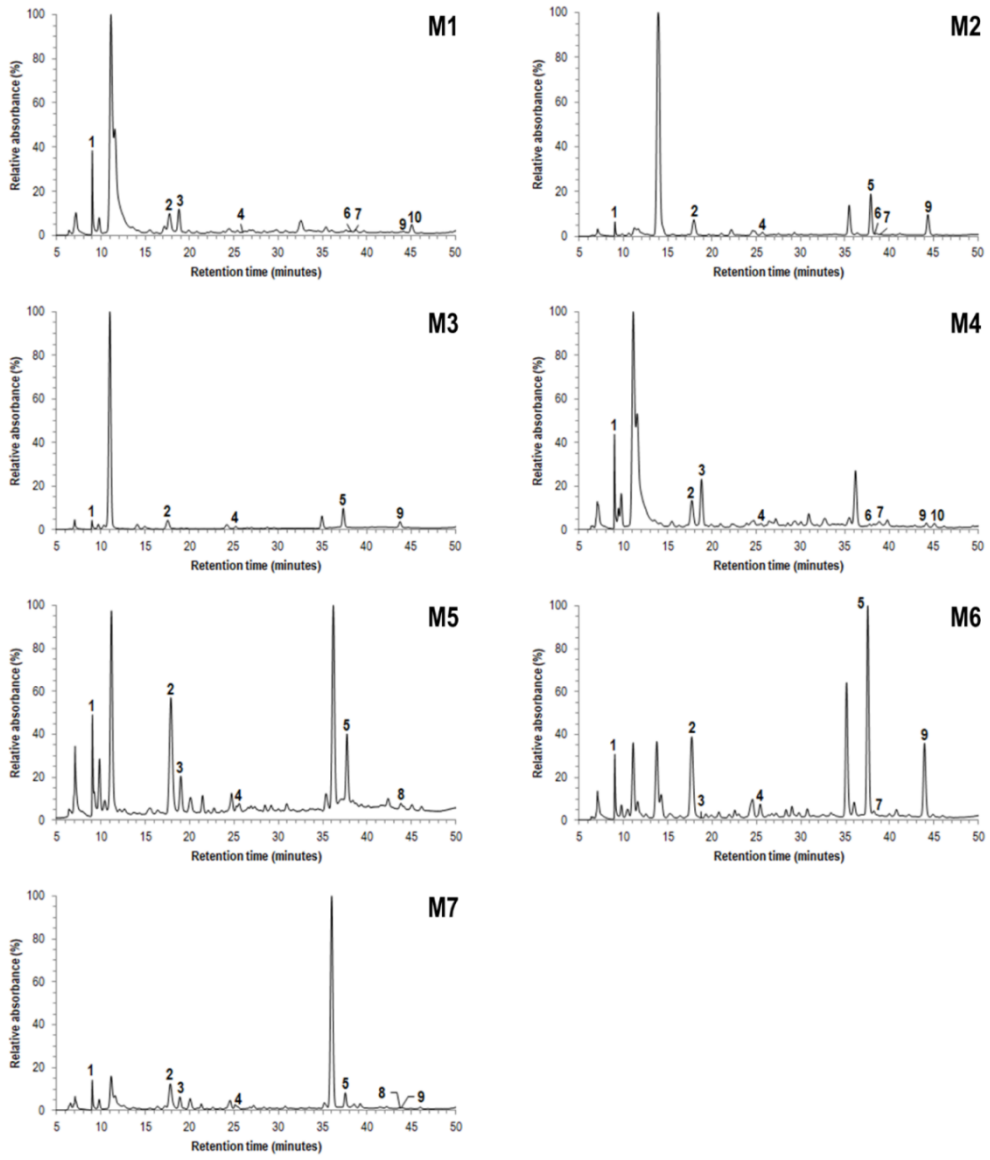

Figure 5.2. LC/DAD chromatographic profiles of the studied honey extracts, recorded at 270 nm. Peak identification in Table 5.6.

Table 5.6. List of compounds identified in each honey sample, using LC/MS analysis.

Compound	R_t (min.)	$(M-H)^-$	SRM*	M1	M2	M3	M4	M5	M6	M7
Gallic acid (1)	9.01	169	169→125	+	+	+	+	+	+	+
4-Hydroxybenzoic acid (2)	17.89	137	137→93	+	+	+	+	+	+	+
Caffeic acid (3)	18.99	179	179→135	+			+	+	+	+
p-Coumaric acid (4)	25.42	163	163→119	+	+	+	+	+	+	+
Abscisic acid† (5)	37.54	263	-		+	+		+	+	+
Luteolin (6)	38.12	285	285→241	+	+		+			
Quercetin (7)	38.77	301	301→179	+	+		+		+	
Apigenin (8)	43.81	269	269→149					+		+
Naringenin (9)	44.38	271	271→151	+	+	+	+		+	+
Kaempferol (10)	45.08	285	285→185	+			+			

Selective Reaction Monitoring (SRM) at a normalized collision energy of 30%. + Abscisic acid identified usingfull scan mode.

3.7. Antioxidant activity

3.7.1. Scavenging DPPH free radicals

The antioxidant properties of honey samples were determined using both entire honey and their phenolic extracts (Table 5.7). All samples had the capacity to reduce the stable violet DPPH radical to yellow DPPH-H, with the 50% of the reduction values (IC_{50}) ranging from M5: IC_{50} = 16.30 mg/mL, as the best values of *E. officinarum* entire honey, to M2: IC_{50} = 80.13 mg/mL) for *E. resinifera* entire honey (13). Concerning the phenolic extract, the IC_{50} values ranged from PE5: IC_{50} = 2.58 mg/mL) to PE3:IC_{50} = 17.64 mg/mL). According to these results, the best antioxidant capacity (the smaller value of IC_{50} = 2.58 mg/mL) was recorded in the phenolic extracts and not in entire honey samples. This agrees with previous authors (28), who have found that the best antioxidant activities were attributed to the phenolic extract. Honey contains many biologically active compounds able to counteract the action of antioxidants, such as polyphenols (27). The phenolic extract (PE5) presented the higher content of phenols (30.77 mg GAE/100 g) and the best antioxidant activity (IC_{50} PE5= 2.58 mg/mL); this correspondence had already been drawn up by Rostislav et al. (26).

Table 5.7. Data presenting the different antioxidant activity (DPPH, NO and superoxide inhibition) of Euphorbia honey samples (M1, M2, M3, M4, M5, M6, M7) and their own phenolic extract (PE1, PE2, PE3, PE4, PE5, PE6, PE7).

Honey samples	Antioxidant activity IC_{50} (mg/mL)		
	DPPH	**NO**	**Superoxide**
M1	52.30 ± 1.74 ^{b}	85.67 ± 0.41 ^{d}	3.86 ± 0.10 ^{b}
M2	80.13 ± 1.11 ^{a}	88.20 ± 0.78 ^{d}	3.71 ± 0.01 ^{b}
M3	55.48 ± 0.73 ^{b}	115.58 ± 1.35 ^{a}	2.82 ± 0.15 ^{c}
M4	38.23 ± 0.31 ^{c}	111.29 ± 2.91 ^{b}	4.26 ± 0.09 ^{a}
M5	16.30 ± 0.41^{d}	76.24 ± 0.55 ^{e}	2.75 ± 0.00 ^{c}
M6	78.50 ± 2.00 ^{a}	94.95 ± 1.99 ^{c}	2.71 ± 0.01 ^{c}
M7	75.83 ± 3.63 ^{a}	116.48 ± 0.36 ^{a}	1.95 ± 0.02 ^{d}
Phenolic extracts	**DPPH**	**NO**	**Superoxide**
PE1	17.16 ± 0.18 ^{b}	20.13 ± 0.19 ^{f}	1.08 ± 0.01 ^{e}
PE2	10.24 ± 0.13 ^{d}	21.16 ± 0.36 ^{e}	0.95 ± 0.00 ^{f}
PE3	17.64 ± 0.12 ^{a}	3.64 ± 0.16^{g}	4.10 ± 0.02 ^{a}
PE4	14.77 ± 0.26 ^{c}	24.66 ± 0.26 ^{d}	3.39 ± 0.00 ^{b}
PE5	2.58 ± 0.04 ^{f}	34.18 ± 0.16 ^{b}	1.24 ± 0.00 ^{d}
PE6	17.54 ± 0.12 ^{ab}	27.82 ± 0.20 ^{c}	1.78 ± 0.05 ^{c}
PE7	4.21 ± 0.13 ^{e}	37.87 ± 0.24 ^{a}	1.09 ± 0.01 ^{e}

The values in the same column followed by the same letter are not significantly different (P< 0.05) by Tukey's multiple range test. a: represent the higher value; b: represent the low value

3.7.2. Nitric oxide (NO) scavenging activity

All the assessed samples were able to scavenge NO free radicals and as for DPPH free radicals, phenolic extracts were also better NO scavengers than the respective entire honeys (Table 5.7). Moreover, the presented results are comparable with a previous publication (12).

Indeed, in this study, the authors have found that NO scavenging activity of *E. officinarum* expressed as IC_{50} was 95.14 mg/mL. In contrast to the DPPH free radicals, NO has ubiquitous presence in the living body and is important in the maintenance of health, nevertheless at high concentrations becomes harmful (33). The capacity for scavenging this reactive nitrogen species may reveals interesting and in the present work, all honey samples showed this activity, mainly due to the compounds of the honey extracts

3.7.3. Superoxide anion radical scavenging ability

All the tested samples presented a high capacity for scavenging superoxide radical anion (Table 5.7). With the exception of M3 honey sample, that had better activity than the respective extract, in the remaining samples, the extracts presented better superoxide radical anion scavenging activity than the whole honey samples, as observed for the other antioxidant activities aforementioned. This characteristic observed in this antioxidant test suggests that the responsible compounds were the phenolic compounds and not the other compounds present in the whole honey. Other authors (34) also demonstrated that phenolic compounds possess potent effects on antioxidant effects due to the ability for scavenging superoxide anion radicals. At the same time, (35) affirms that the polyphenolic contents in honey are linked to high superoxide scavenging activity. The Zantaz honey (*Bupleurum spinosum* pollen as predominant species) from Morocco (35), had recorded an IC_{50} values of 50.91 mg/mL for the superoxide activity, a very low activity compared to that found in our *E. officinarum* honey M7:IC_{50} = 1.95 mg/mL.

3.8. Enzymatic activities

3.8.1. Inhibition of acetylcholinesterase activity

The entire honeys and their extracts studied in this work were characterized by the ability to inhibit acetylcholinesterase enzyme (Table 5.8). Acetylcholinesterase (AChE) is a specific enzyme which breaks down the acetylcholine, a neurotransmitter in the nerve synapse (36).

Table 5.8. Data presenting the different enzymatic activities (Acetylcholinesterase, lipoxygenase, tyrosinase and xanthine oxidase inhibition) of Euphorbia honey samples (M1, M2, M3, M4, M5, M6, M7) and their own phenolic extract (PE1, PE2, PE3, PE4, PE5, PE6, PE7).

Honeys samples	Enzymatic activities IC_{50} (mg/mL)			Percentage inhibition (%)
	Acetylcholinesterase	**Lipoxygenase**	**Tyrosinase**	**Xanthine oxidase**
M1	154.98 ± 5.75 [a]	48.85 ± 0.63 [b]	82.61 ± 6.35 [b]	85.27 ± 0.31 [bc]
M2	44.65 ± 8.33 [b]	32.72 ± 0.35 [e]	11.46 ± 1.80 [e]	71.74 ± 1.89 [d]
M3	164.49 ± 8.50 [a]	46.77 ± 0.39 [c]	54.90 ± 3.23 [c]	94.92 ± 0.39 [a]
M4	16.77 ± 0.34 [c]	43.01 ± 0.06 [d]	97.06 ± 5.89 [a]	84.75 ± 0.27 [c]
M5	4.52 ± 0.88 [c]	43.43 ± 1.22 [d]	39.49 ± 1.32 [d]	66.34 ± 1.03 [e]
M6	16.30 ± 1.56 [c]	29.04 ± 0.33 [f]	67.27 ± 3.16 [c]	87.66 ± 0.66 [b]
M7	3.90 ± 0.60 [c]	51.54 ± 0.20 [a]	42.21 ± 6.25 [d]	48.78 ± 0.73 [f]
Phenolic extracts	**Acetylcholinesterase**	**Lipoxygenase**	**Tyrosinase**	**Xanthine oxidase**
PE1	1.35 ± 0.01 [d]	0.43 ± 0.00 [c]	16.27 ± 0.03 [d]	97.69 ± 0.38 [b]
PE2	7.76 ± 0.51 [bc]	0.32 ± 0.00 [f]	15.28 ± 0.17 [d]	96.54 ± 0.38 [c]
PE3	8.09 ± 0.04 [b]	0.50 ± 0.00 [b]	40.15 ± 0.60 [b]	99.66 ± 0.08 [a]
PE4	13.62 ± 0.22 [a]	0.51 ± 0.00 [a]	44.71 ± 0.00 [a]	96.15 ± 0.08 [c]
PE5	1.13 ± 0.06 [d]	0.51 ± 0.00 [a]	37.89 ± 1.21 [bc]	90.91 ± 0.63 [e]
PE6	13.32 ± 0.32 [a]	0.38 ± 0.00 [d]	41.13 ± 2.86 [b]	94.76 ± 0.08 [d]
PE7	7.10 ± 0.32 [c]	0.36 ± 0.00 [e]	35.76 ± 0.29 [c]	48.56 ± 0.22 [f]

The values in the same column followed by the same letter are not significantly different ($P < 0.05$) by Tukey's multiple range test. a: represent the higher value; b: represent the low value.

A great inhibitory activity was observed in the entire honey samples (IC_{50} values from M7: IC_{50} = 3.90 mg/mL to M3: IC_{50} = 164.49 mg/mL, but not as much as their phenolic extract. *Euphorbia officinarum* is the honey which recorded the best enzymatic inhibition activity not only for its pure honey but also for the phenolic extracts as compared with the *E. resinifera* honey samples. The activities found can be partially attributed to the total phenolics. Besides, our results show that M7: IC_{50} = 3.90 mg/mL honey sample, which recorded the best anti-acetylcholinesterase activity, possessed a value of M7 = 46.14 mg GAE/100 g as the rate of total phenol as well as better scavenging ability of superoxide anion radical (IC_{50} = 1.95 mg/mL). Concerning the phenol extract, the same trend was observed. Similar behaviour was noticed for the PE5: IC_{50} = 1.13 mg/mL which possesses the best AChE inhibition activity, the higher TPC (PE5 = 30.74 mg GAE/100 g) and the best DPPH antioxidant activity (PE5: IC_{50} = 2.58 mg/mL). As indicated by some authors (37,38), the ability of honey and their methanolic extracts to inhibit the AChE enzyme is related tothe presence of a higher total phenol content and its antioxidant potency

3.8.2. Inhibition of lipoxygenase activity

This study determined the anti-inflammatory activities of various samples of Moroccan *Euphorbia* honeys and their methanolic extracts through the anti-lipoxygenase activity.The results of the entire honeys samples and their phenolic extracts are summarized in Table 5.8. The anti-lipoxygenase activity was observed to vary depending on the type of *Eu-phorbia* species source. The *E. resinifera* entire honeys (M2 and M6) had the highest capacity for inhibiting the lipoxygenase activity. Concerning the respective phenolic fractions, only PE2 was also better than the remaining phenolic fractions, although PE6 also presented high ability but along with PE7 (*E. officinarum*). The inhibition of lipoxygenase has been considered as an indicator of anti-inflammatory and antioxidant activities (20) because lipoxygenase-generated free radicals disrupt membrane selective permeability, through peroxidation of membrane phospholipids (39). Samples having high total phenol contentand antioxidant activity will surely have an anti-inflammatory activity (39).

3.8.3. Inhibition of tyrosinase activity

The tyrosinase inhibitory activity of honey and the respective honey methanolic extracts is shown in Table 5.8. The highest inhibition activity of honey was found in *E. resinifera* and respective extract (PE2). Moreover, this sample is the only that entire honey has higher activity than the respective phenolic fraction (Table 5.8). Tyrosinase is well-known as a key enzyme in melanin biosynthesis (40). Melanin production in human kin represents a primary defense mechanism against the UV light, but the excessive accumulation and formation of epidermal pigmentation can cause various disorders (40), therefore the capacity of inhibition of tyrosinase enzyme that a natural product possess can be useful. According to Akin et al. (41), the antioxidant activity of natural sources plays a very important role in the inhibition of tyrosinase. For example, PE2 had good ability for scavenging superoxide radical anions

(IC_{50} = 0.95 mg/mL) and also for inhibiting tyrosinase activity (IC_{50} = 15.28 mg/mL) According to Petrillo et al. (40) Sardinian honeys showed the highest anti-tyrosinase activity (IC_{50}: 64.3 ± 1.6 mg/mL), lower as compared to *Euphorbia* honey sample (M2 and PE2) of the present work, nevertheless closer to theremaining samples.

3.8.4. Inhibition of xanthine oxidase activity

The entire honey showed a percentage of inhibition ranging from M7 = 48.78% to M3 = 94.92% and the phenolic extracts from PE7 = 48.58 to PE3 = 99.66%. *E. officinarum* pure honey and phenolic extract exhibited the highest percentage of inhibition: >99% for both of them. According to some authors (42), honey is an important inhibitor of xanthine oxidase depending on the floral source and the phenolic contents. Moreover, the ROS are generated by the reaction catalyzed by xanthine oxidase, which catalyzes the oxidation of hypoxanthine to xanthine and at the end the uric acid, responsible for several diseases (40). The blocking of uric acid production is based on blocking the key enzyme, xanthine oxidase (43), and according to several studies this blocking can be induced by substances with a high antioxidant power such as the phenolic compounds. In this work, *E. offinarum* honey and phenolic extracts showed a varied antioxidant power (Table 5.7) and phenolic content (Table 5.4), which gives them the property to block the formation of uricacid from derivatives obtained by xanthine oxidase enzyme.

3.9. Correlations between antioxidant activities and enzymatic activities

As shown in Table 5.9, the phenolic compounds were strongly negatively correlated with the nitric oxide scavenging activity of entire honey samples ($r = -0.56$, $p < 0.01$), that is, higher phenolic content, lower the IC_{50} values, therefore better activity. A correlation be-tween phenolic content and the remaining activities were not observed. On the other hand, the IC_{50} values of DPPH and NO free radical scavenging activities negatively correlated with total phenolics of the extracts but the anti-lipoxygenase and anti-tyrosinase positively correlated with phenolic content of the extracts. This suggests that the antioxidant activity of the phenolic fraction enhances with the increase of phenolic content but playing an inverse role on the enzymatic inhibitory activities.

Table 5.9. The correlations between the results of TPC, antioxidant activities (DPPH, NO, superoxide scavenging activities) and inhibition of acetylcholinesterase, lipoxygenase and tyrosinase activities of Euphorbia honey samples and Euphorbia honey phenolic extract.

				***Euphorbia* honeys samples**			
Correlation	**TPC**	**DPPH**	**NO**	**Superoxide**	**ACTE**	**Lipoxygenase**	**Tyrosinase**
TPC	1	-0.23	-0.56**	0.07	0.30	-0.37	0.22
DPPH	-0.23	1	0.31	-0.23	0.01	-0.36	-0.30
NO	-0.56**	0.31	1	-0.22	0.08	0.35	0.24
Superoxide	0.06	-0.23	-0.22	1	0.25	-0.17	0.41
ACTE	0.30	0.01	0.08	0.25	1	0.34	0.20
Lipoxygenase	-0.37	-0.36	0.35	-0.17	0.34	1	0.22
Tyrosinase	0.22	-0.30	0.24	0.41	0.20	0.22	1
				***Euphorbia* honey phenolic extract**			
TPC	1	-0.61**	-0.50*	-0.04	-0.22	0.50 *	0.56**
DPPH	-0.61**	1	-0.72**	-0.53*	0.43	0.13	-0.13
NO	-0.49*	-0.719**	1	-0.66**	-0,01	-0.27	0.14
Superoxyde	-0.04	0.53*	-0.66**	1	0.5*	0.66**	0.57**
ACTE	-0.22	0.43	-0.01	0.5*	1	-0.09	0.57**
Lipoxygenase	0.50*	0.13	-0.28	0.65**	-0.09	1	0.42
Tyrosinase	0.56**	-0.13	0.14	0.57**	0.58**	0.42	1

**Correlation is significant at the $p < 0.01$ level. *Correlation is significant at the $p < 0.05$ level.

This study shows that the antioxidant capacity of *Euphorbia* honey comes from the phenolic compounds present in whole honey, with the exception of superoxide anion radicals scavenging activities were no correlation could be found. On the other hand, the phenolic content has a negative effect on the enzymatic inhibitory activities since a positive correlation was observed between the phenolic content and the activities found.

3.10. Comparison of antioxidant activities and enzymes' inhibitory activities with other Moroccan honeys

The antioxidant activities and other biological properties of Moroccan honeys from diverse botanical source have been studied. Concerning the antioxidant activity, several methods have been used predominating the ability for scavenging some type of free radicals, such as DPPH, superoxide and nitric oxide (Table 5.10). In this Table are those results obtained from the same type of assay used in the present work, in order to better compare the results. Moreover, in this Table only are depicted the lowest and the highest IC_{50} values found by the authors although much more honeys can have been studied. The results show that our values are within those already reported, with the exception of superoxide anion free radical which our results are much better than those reported by Aazza et al. (12). The enzyme inhibitory activities were for the first time reported by this team (13) for one sample of *E. officinarum* (M3) and one sample of *E. resinifera* (M2) which are completed in the present work with other *Euphorbia* honey samples. In all cases, the phenolic fraction of all honeys seem to have

a role in all activities found, since in all cases the activities were better than the entire honeys, although in some cases, a positive correlation between the total phenol of the honey extract and the activities were found, meaning these results that beyond the total phenolic content, the type of compound is also important.

Table 5.10. IC50 values (mg/mL) found for the assays DPPH, NO and superoxide found in other works on Moroccan honeysand those found in the present work.

Honey Type	DPPH	NO	Superoxide	Reference
Thymus spp	ND	21.47	ND	(12)
Euphorbia resinifera		95.14		
Bupleurum spinosum	15.34	125.89	50.91	(35)
Ceratonia siliqua	12.54–23.52	ND	ND	(44)
Thymus vulgaris	5.57			
Peganum harmala	48.67	ND	ND	(45)
E. resinifera	15.34			
Bupleurum spinosum	13.57–45.34	ND	ND	(46)
E. officinarum	16.30–75.83	76.24–116.48	1.95–4.26	Present work
Euphorbia resinifera	78.50–80.13	88.20–94.95	2.71–3.71	Present work

4. Conclusion

All honey samples were found to be within the acceptable limit of the international standards. *E. resinifera* honeys presented lower moisture, HMF but higher reducing sugar percentage than *E. officinarum* honeys. The mineral analyses showed that the main dominant mineral element in the two *Euphorbia* honey types is potassium (K). Phenolic compounds gallic acid, 4-hydroxybenzoic acid and *p*-coumaric acid were detected in all extracts, although in different ratios, whereas kampferol was only present in two samples. The methanolic extracts of the honey recorded the highest values in all the biological activities, in comparison with the corresponding entire honey. Moreover, *E. resinifera* honeys had better anti-inflammatory activities than the *E. officinarum* honeys, while in the remaining biological activities it was not possible to observe differences between the two monofloral honey types. To conclude, this study revealed that *Euphorbia* honey may be a source of antioxidant molecules. In addition, it may provide new compounds useful for the symptom treatment of various diseases (e.g., gout, hyperpigmentation or Alzheimer) or delay its progression.

Author Contributions:

Conceptualization, M.G.M., S.A. and L.E.G.; methodology, O.B., S.E.-G.,A.M., V.B.P., J.C. and B.R.; software, O.B., S.E.-G., A.M., V.B.P., J.C. and B.R.; investigation, O.B. andA.M.; writing—original draft preparation, O.B. and A.M., funding acquisition, M.G.M., M.C.C., S.R.,C.B.D. and L.M.E.; writing—review and editing, M.G.M., L.M.E., S.R. and J.C. All authors have readand agreed to the published version of the manuscript.

Funding:

This research was funded by the Fundação para a Ciência e a Tecnologia I.P. (FCT), Portugal: UIDB/05183/2020, UIDB/04326/2020, UIDB/00690/2020, UID/MAR/00350/2020, UIDB/04449/2020,UIDP/04449/2020, CEECIND/00791/2017.

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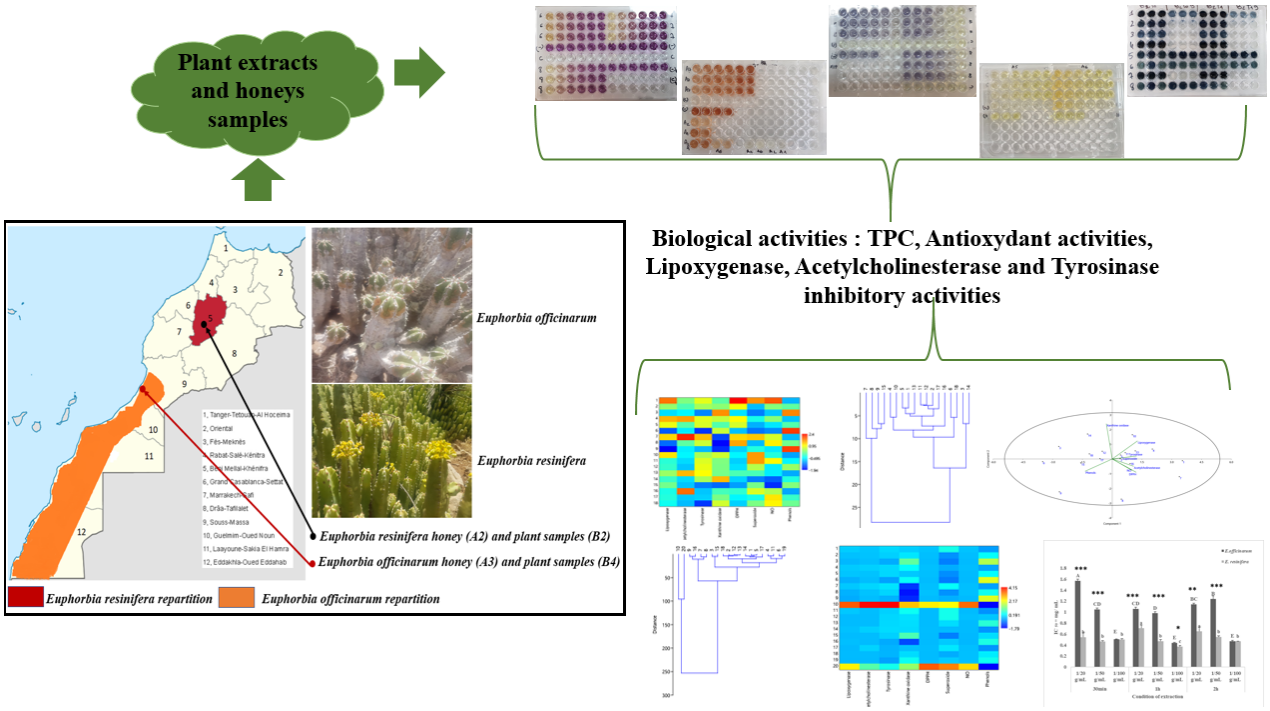
Chapter 6

Antioxidant activity and enzyme inhibitory potential of *Euphorbia resinifera* and *Euphorbia officinarum* honeys from Morocco and plant aqueous extracts



6

Chapter VI: Antioxidant activity and enzyme inhibitory potential of Euphorbia resinifera and Euphorbia officinarum honeys from Morocco and plant aqueous extracts*

Résumé

Les produits naturels peuvent être appliqués dans un large éventail de domaines, de l'agriculture aux industries alimentaires et pharmaceutiques. Dans cette étude, les propriétés antioxydant et la capacité à inhiber certaines activités enzymatiques des extraits aqueux et des miels d'Euphorbia resinifera et d'Euphorbia officinarum ont été évaluées. Les caractéristiques physico-chimiques ont également été évaluées.

Des quantités plus élevées de fer, de cuivre et d'aluminium ont été détectées dans le miel d'E. Officinarum, ce qui peut indiquer une pollution de l'environnement autour des ruches ou un stockage inadéquat des échantillons de miel. Cet échantillon de miel a montré des quantités plus élevées de phénols totaux et une meilleure capacité à piéger les radicaux libres anions Superoxyde et les radicaux libres DPPH par rapport au miel d'E. Resinifera, mais une plus faible capacité à inhiber la lipoxygenase, l'acétylcholinestérase, la tyrosinase et la xanthine oxydase.

Le rapport masse végétale/volume de solvant (1:100) et le temps d'extraction (1 à 2 h) étaient associés à des phénols totaux plus élevés et à de meilleures activités antioxydante et inhibitrices de la lipoxygenase, de l'acétylcholinestérase et de la tyrosinase, quelle que soit l'espèce végétale. Les extraits aqueux avaient des activités in vitro systématiquement plus élevées que les échantillons de miel respectifs.

Mots clés : Antioxydant, Acétylcholinestérase, Lipoxygénase, Tyrosinase, Xanthine oxydase, Produits naturels.

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* Adapté de Oumaima Boutoub, Soukaina El-Guendouz, Letícia M. Estevinho, Vanessa B. Paula, Smail Aazza, Lahsen El Ghadraoui, Brígida Rodrigues, Sara Raposo, Jorge Carlier, Maria C. Costa and Maria G. Miguel. Antioxidant activity and enzyme inhibitory potential of *Euphorbia resinifera* and *Euphorbia officinarum* honeys from Morocco and plant aqueous extracts. Journal: Environmental Science and Pollution Research. **2020**, DOI: 10.1007/s11356-020-10489-6

6

Antioxidant activity and enzyme inhibitory potential of *Euphorbia resinifera* and *Euphorbia officinarum* honeys from Morocco and plant aqueous extracts*

Abstract

Natural products may be applied in a wide range of domains, from agriculture to food and pharmaceutical industries. In this study, the antioxidant properties and the capacity to inhibit some enzymatic activities of *Euphorbia resinifera* and *Euphorbia officinarum* aqueous extracts and honeys were assessed. The physicochemical characteristics were also evaluated. Higher amounts of iron, copper and aluminium were detected in *E. officinarum* honey, which may indicate environmental pollution around the beehives or inadequate storage of honey samples. This honey sample showed higher amounts of total phenols and better capacity for scavenging superoxide anion free radicals and DPPH free radicals as compared with *E. resinifera* honey, but poorer capacity for inhibiting lipoxygenase, acetylcholinesterase, tyrosinase and xanthine oxidase. The ratio plant mass:solvent volume (1:100) and extraction time (1 - 2 h) were associated with higher total phenols and better antioxidant activities and lipoxygenase, acetylcholinesterase and tyrosinase inhibitory activities, regardless of the plant species. The aqueous extracts had systematically higher in vitro activities than the respective honey samples.

Keywords: Antioxidant, Acetylcholinesterase, Lipoxygenase, Tyrosinase, Xanthine oxidase, Natural products

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* Adapte from Oumaima Boutoub, Soukaina El-Guendouz, Letícia M. Estevinho, Vanessa B. Paula, Smail Aazza, Lahsen El Ghadraoui, Brígida Rodrigues, Sara Raposo, Jorge Carlier, Maria C. Costa and Maria G. Miguel. Antioxidant activity and enzyme inhibitory potential of *Euphorbia resinifera* and *Euphorbia officinarum* honeys from Morocco and plant aqueous extracts. Journal: Environmental Science and Pollution Research. **2020**, DOI: 10.1007/s11356-020-10489-6

1. Introduction

Euphorbia officinarum L. and *E. resinifera* O. Berg are dicotyledonous plants of the Euphorbiaceae family (1). This family contains around 300 genera of plants (2) being one of the largest and most cosmopolitan families in sub-branching angiosperms (3,4). The floristic diversity in Morocco (8) allowed the use of a wide variety of plants for therapeutic and medicinal purposes since hundreds of years. *E. resinifera* and *E. officinarum* are one of the oldest "drugs" in the Western medical tradition, being much used by Moroccan herbalists and therapists (5). "Zaggoume", the Arabian name for *E. resinifera*, is an endemic species of Morocco mainly distributed in the middle of the country, in Azilal and Beni Mellal regions (Middle Atlas), with some scattered populations in the High Atlas and Anti-Atlas Mountains (6). "Daghmouss", the Arabian name for *E. officinarum*, is an endemic species of North Africa; in Morocco, this plant is distributed from the north of the Souss River until the Moroccan sahara reaching the region of Zemmour (7).

Morocco is a favourable territory for beekeeping and honey production owing to its floral resources and climate (8). The number of hives and beekeepers is estimated at 375,000 and 35,000, respectively. The total annual honey production is estimated at 3500 tonnes (9). The Euphorbia honey is considered the most precious by the consumer (9).

To date, most studies on *Euphorbia* species are focused on its latex (10-13)), with little emphasis on the other parts of the plant. The main goal of the present study was to compare some *in vitro* biological properties (inhibition of xanthine oxidase, lipoxygenase, acetylcholinesterase and tyrosinase enzymes' activities as well as antioxidant activities) of the aqueous extracts of the aerial part of *E. resinifera* and *E. officinarum* and of those of honey samples from the same monofloral origin. The physicochemical characterization of the two monofloral honey types was also studied.

2. Experimental

2.1. Honey samples

Monofloral honey samples from E. resinifera and E. officinarum were acquired from the beekeepers in Morocco and kept under ambient temperature in the shelter of light. Table 6.1 depicts the coordinates where honey samples were collected as well as their melissopalynological profiles.

2.2. Plant samples

The aerial part of the plants (*E. resinifera O*. Berg and *E. officinarum*L.) was collected in July 2018 directly from the fields of Beni Mellal and Tiznit, Morocco (Figure 6.1). Dried plant material from both species was deposited as authenticated vouchers in the Herbarium of the Universidade do Algarve (acronym ALGU), with the accession numbers 15745/ALGU and 15746/ALGU, respectively.

Table 6.1. Places of collection, year of production and the most predominant pollen of two *Euphorbia* honey samples from Morocco

Honey type	Pollen species (percentages, %)	Production region	Coordinates	Production year
Euphorbia resinifera	*E. resinifera* 48.7 ± 1.1 *Caesalpinia pulcherrima* 21.8 ± 1.3 *Malvus domestica* 10.2 ± 0.4 *Cistus crepis* 7.9 ± 0.9 *Populus nigra* 4.0 ± 0.3 *Genista hirsuta* 2.9 ± 0.2 *Populus alba* 1.9 ± 0.2 *Ilex aquifolium* 2.6 ± 0.3	Beni Mellal-Khénifra	32° 20′ 22″ N, 6° 21′ 39″ W	2018
Euphorbia officinarum	*E. officinarum* 52.1 ± 1.6 *Caesalpinia pulcherrima* 11.8 ± 0.7 *Arbutus unedo* 6.1 ± 1.2 *Populus alba* 5.8 ± 0.8 *Pinus pinaster* 5.0 ± 0.2 *Eucalyptus globulus* 3.3 ± 0.6 *Malvus domestica* 3.0 ± 0.2 *Thymus lotocephalus* 2.4 ± 0.3 *Quercus suber* 2.0 ± 0.1 *Eucalyptus cinereae* 1.9 ± 0.2 *Populus nigra* 1.8 ± 0.3 *Caesalpina spinosa* 1.7 ± 0.1 *Cistus albidus* 1.7 ± 0.2 *Trifolium arvense* 1.5 ± 0.3	Tiznit- Souss-Massa	29° 42′ 00″ N, 9° 43′ 37″ W	2018

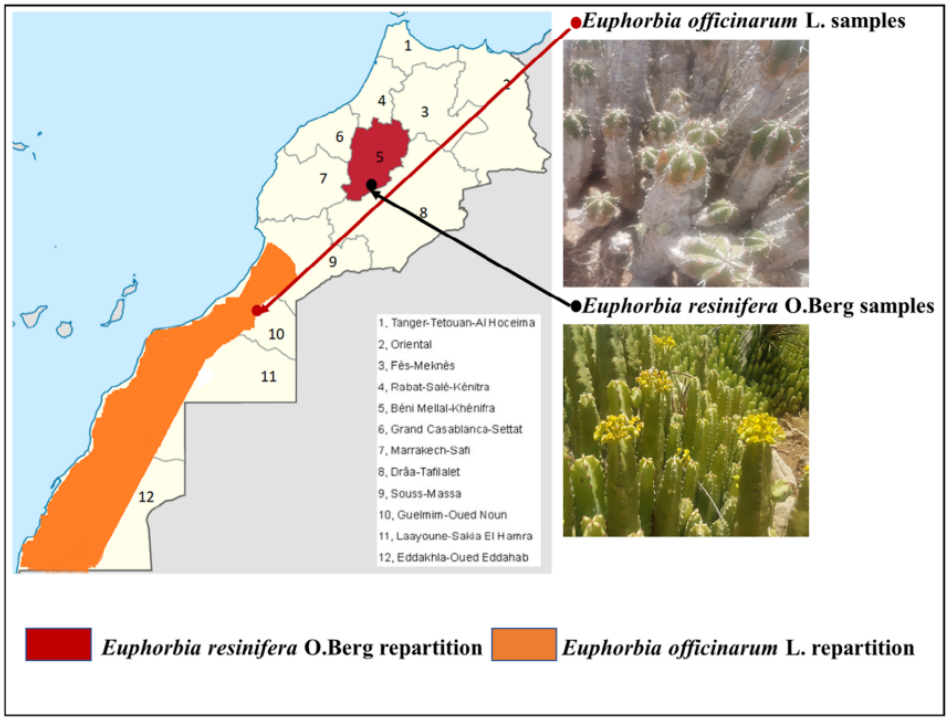


Figure 6.1. Range of the 2 plants (*E. resinifera* O. Berg, *E. officinarum L.*) as well as the distribution of the samples studied in Morocco

2.3. Extract preparation

Plant material was well dried at room temperature, in the dark and afterwards 1 g from the aerial parts of each species was extracted with 20 mL (50 mg/mL), 50 mL (20 mg/mL) and 100 mL (10 mg/mL) of distilled water (w/v) by decoction. For each volume of water used, three decoction times were assayed (30 min, 1 h and 2 h). Each sample was centrifuged at 5,000 rpm, for 15 min. The supernatant was recovered and kept in – 20 °C until further analysis.

2.4. Physicochemical analysis of honey

The parameters pH, free acidity, lactonic acidity, total acidity, ash content, electrical conductivity, moisture, proline content, diastase activity, hydroxymethylfurfural (HMF) and reducing sugars were determined according to methodologies previously described in detail (14).

2.5. Melissopalynological analysis

The analysis of the honey samples' pollen qualitative and quantitative spectrum was performed according to the International Commission for Bee Botany (ICBB), as previously described (15). Pollen identification and count were carried out using a light microscope (Leitz Messtechnik GmbH; Wetzlar, Germany) with 400× and 1000× objectives.

2.6. Estimation of honey colour

The colour was determined by measuring the absorbance of the honey solution (1 g in 2 mL of distilled water) at 635 nm (A_{635}), using a UV-Vis spectrophotometer (Ultrospec™ 1100 pro UV/visible spectrophotometer). The mm Pfund values of honey samples were obtained using the following algorithm: mm Pfund = − 38.7 + 371.39 × A_{635}. (43)

2.7. Elemental analysis

Nine elements were quantified (Fe, Zn, Mn, Cu, Al, Ca, K, Mg, Na) according to the method previously detailed (16). Ca, Mg, Mn, Zn, Cu and Fe were measured by flame atomic absorption spectroscopy (novAA 350, Analytik Jena, Germany), while, Na, K and Al by microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent, USA).

2.8. Carbohydrate content

For carbohydrate determination, the method previously described by (17) with few modifications was followed. In brief, 5 g of honey was dissolved in water and acetonitrile (75:25, v/v) using a 100-mL volumetric flask. The solution was filtered (0.45 μm) and stored at 4 °C until analysis. The quantification was done through highperformance liquid chromatography, using a chromatograph (Hitachi LaChrom Elite HPLC, Japan) equipped with a refractive index detector (Hitachi L-2490, Japan). A Purospher STAR NH2 (5 μm particle size) (Merck, Germany) column was used with an isocratic elution using as mobile phase acetonitrile and water (85:15, v/v) at room temperature. Monosaccharides and disaccharides were analyzed at a flow rate of 1 and 1.3 mL/min, respectively. Injection volume was set at 20 μL. The quantification method of every carbohydrate was made using standard solutions at different concentrations (0.5–50 mg/mL), which were injected in the same conditions of honey samples. A calibration curve was done (concentration versus peak area) for the quantification of carbohydrates present in honey samples.

2.9. Total phenol content

The total polyphenol content in honey and plant samples was determined as stated by (18). The reducing sugars interfere with Folin-Ciocalteu's phenol reagent; therefore, a solution with the same concentrations of glucose and fructose detected in honeys was used as control. The difference of absorbances observed was used to determine the total polyphenol content which was expressed as mg gallic acid equivalents (GAE) per g for plant extract and GAE/100 g for honey.

2.10. Antioxidant activity

2.10.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

DPPH free radical scavenging activity was assessed as described by (19) in which 25 μL of the plant extracts or 200 μL of honey samples was added to 250 μL of DPPH solution (63.4 μM) and incubated for 30 min at room temperature; the absorbance was measured at 517 nm. The result was calculated using the following formula: Inhibition = $((A_0-A_1)/A_0) \times 100)$, with A_0 is the

absorbance of the control and for A_1 is the absorbance of the sample. The sample concentration providing 50% inhibition (IC_{50}) was achieved by plotting the inhibition percentage against samples' concentrations. Butylated hydroxytoluene (BHT) was used as standard (0.03–1 mg/mL).

2.10.2. Nitric oxide scavenging activity

The nitric oxide (NO) scavenging activity was carried out according to the manufacturer's instructions (Griess reagent kit 2003) (44). The result was calculated using the following formula: Inhibition = $((A_0-A_1)/A_0) \times 100$), with A_0 is the absorbance of the control and for A_1 is the absorbance of the sample. The concentration of the sample that allows 50% inhibition (IC_{50}) was obtained by drawing the percentage of inhibition curve. Curcumin was used as standard (0.03–1 mg/mL).

2.10.3. Scavenging ability of superoxide anion radical

The scavenging ability of superoxide anion radical was assayed as reported by (19). The result was calculated using the following formula: Inhibition = $((A_0- A_1)/A_0) \times 100$), with A_0 is the absorbance of the control and for A_1 is the absorbance of the sample and the IC_{50} value was determined as reported previously. Ascorbic acid was used as standard (0.03–1 mg/mL).

2.11. Enzymatic activities

2.11.1. Inhibition of acetylcholinesterase

The acetylcholinesterase inhibition was carried out with few modifications as reported by El-Guendouz et al. (18). About 50 µL of plant extract or 300 µL of honey solution was used in the assay. The percentage of inhibition of acetylcholinesterase activity was determined and the IC_{50} value was calculated. Galantamine was used as standard (0.03–1 mg/mL).

2.11.2. Inhibition of lipoxygenase

The lipoxygenase assay was used as an indicator of anti-inflammatory and antioxidant activity (20). The inhibition action of honey solution and plant extract was determined as previously reported (19), with some modifications. In short, 100 µL of plant extract or 150 µL of the honey solution was used in the assays. The inhibitory effect of the test was calculated by comparison with negative control: Inhibition % = $((A_0-A_1)/A_0) \times 100$, with A_0 is the absorbance of the blank sample and A_1 is the absorbance of the sample. The results were expressed as IC_{50} value. Nordihydroguaiaretic acid (NDGA) was used as standard (0.03–1 mg/mL).

2.11.3. Inhibition of tyrosinase

The tyrosinase inhibitory activity was determined based on the protocol proposed by (18), with slight modifications. The total assay mixture consisted on adding 50 µL of honey solution or plant extract, mixed with 170 µL of phosphate buffer (50 mM; pH = 6.5) and 20 µL of tyrosinase enzyme (100 U/mL); the mixture was incubated for 40 min. After this period, 60 µL of L-Dopa (5 mM) was added. The reading was done at 492 nm, the inhibition percentage of the enzyme was

calculated, and the IC50 values were compared. Kojic acid was used as standard (0.006–1 mg/mL).

2.11.4. Inhibition of xanthine oxidase

The inhibitory activity of plant extracts and honey solutions on xanthine oxidase was determined as described by (18), with minor modifications. For that, 50 µL of plant extract or 150 µL of honey solution was used in the assays. The percentage of inhibition of xanthine activity was calculated and the IC_{50} was determined.

2.12. Statistical analysis

Statistical analysis was performed with the Software Package of Statistics for the Social Sciences (SPSS) 25.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were made with a one-way analysis of variance followed by multiple Tukey's comparisons. The level of significance was set at 5%. Correlations between phenol and antioxidant activity levels and enzyme inhibitory activities were obtained by the Spearman (r) correlation coefficient at a 95% level of significance. To run PCA (Principal Component Analysis), the PAST statistics version 4 software (Øyvind Hammer, Natural History Museum, University of Oslo) was used. Prior to each PCA running, the data was normalized by subtracting the mean value and dividing by the standard deviation of each of the given values. The same procedure and program were used to build the matrix plot. The dendrogram was obtained based on correlation and using Ward's method.

3. Results

3.1. Melissopalynological and general physicochemical properties

The predominant pollen grain analysis allowed to classify the samples as being monofloral honeys from *E. resinifera* and *E. officinarum* honeys (Table 6.1). In the case of *E. resinifera* honey, a secondary pollen type was identified: *Caesalpinia pulcherrima* (L.) Sw that exceeded 20%. In the *E. officinarum* honey, the difference between the percentageof the predominant pollen grains of *E. officinarum* (52.1%) and the secondary pollen grains of *C. pulcherrima* (11.8%) was higher (Table 6.1).

In this study, pH values for both monofloral honey types were 4.1 (Table 6.2). The free acidity values found in the present work did not exceed 10.6 meq/kg for both honey types (Table 6.2), and were slightly lower than 18.2 meq/kg. Regarding lactonic acidity, *E. officinarum* honey exhibited higher value of 10.96 meq/kg, compared with that of *E. resinifera* honey (7.68 meq/kg). Finally, the values recorded for the total acidity were as follows: 17.76 meq/kg for *E. resinifera* honey and 21.60 meq/kg for *E. officinarum* honey (Table 6.2).The values of moisture were 18.69% for *E. resinifera* and 19.00% for *E. officinarum* (Table 6.2). The values of electrical conductivity obtained in the present study were 379 µS/cm for *E. resinifera* and 342 µS/cm for *E. officinarum* (Table 6.2).

The values of diastase activity were above 8.0 Shade units/g (Table 6.2). In this study, the amount of proline detected in *E. officinarum* honey was 730 mg/kg while *E. resinifera* honey presented greater amount of 954 mg/kg (Table 6.2). The two studied honey samples presented the same ash content (0.14%) (Table 6.2). *E. officinarum* honey had 89.4 mg/kg (Table 6.2); the value surpasses the limit permitted by the European regulations. Regarding *E. resinifera* honey, the value found was 2.30 mg/kg (< 40 mg/kg). Concerning honey colour, both samples had a mm Pfund > 114, corresponding to a dark amber colour (colour Pfund scale =408 mm for *E. resinifera* and 295 mm for *E. officinarum*) (Table 6.2).

For *E. resinifera*, the percentage of reducing sugar was 66.7% and for *E. officinarum* was 61.7%; therefore, they are within the range of the quality standards (Table 6. 2).The results of the sugars profiling are summarized inTable 6.2. According to the results depicted in Table 6.2, the *E. resinifera* honey had higher amount of fructose (37.0 ± 0.8 g/100 g) when compared with *E. officinarum* honey (34.9 ± 0.1 g/100 g). Concerning glucose in both honey samples, the amounts were 30.2 ± 30 and 34.1 ±3.8 g/100 g for *E. resinifera* and *E. officinarum* honeys, respectively. In honey samples, it was also possible to find sucrose, trehalose, maltose and turanose (Table 6.2); nevertheless, the levels were different depending on the honey type. *E. officinarum* honey had higher amounts of trehalose, maltose and turanose but lower amounts of sucrose than *E. resinifera* honey.

The analysis of the mineral elements in both honey samples revealed the predominance of potassium with a value of 394 mg/kg in *E. officinarum* honey and 334 mg/kg in E. *resinifera* honey (Table 6.2). Relative high amounts of Fe, Cu and Al were observed *in E. officinarum* honey (Table 6.2)

3.2. Phenol content and antioxidant activity

The phenolic content in the *E. honeys* were as follows: *E. officinarum* honey (61.8 mg GAE/100 g) and *E. resinifera* honey (57.6 mg GAE/100 g) (Table 6.2).

Since honey contains several phenolic compounds with antioxidant capacity, it is interesting to evaluate the use of different antioxidant methods to test their antioxidant capabilities. Thus, three antioxidant tests were performed in this study (DPPH, superoxide and NO radicals scavenging capacity). In the present work, *E. officinarum* honey presented lower IC_{50}values in the DPPH and superoxide radical scavenging activity (55.5 and 2.80 mg/mL, respectively) compared with *E. resinifera* honey (80.1 and 3.70 mg/mL, respectively) (Table 6.2), therefore with higher ability for scavenging those free radicals. In contrast, in what concerns the ability for scavenging the NO free radicals, *E. resinifera* honey presented lower IC_{50} value (88.2 mg/mL) than *E. officinarum* honey (116 mg/mL) (Table 6.2).

3.3. Inhibition of lipoxygenase, acetylcholinesterase, tyrosinase and xanthine oxidase activities

The inhibitory action of *E. resinifera* honey on lipoxygenase, acetylcholinesterase, tyrosinase and xanthine oxidase activities was better, with lower IC_{50} values (32.7, 44.7, 11.5 and 71.7 mg/mL, respectively), than *E. officinarum* honey, which exhibited higher IC_{50} values (46.8, 165, 54.9 and 94.9 mg/mL, respectively) (Table 6.2). These results showed a negative correlation between total phenols and IC_{50} values, that is, higher amounts of phenols promoted the enzyme inhibition.

Table 6.2. Physicochemical parameters determined to honey samples

	Euphorbia resinifera	*Euphorbia officinarum*
pH	4.10 ± 0.0 [a]	4.10 ± 0.0 [a]
Moisture (%)	18.6 ± 0.0 [b]	19.0± 0.0 [a]
Free Acidity (meq/kg)	10.1 ± 0.8 [a]	10.6 ± 1.3 [a]
Lactonic Acidity (meq/kg)	7.70 ± 1.3 [b]	11.0 ± 0.9 [a]
Total Acidity (meq/kg)	17.8 ± 1.4 [a]	21.6 ± 2.2 [a]
Conductivity (µS/cm)	379 ± 0.6 [a]	342± 1.5 [b]
Diastase (Shade units/g)	37.4 ± 1.5 [a]	13.2± 1.3 [b]
Proline (mg/kg)	954 ± 36.5 [a]	730 ± 43 [b]
Ash (%)	0.10± 0.0 [a]	0.10± 0.0 [a]
HMF (mg/kg)	2.30 ± 0.3 [b]	89.4± 8.6 [a]
Colour Pfund scale (mm)	408 ± 1.9 [a]	295± 2.0 [b]
	Dark Amber	**Dark Amber**
Reducing sugars (%)	66.6 ± 2.3[a]	61.6 ± 0.6 [b]
Fructose (g/100 g)	37.0 ±0.8[a]	34.9 ± 0.1[b]
Glucose (g/100 g)	34.1 ± 3.8[a]	30.2 ± 3.0[a]
Sucrose (g/100 g)	5.50 ± 2.5[a]	4.30 ± 0.2[a]
Turanose (g/100 g)	2.10 ± 0.9[a]	2.80 ± 0.4[a]
Maltose (g/100 g)	2.30 ± 0.7[b]	3.80 ± 0.2[a]
Trehalose (g/100 g)	2.80 ± 0.5[a]	4.00 ± 0.8[a]
Mineral analysis (mg/kg)		
Fe	10.3 ± 0.3 [b]	333 ± 10.2 [a]
Zn	1.70 ± 0.2 [a]	1.80 ± 0.1 [a]
Mn	1.10± 0.0 [b]	1.50 ± 0.2 [a]
Cu	< LOD	110 ± 21.7 [a]
Al	11.9 ± 0.5[b]	64.3 ± 6.8 [a]
Ca	117 ± 1.1 [a]	70.1 ± 0.4 [b]
K	334 ± 9.4 [b]	394 ± 0.1 [a]
Mg	40.2 ± 0.7 [a]	35.0 ± 2.2 [b]
Na	40.2 ± 0.1 [a]	36.9 ± 0.0 [b]
Polyphenols (mg GAE/100g)	54.5 ± 1.7 [b]	61.7 ± 2.9 [a]
DPPH IC_{50} (mg/mL)	80.1 ± 1.1 [a]	55.5 ± 0.7 [b]
Superoxide IC_{50} (mg/mL)	3.70 ± 0.0 [a]	2.80 ± 0.2 [b]
Nitric oxide IC_{50} (mg/mL)	88.2 ± 0.8 [b]	116 ± 1.4 [a]
Lipoxygenase IC_{50} (mg/mL)	32.7 ± 0.4 [b]	46.8 ± 0.4 [a]
ACTI IC_{50} (mg/mL)	44.7 ± 8.3 [b]	165 ± 8.5 [a]
Tyrosinase IC_{50} (mg/mL)	11.5 ± 1.8[b]	54.9 ± 3.2 [a]
Xanthine oxidase IC_{50} (mg/mL)	71.7 ± 1.9 [b]	94.9 ± 0.4 [a]

Values in the same row followed by the same letter are not significantly different ($P < 0.05$) by Student's t-test. [a] : represent the higher value ; [b] : represent the low value; LOD (limit of detection) = 0.786 mg/ mL

3.4. Phenols' content and antioxidant activity of the aqueous extracts of Euphorbia plants

Generally, the aqueous extracts of *E. officinarum* had higher concentrations of total phenols than the ones of *E. resinifera* (Fig. 6.2), independently of the extraction time and the ratio plant material (d.w.)/volume of extraction solvent. The ratio mass of *E. officinarum*: solvent volume of 1:100 and extraction time of 1 h or 2 h provided extracts with higher concentrations of total phenols (11.8 mg GAE/g in both cases). In what concerns *E. resinifera*, the best extraction conditions were 1:100 and 30 min and 1 h (7.50 and 7.20 mg/g, respectively) (Fig. 6.2). Generally, and in both plant extracts, the lowest ratio originated extracts with lower amounts of phenols.

Antioxidant activity assayed through DPPH method, revealed that with the exception of the ratio 1:100, in the time periods of 30 min and 2 h, *E. resinifera* extracts had lower IC_{50} values (IC_{50} = 0.370 mg/mL), therefore higher capacity for scavenging the DPPH free radicals (Figure 2b). Nevertheless, in what concerns the capacity for scavenging superoxide anion radicals, the best activity was found in *E. officinarum* extract of 1:100 ratio and after 1 h of extraction (IC_{50} = 0.17 mg/mL). In addition, 1 h of extraction originated extracts of *E. officinarum* with good ability for scavenging the superoxide radical anions, in opposition to the extracts of *E. resinifera* that showed the poorest superoxide scavenging activity (Figure 6.2c). The best NO scavenging activity was found for *E. resinifera* extract of 1:100 ratio and after 2 h of extraction. Moreover, this time period provided the extracts of *E. resinifera* with the lowest IC_{50}, therefore with the best activities. Among the *E. officinarum* extracts, the ones yielded using the ratio 1:100 for 1 h of extraction were the best (IC_{50} =0.39 mg/mL) for scavenging the NO free radicals (Figure 6.2d)

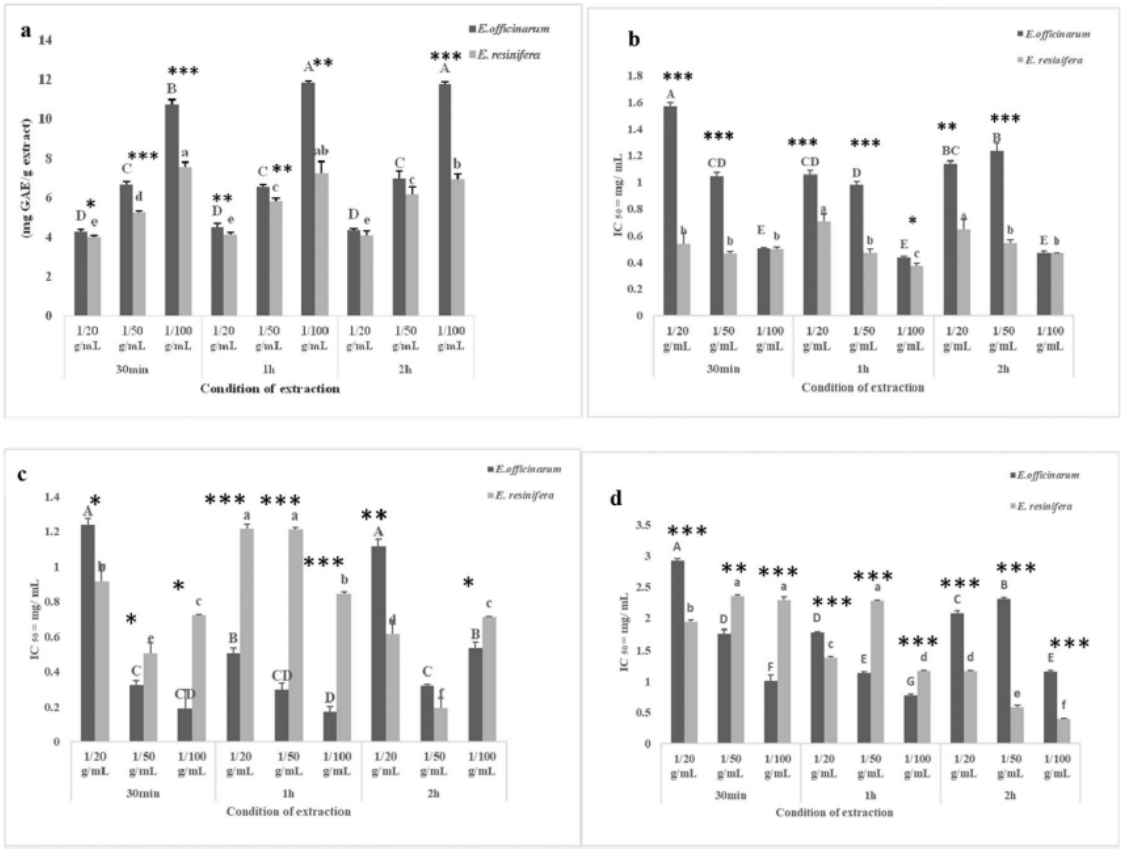


Figure 6.2. Total phenols and antioxidant activities of *Euphorbia resinifera* O. Berg and *Euphorbia officinarum* L. water extracts; (**a**): Phenol compounds estimations; (**b**): DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity; (**c**): Scavenging ability of superoxide anion radical; (**d**): Nitric oxide (NO) scavenging activity. Capital letters: present the statistical analysis of *Euphorbia officinarum* (p value <0,005) Minimal letters: present the statistical analysis of *Euphorbia resinifera* (p value <0,005), P value by Student's T-test: - * ($p<0.05$)- ** ($p<0.01$); *** ($p<0.001$).

3.5. Enzymatic inhibitory activities of the aqueous extracts of Euphorbia plants

The extracts of *E. officinarum* showed the best anti-lipoxygenase activity (Figure 6.3a) when the ratio was 1:100 and the extraction time was 2 h (IC_{50} = 0.57 mg/mL), immediately followed by those extracts with the ratio 1:100 and 1 h of extraction and 1:50 and 2 h of extraction (IC_{50} = 0.99 mg/mL and IC_{50} = 1.07 mg/mL, respectively). Nevertheless, this species also presented the worst activities in the following extracts (1:20 and 30 min of extraction; 1 h and 2 h of extraction) (Figure 6.3a). In contrast to the inhibitory activity of lipoxygenase, in the inhibition action on acetylcholinesterase, the *E. resinifera* possess almost always better activities than E. *officinarum*, independently on the ratio or extraction time (Figure 6.3b). The sole exception was the extract of 1:20 ratio and 30 min of extraction. The worst activities were found when the ratio was 1:20, independently on the species or time extraction (Figure 6.3b). Generally, *E. resinifera* extracts

presented better capacity for inhibiting tyrosinase activity, with only one exception (1:100 ratio and 1 h of extraction). In this case, *E. officinarum* had the lowest IC_{50} value (1.46 mg/mL), and therefore the best activity. This IC_{50} was similar to that observed for *E. resinifera* ratio 1:100 and 30 min of extraction (IC_{50} = 1.49 mg/mL). Generally, the trend of inhibitory activity was similar to that registered for acetylcholinesterase inhibitory activity, that is, the highest the ratio (plant material:solvent extraction), the best inhibitory activities were found (Figures 6.3b and 6.3c). Concerning xanthine oxidase inhibitory activity, *E. officinarum* extracts obtained after 2 h of extraction and in all ratio assayed (1:20, 1:50 or 1:100) had the lowest IC_{50} values (23.6, 18.7 and 21.6 mg/mL, respectively), which means a best ability for inhibiting the xanthine oxidase activity (Figure 3d). Regarding *E. resinifera*, only the extract 1:100 ratio and 2 h of extraction had significantly better activity than the remaining *E. resinifera* extracts (IC_{50}= 26.1 mg/mL) (Figure 6.3d)

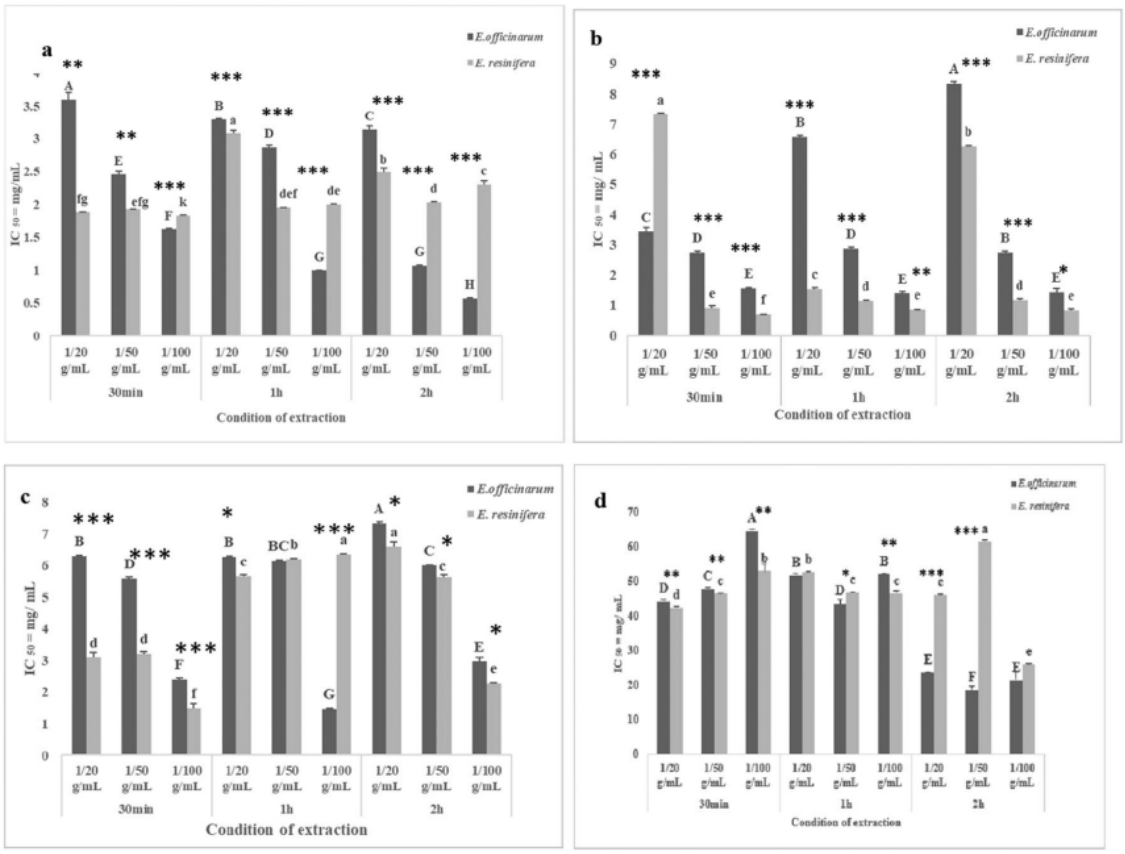


Figure 6.3. Enzyme's inhibitory activities of *Euphorbia resinifera* O.Berg and *Euphorbia officinarum* L. water extracts; (**a**): Inhibition of lipoxygenase activities; (**b**): Inhibition of acetylcholinesterase activities; (**c**): Inhibition of tyrosinase activities; (**d**): Inhibition of xanthine oxidase activities.

Capital letters: present the statistical analysis of *Euphorbia officinarum* (p value <0.005). Minimal letters: present the statistical analysis of *Euphorbia resinifera* (p value <0,005) P value by Student's T-test: - * (p<0.05); ** (p<0.01); *** (p<0.001)

Table 6.3. Pearson correlation coefficients between total phenols and antioxidant activities and enzyme inhibitory activates of Euphorbia extracts

E. officinarum	**Phenol**	**Lipoxygenase**	**Acetylcholinesterase**	**Tyrosinase**	**Xanthine oxidase**	**DPPH**	**NO**	**Superoxide**
	1	- 0.878**	- 0.774**	- 0.949**	0.145	-0.901**	-0.778**	-0.622**
E. resinifera	1	- 0.464*	- 0.697**	- 0.356	-0.235	-0.704**	-0.163	-0.308
E. officinarum	1	- 0.756**	- 0.531**	- 0.629**	-0.030	-0.413**	-0.578**	-0.452**

**Correlation is significant at the P < 0.01 level; *Correlation is significant at the P < 0.05 level

3.6. Comparison of studied activities between Euphorbia honey samples and aqueous Euphorbia extracts

When comparing the activities found in honey samples and those in aqueous extracts of the plants, the results observed for aqueous plant extracts were always better than those found in aqueous honey samples (Figure 6.4a), which can partially be explained by the lowest amounts of phenols detected in honey samples (around 10–20 times lower than the plant extracts). However, in some cases, the activities of honey samples were not 10 or 20 times lower than the activities of plant extracts. For example, the minimal and maximal IC_{50} values of DPPH and NO scavenging activities, and acetylcholinesterase inhibitory activity of *Euphorbia* plants extracts were 0.37 - 1.57;0.39 - 2.92; and 0.70 - 8.36 mg/mL, respectively, which are more than 20 times lower than the respective IC_{50} values of honey (Table 6.2). These results mean that the activities of the plant extracts are more than 20 times greater than the activities of the honeys. Therefore, it might exist in some compounds, not determined in this work, present in honey samples that impair the biological properties. On the other hand, the capacity for scavenging the superoxide radical anions or the anti-acetylcholinesterase and anti-lipoxygenase activities of the aqueous *Euphorbia* extracts were approximately 10 times higher than those verified in the honey samples. On the anti-xanthine oxidase activity, the differences observed in plant extracts and honey samples were not as great as observed inthe remaining biological properties (Figure 6.4a).

When comparing *E. officinarum* and *E. resinifera* honey samples with those of the respective plant extracts, it was possible to verify that the activities of plant extracts were higher (blue colour means lower IC_{50}, thus higher inhibitory capacity or scavenging activity depending on the case) than honey samples. Xanthine oxidase inhibition activity of all samples except 7, 8, 9 and 16 was poorer (higher IC_{50}) thanthe remaining activities (Figure 6.4a). This permitted grouping these four samples in one cluster (Figure 6.5a): the 2 samples of honey which stood out of the remaining samples by their weaker activities in almost all samples constituted another cluster; samples 3 and 15 constituted a third cluster with lower anti-xanthine oxidase activity than the remaining plant

extracts but even superior to the honey extracts; and the remaining samples constitute a fourth cluster (Figure 6.5a). According to the results, it seems that the extraction observed for *E. officinarum (*7, 8 and 9) and the extraction 16 for *E. resinifera* were the most adequate for obtaining better anti-xanthine oxidase.

Since the values of honey samples were very different from those obtained for plant extracts, only the plant extract parameters were considered in Figure 4b. The matrix plot allowed concluding that *E. resinifera* extracts had better anti-acetylcholinesterase activity and DPPH free radical scavenging activity (Figure 6.4b). The samples 7, 8, 9 and 15 (former 16) corresponded to the samples with the best anti-xanthine oxidase activity; samples 8 and 9 had also the best anti-lipoxygenase activity, along with sample 6. Samples 3, 6 and 9 (*E. officinarum*) and 15 and 18 had the best anti-tyrosinase activity (Figure 6.4b). Figure 6.5b illustrates the dendrogram obtained only considering the values of plant extracts. In this case, the samples 7, 8, 9 and 15 (former 16) constitute a cluster as observed when honey samples were also considered. It is the same for the samples 3 and 14 (former 15) that constitute other cluster (Figure 6.5b).

Figure 6.6a depicts a plot of two principal components (PC1 and PC2) from all analyzed parameters for *Euphorbia* plant extracts and *Euphorbia* honeys. The Figure shows 2 outliers corresponding to honey samples (10 and 20) occupying the positive part of the PC1. All plant extracts occupied the negative part of PC1. For this reason, those two honey samples were removed, and the data again analyzed (Figure 6.6b). The two components rep-resented 47.63% and 16.06% for PC1 and PC2, respectively. The plot shows that NO and DPPH scavenging activities are strongly related, as well as superoxide scavenging activity and anti-tyrosinase activity. The phenol content is strongly and negatively correlated with anti-lipoxygenase activity and less with anti-tyrosinase and anti-acetylcholinesterase activities, and superoxide, NO and DPPH scavenging activities. The samples 3, 6, 9; and 12, 15 and 18 occupied the negative part of PC1, that is, these samples of *E. officinarum* (3, 6, 9) and *E. resinifera* (12, 15, 18) were all of them extracted using a ratio mass/volume (1/100), independently on the extraction time (1/2 h, 1 h and 2 h). In this negative part of PC1, were also placed the samples of *E. resinifera* (14 and 17) corresponding to the ratio 1/50 extracted for 1 h. In general, this PCA permitted distinguishing the samples with higher extraction efficiencies, with a ratio of 1/100, from the remaining extraction conditions, for both plant species (Figure 6.6b).

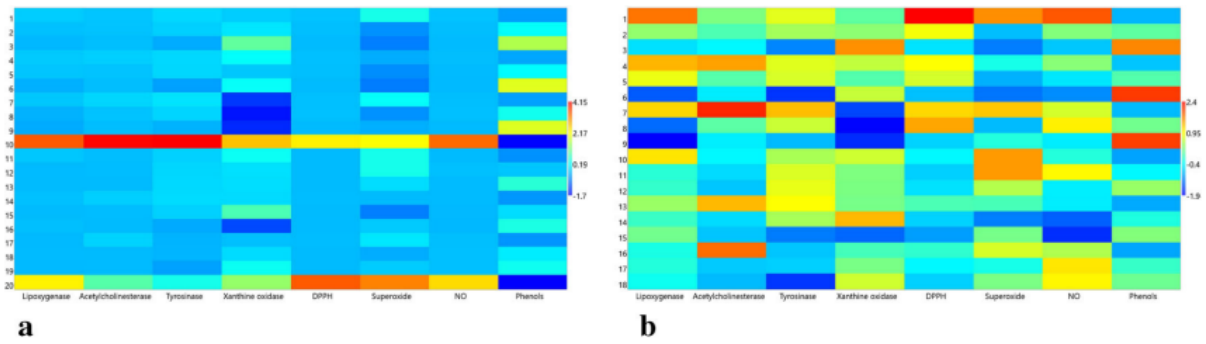


Figure 6.4a. Two-dimensional plot of the data matrix (blue for the lowest value, red for the highest value) for: inhibitory capacities of lipoxygenase, acetylcholinesterase, tyrosinase and xanthine oxidase (IC_{50} values); for DPPH free radical, Superoxide anion radical and Nitric Oxide (NO) scavenging abilities (IC_{50} values) and total phenols (mg GAE/g). 1-9 - extracts of *Euphorbia officinarum* plants (1 – 3: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 4 -6: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1h; 7- 9 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2h); 11-19 extracts of *Euphorbia resinifera* plants (11 – 13: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 14 - 16: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1 h; 17- 19 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2 h); 10 and 20 - *Euphorbia officinarum* and *Euphorbia resinifera* honey samples, respectively.

Figure 6.4b. Two-dimensional plot of the same data matrix as in figure 4a, but without the honey samples (10 and 20).

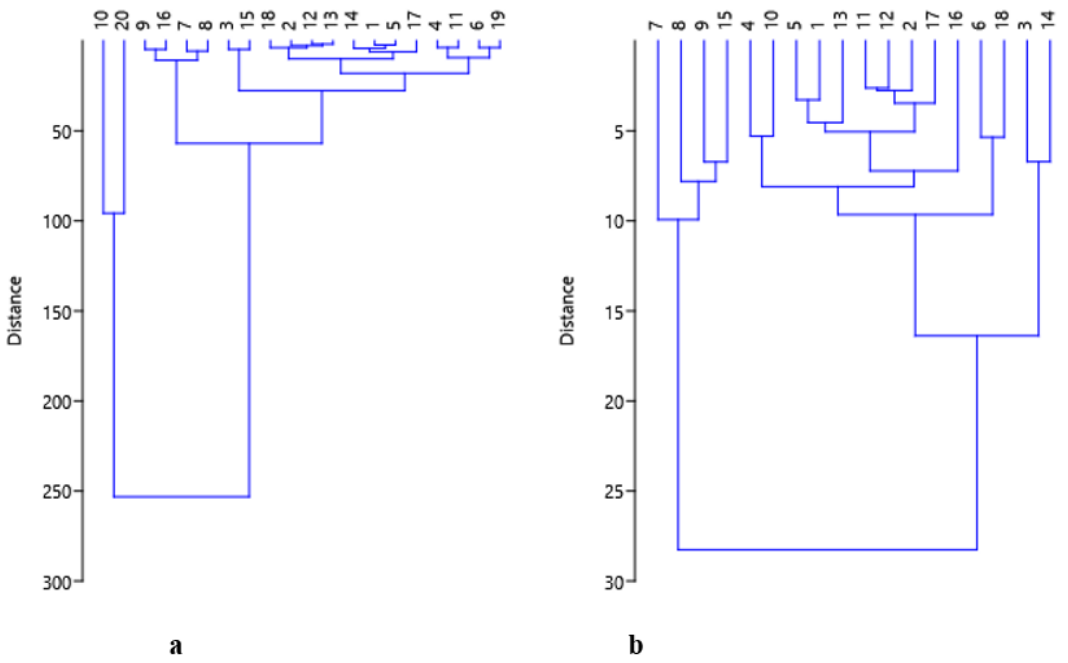


Figure 6.5a. and 6.5b. Dendrogram obtained by cluster analysis of the phenol content and activities, based on correlation and using Ward's method.

Figure 6.5a. 1-9 - extracts of Euphorbia officinarum plants (1 – 3: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 4 -6: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1h; 7- 9 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2h); 11-19 extracts of Euphorbia resinifera plants (11 – 13: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 14 - 16: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1 h; 17- 19 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2 h); 10 and 20 - Euphorbia officinarum and Euphorbia resinifera honey samples, respectively.

Figure 6.5b. 1-9 - extracts of Euphorbia officinarum plants Legend: 1-9 - extracts of Euphorbia officinarum plants (1 – 3: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 4 -6: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1h; 7- 9 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2h); 10-18 extracts of Euphorbia resinifera plants (10 – 12: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 13 - 15: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1 h; 16- 18 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2 h).

4. Discussion

4.1. General physicochemical properties

Physicochemical properties of honeys are important indicatorsof the quality and origin of this natural product (21). The pH is an important parameter during honeys' extraction and the storage, influencing the texture as well as the stability of the product (9). Usually the pH of honey is < 4.0, which is an important factor preventing microorganisms' growth (21). In this study, pH values were similar to that found by (9) in eleven *E. officinarum* honey samples where pH values ranged from 4.0 to 3.8.

Organic acids are responsible for the acidity of honey and influence its taste (21). According to the values given by the Codex Alimentarius, the free acidity of honey must not exceed 50 milliequivalents of acid per 1000 g (Codex Alimentarius Commission 1999) (22) .The free acidity values found in the present work were slightly lower than that previously reported (18.2 meq/kg for *E. resinifera* honey) (8). In another study (23), the results were significantly higher (33 meq/kg) than our results. Concerning *E. officinarum* honey, the results obtained by (9) were higher (53.22 meq/kgand 80.28 meq/kg) than our results for the same type of honey. In the work of (31), higher lactonic acidity values ranged from 14.70 to 53.92 meq/k g (mean = 24. 4 meq/kg) were reported for *E. resinifera* honey. On the other hand, the results found by (9) showed that *E. officinarum* honey presented lower lactonic acidity value than the one we found in the present study (2.58 meq/kg).

Water content is the second most important parameter ofhoney and its content can vary from 15 to 23% (EU 2002)(24). Inour study, the values of moisture were within the standardsproposed by the European legislation) (EU 2002) (24). A previous-ly published study (23) has shown that watercontent of *Euphorbia resinifera* was 20.00 and 18.00%. On the otherhand, (9) has found 18.50% for *Euphorbia officinarum* , a value similar to that one found in the presentwork (19%).

The electrical conductivity of honey usually depends on the content of minerals, organic acids and protein (21). The results of electrical conductivity found in this work are in agreement with the values proposed by the European legislation (< 800 µS/cm) (EU 2002) (24). In another study (8), *E. resinifera* honey showed values (761µS/cm) higher than the ones of this work. For *E. officinarum* honey, a study performed by (9) has also shown a value superior (561.18 µS/cm) than the one we found.

The predominant enzyme in honey is diastase which allows the degradation of starch to maltose; this enzyme is relatively sensitive to heat and storage (21). The diastase activity is one of the important indicators of storage condition. It is used to confirm the honey freshness (8). Both honey

samples were above the minimum required by European legislation (2002) (24), which is in agreement with the values reported by (8) for honeys produced from different plants in Morocco.

The amino acids' content assessed as proline content of honey varied between 0.05 and 0.1%, being proline the most abundant (21). Both samples contained more than the minimum acceptable for proline concentration (200 mg/kg) (25).

The ash content, as reported by (6), is traditionally used to determine the honey type (nectar or honeydew). According to the European legislation (2002) (24), the value of ash content in honey must not exceed 0.6%, whereby the honey samples with values lower than this percentage is not a honeydew.

The HMF (hydroxymethylfurfural) is a compound formed slowly during honey natural storage and quickly when the honey is heated (21). The Codex Alimentarius (1999) (22) requires that the HMF content must not exceed 40 mg/kg in honey. Concerning *E. officinarum* honey, the value surpasses the limit permitted by the European regulations. According to (9), *E. officinarum* honey had 85.48 mg/kg, that is, close to the value that we found. Regarding E. *resinifera* honey, the value found was 2.30 mg/kg (< 40 mg/kg), a value that meets the prescribed standards of the Codex Alimentarius (1999) (22). In a study presented by (6), *E. resinifera* honeys showed lower values of HMF ranging from 0.4 to 16.8 mg/kg.

Honey colour is an indirect indicator of its content of polyphenols, terpenes and carotenoids (26). Both samples had a mm Pfund > 114, corresponding to a dark amber colour.

The reducing sugar content in honey varies due to storage conditions, enzymatic activity, the reversal of acids and harvest period (27). The European standard for a quality honey recommends values above 60% for content of reducing sugars (fructose, glucose and maltose) (EU 2002) (24); therefore, both samples are within the range of the quality standards. Honey consists mostly of the monosaccharides glucose and fructose (28). According to (4), the fructose/glucose ratio is able to influence the crystallization of honey, and more precisely crystallization is prevented by fructose, but promoted by glucose. This ratio was 1.08 for *E. resinifera* honey and 1.15 for *E. officinarum* honey (Table 6.2). Such results may indicate that *E. resinifera* honey is more likely to crystalize than *E. officinarum* honey. The amounts of these two monosaccharides were within the range found by (6) for *E. resinifera* honey.

According to Elamine et al. (29), potassium prevails in honey samples followed by calcium and sodium. In the present work, this was observed only for *E. resinifera* honey (Table 6.2), while for *E, officinarum* honey, the second and third elements were Fe (332.5 mg/kg) and copper (109.7 mg/kg). The high amounts of Fe, Cu and Al in *E. officinarum* honey may be related to some environmental contamination by these elements in the area surrounding beehives or even the

inadequate use of metallic containers for honey storage, although the importance of the origin area on the elemental composition of honeys (30).

4.2. Phenol content and antioxidant activity

Generally, and following research done on the Sahara honeys, the total phenolic contents found ranged between 72.0 and 97.9 mg GAE/100 g of honey (45). In another study done on *Euphorbia* Turkish honey (31), the authors reported 278.98 mg GAE/100 g, a value higher than those found in the present work. In another work (32), where the authors studied several types of Moroccan honeys, much higher phenolic content (518.92 mg GAE/100 g) was found for *E. resinifera* honey when compared with the results of this work (Table 6.2).

Concerning the capacity for scavenging free radicals, our data are in close agreement with other authors (33) who have found DPPH IC_{50} in the range of 24.46–81.82 mg/mL for Turkish *Euphorbia* honeys. Nevertheless, some authors (34) showed better capacity for scavenging DPPH free radical of *E. resinifera* honey (IC_{50} = 7.91 mg/mL) than our sample (Table 6.2). The results reported by Elamine et al. (29) have shown that the capacity for scavenging the superoxide anion radicals of Moroccan *Bupleurum spinosum* honey, with the highest IC_{50} value (51.31 mg/mL), was weaker than that of Moroccan *Euphorbia* honey. The IC_{50} values found for the NO assay were similar to those already reported (32) for *E. resinifera* honey (IC_{50} = 95.14 mg/mL) and for Moroccan *B. spinosum* honey (IC_{50} = 118.38 mg/mL) (29). These differences observed can be attributed to the botanic origin as well as the climatic conditions where samples were collected (29,35). Higher amounts of phenols in *E. officinarum* honey determined its better ability for scavenging DPPH and superoxide radical anions (lower IC_{50} values), nevertheless with a negative effect on the ability for scavenging NO free radicals, as revealed by the best activity found in *E. resinifera* honey (Table 6.2)

4.3. Inhibition of lipoxygenase, acetylcholinesterase, tyrosinaseand xanthine oxidase activities

The negative correlation between total phenols and IC_{50} values, that is, higher amounts of phenols promoted the enzyme inhibition, is expected and even observed insome cases for natural products (18). The biological activities cannot solely be attributed to the phenols, even to individual ones, when weare working with complex systems such as food matrices in which honey is included. Several components other than phenols in different proportions can contribute to the properties found. Moreover, such components and their relative proportions can change depending on uncontrolled several factors (e.g. climate, soil...). In addition, only two samples of honey were studied; therefore and although one of them presented better results than the other one, it is just better to say that both present capacity for inhibiting those enzymes. Much more samples would

be needed to be sure about the differences observed in the present work. However, it will be always difficult due to the limited areas of *E. resinifera* honey production.

Other types of monofloral honeys (citrus, lavender) or different geographical origins have been reported as possessing anti-lipoxygenase activity (20), anti-acetylcholinesterase activity (36), anti-tyrosinase activity (37,38) and anti-xanthine oxidase activity (37; 39).

4.4. Phenols' content and antioxidant activity of the aqueousextracts of Euphorbia plants

Generally, and in both plant extracts, the lowest ratio originated extracts with lower amounts of phenols: the higher the ratio, the higher the phenol concentration. These results may reveal that lower volume of water (extraction solvent) was not enough for extracting higher amounts of phenols; probably the components extracted had low solubility in water (e.g. aglycones) which were only extracted and, therefore, solubilized in higher volumes of water. For this reason, other authors (5) had found better extraction results than those found in the present work, but using other extraction solvent (ethyl acetate).

The lowest values of IC_{50} found in the present work for the three assays (DPPH, superoxide and NO scavenging activity) were always higher than those verified for the standards used for comparison (IC_{50} = 0.022, IC_{50} = 0.013 and IC_{50} = 0.229 mg/mL, for BHT, ascorbic acid and curcumin, respectively). With the exception of the IC_{50} value for curcumin, which was approximately the half of that verified for the best extract (IC_{50} = 0.393 mg/mL), the remaining standards were approximately ten times lower than the best extracts for scavenging DPPH or superoxide free radicals. The capacity for scavenging DPPH free radicals previously reported (40) for methanolic extracts of *E. hirta* leaves (1 mg/mL had a percentage of inhibition of 73%) was poorer than that observed in the present work. However, for methanolic extract of *E. resinifera* roots, (5) described significantly higher activity (IC_{50} = 0.010 mg/mL).

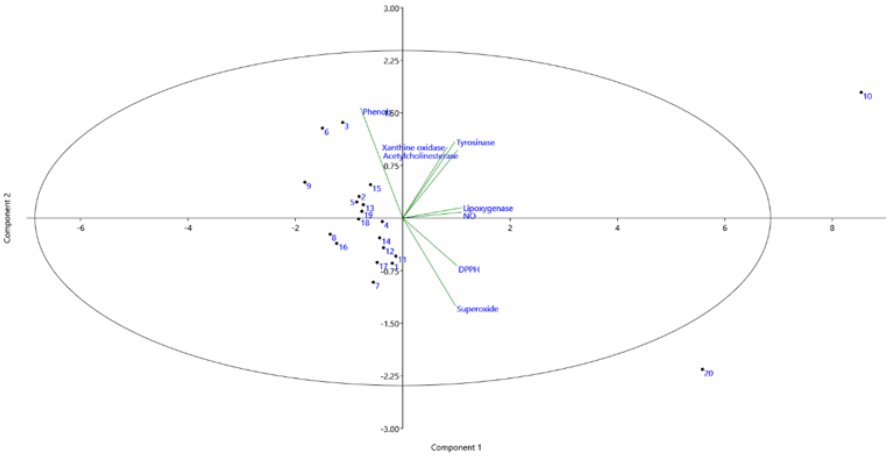
4.5. Enzymatic inhibitory activities of the aqueous extractsof Euphorbia plants

The capacity for inhibiting the xanthine oxidase activity in the present work was much lower than those verified for extracts obtained using other solvents and from other species of *Euphorbia* (IC_{50} = 0.1 mg/mL) (41,42).

Galantamine, kojic acid and nordihydroguaiaretic acid (NDGA) were used as standards to compare their activities with the ones of the samples, and all of them presented significantly better activities than the sample extracts, presenting lower IC_{50} values (IC_{50} = 0.001, 0.006 and 0.003 mg/mL,respectively).

The importance of phenols for the antioxidant activity of the extracts, particularly for the capacity for scavenging DPPH free radicals, is evident in both species extracts, through the negative

correlation between the content phenols and the IC_{50} values (Table 6.3), that is, the highest concentrations of phenols the best scavenging DPPH free radicals. The same can be observed in what concerns the inhibition of lipoxygenase and acetylcholinesterase activities; nevertheless, the role of phenols on the inhibitory action on xanthine oxidase is absent in both extracts (Table 6.3). Non-phenolic compounds can be responsible for the xanthine oxidase inhibitory activities of the extracts. The correlation between the phenol content and the capacity for scavenging NO and superoxide radicals or between the phenol content and the inhibitory activity on tyrosinase was also negative in both extracts, nevertheless only with statistical significance in the *E.officinarum* extracts. Therefore, particularly in the case of *E. resinifera* extracts, not just phenols but also other components play an important role on the properties detected. When both data of *E. officinarum* and *E. resinifera* are treated as a whole, only the correlation between phenol content and anti-xanthine oxidase activity is nonexistent, as expected since when the results were treated separately, they did not show any correlation type (Table 6.3).



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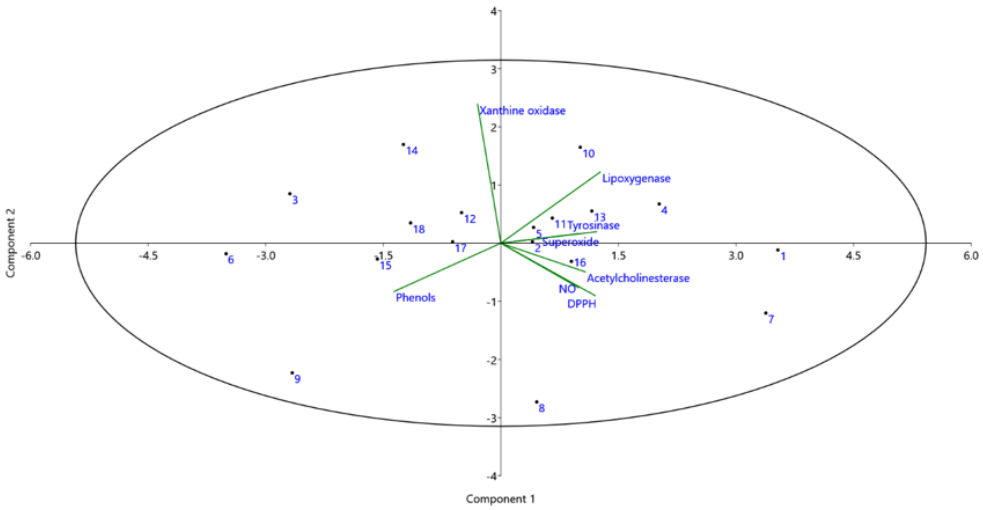

b

Figure 6.6a. Plot of two principal compounds analyses (PCA). 1-9 - extracts of *Euphorbia officinarum* plants (1 – 3: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 4 -6: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1h; 7- 9: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2h); 11-19 extracts of *Euphorbia resinifera* plants (11 – 13: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 14 - 16: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1 h; 17- 19 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2 h); 10 and 20 - *Euphorbia officinarum* and *Euphorbia resinifera* honey samples, respectively.

Figure 6.6b. Plot of two principal compounds analyses (PCA). 1-9 - extracts of *Euphorbia officinarum* plants . 1-9 - extracts of *Euphorbia officinarum* plants (1 – 3: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 4 -6: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1h; 7- 9 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2h); 10-18 extracts of *Euphorbia resinifera* plants (10 – 12: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 30 min; 13 - 15: 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 1 h; 16- 18 : 1/20, 1/50, 1/100 g/mL, respectively for an extraction period of 2 h).

5. Conclusion

The results of this work have shown that *E. officinarum* and E. *resinifera* honeys were within the limits established by the European legislation. However, *E. officinarum* honey revealed high HMF content, which may indicate inadequate heating and/or storage conditions. The amounts of iron, copper and aluminum were also detected in relatively high amounts in *E. officinarum* honey, which may be related to the environmental pollution around the beehives or inadequate storage. Such results suggest the need to improve the production and storage conditions of honey. In addition, and in what concerns the extracts of plants visited by bees (*E. resinifera* and *E. officinarum*), it highlights the importance of refining extraction procedure for *Euphorbia* plant: the volume of solvent, the ratio and the extraction time in order to obtain aqueous extracts with better *in vitro* antioxidant and enzyme inhibitory properties (anti-lipoxygenase, anti-acetylcholinesterase and anti-tyrosinase). The ratio plant mass:solvent volume (1:100) and extraction time (1 - 2 h) were associated with higher total phenols and better antioxidant activities and lipoxygenase, acetylcholinesterase and tyrosinase inhibitory activities, regardless of the plant species. Lastly, the study suggests that the differences in the activities found between the honey samples (lower activity) and *Euphorbia* extracts cannot be attributed just to the levels of phenols in the samples since the difference in concentrations of these compounds in both samples was not proportional to the differences found in the *in vitro* activities.

Acknowledgements

The authors acknowledge Manuela David, curator of ALGU Herbarium, in supervising tasks involving the identification, preparation and deposit of the voucher specimens.

Funding information

The authors would like to acknowledge the support granted from FCT under the projects UID/MAR/00350/2020; UIDB/05183/2020; UIDB/04326/2020 and UID/AGR/00690/2019

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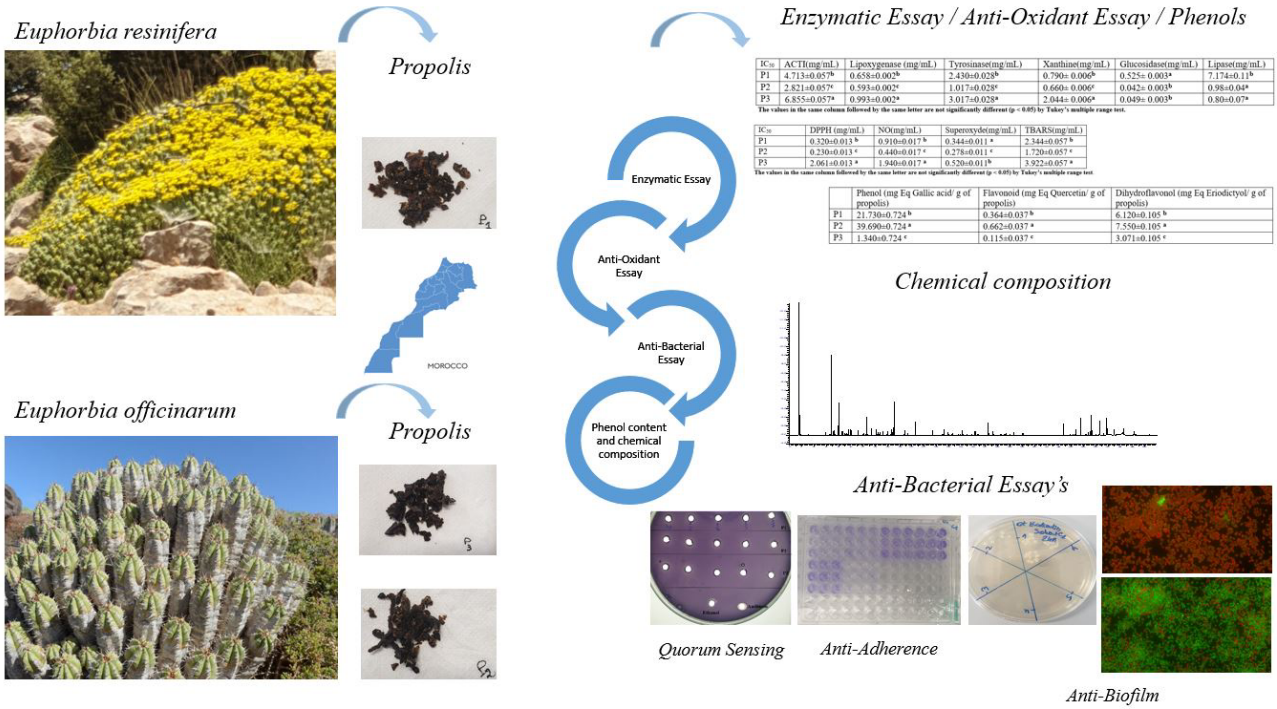
Chapter 7

Euphorbia resinifera and *Euphorbia officinarum* Moroccan Propolis: some *in vitro* biological properties



IC_{50}	ACTI(mg/mL)	Lipoxygenase (mg/mL)	Tyrosinase(mg/mL)	Xanthine(mg/mL)	Glucosidase(mg/mL)	Lipase(mg/mL)
P1	4.713±0.057^{b}	0.658±0.002^{b}	2.430±0.028^{b}	0.790± 0.006^{b}	0.525± 0.003^{a}	7.174±0.11^{b}
P2	2.821±0.057^{a}	0.593±0.002^{a}	1.017±0.028^{a}	0.660± 0.006^{a}	0.042± 0.003^{b}	0.98±0.04^{a}
P3	6.855±0.057^{a}	0.993±0.002^{a}	3.017±0.028^{a}	2.044± 0.006^{a}	0.049± 0.003^{b}	0.80±0.07^{a}

The values in the same column followed by the same letter are not significantly different ($p < 0.05$) by Tukey's multiple range test.

IC_{50}	DPPH (mg/mL)	NO(mg/mL)	Superoxyde(mg/mL)	TBARS(mg/mL)
P1	0.320±0.013 ^{b}	0.910±0.017 ^{b}	0.344±0.011 ^{a}	2.344±0.057 ^{b}
P2	0.230±0.013 ^{a}	0.440±0.017 ^{a}	0.278±0.011 ^{a}	1.720±0.057 ^{a}
P3	2.061±0.013 ^{a}	1.940±0.017 ^{a}	0.520±0.011^{b}	3.922±0.057 ^{a}

The values in the same column followed by the same letter are not significantly different ($p < 0.05$) by Tukey's multiple range test.

	Phenol (mg Eq Gallic acid/ g of propolis)	Flavonoid (mg Eq Quercetin/ g of propolis)	Dihydroflavonol (mg Eq Eriodictyol/ g of propolis)
P1	21.730±0.724 ^{b}	0.364±0.037 ^{b}	6.120±0.105 ^{b}
P2	39.690±0.724 ^{a}	0.662±0.037 ^{a}	7.550±0.105 ^{a}
P3	1.340±0.724 ^{c}	0.115±0.037 ^{c}	3.071±0.105 ^{c}

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Chapter VII: Chemical characterization and biological properties assessment of Euphorbia resinifera and Euphorbia officinarum Moroccan propolis *

Résumé

Cette étude visait à caractériser le profil pollinique, volatil, phénol et minéral, ainsi que les activités antioxydantes, d'inhibition d'enzymes et antimicrobienne de trois échantillons de propolis d'*Euphorbia* collectés au Maroc. L'analyse du pollen a montré que le pollen *d'E. resinifera* dominait dans l'échantillon P1 (58 %) et le pollen d'*E. officinarum* dans les échantillons P2 et P3 (44 %). Les volatiles d'*Euphorbia propolis* étaient dominés par les hydrocarbures monoterpéniques (35 % dans P1 et 31 % dans P2), l'α-pinène étant le composant principal dans les deux cas (16 % dans P1 et 15 % dans P2). Le calcium (Ca) était l'élément minéral le plus important dans les échantillons de propolis d'*E. resinifera* (P1) et d'*E. officinarum* (P2 et P3). Des quantités plus élevées de phénols, de flavonoïdes et de dihydroflavonoïdes ont été trouvées dans l'échantillon d'*E. officinarum* P2, ce qui correspondait à une meilleure activité antioxydante déterminée par la capacité à éliminer le 2,2-diphényl-1-picrylhydrazyl (DPPH) et l'oxyde nitrique (NO) radicaux libres, radicaux anions superoxydes et pour prévenir la peroxydation lipidique grâce au test des substances réactives à l'acide thiobarbiturique (TBARS). P1 et P3 ont montré la meilleure capacité à inhiber l'activité de la glucosidase, tandis que l'échantillon P2 a montré la meilleure capacité à inhiber les activités de l'acétylcholinestérase, de la lipoxygénase, de la tyrosinase et de la xanthine oxydase. La valeur de la concentration minimale inhibitrice (CMI) variait entre 50 et 450 µL/mL, déterminée contre les bactéries Gram-positives et Gram-négatives. La propolis d'Euphorbia était capable d'inhiber le quorum sensing du biocapteur Chromobacterium violaceum CV026 et de nuire à l'adhérence et de perturber le biofilm bactérien, y compris celui des pathogènes bactériens résistants.

Mots clés : Propolis, *Euphorbia resinifera*, *Euphorbia officinarum*, Antioxydant, ROS, Volatiles, anti-diabétique, Anti-obésité, Hyperpigmentatine, Anti-Alzheimer, TBARS, Anti-Quorum sensing, Anti-Adherence, Anti-Biofilms, Chromobacterium violaceum, Staphylococcus aureus , S. aureus résistant à la méthicilline, Escherichia coli

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Chemical characterization and biological properties assessment of *Euphorbia resinifera* and *Euphorbia officinarum* Moroccan propolis *

Abstract

This study aimed at characterizing the pollen, volatile, phenol and mineral profile, as well as the antioxidant, inhibition of the enzyme, and antimicrobial activities of three *Euphorbia* propolis samples collected in Morocco. Pollen analysis showed that *E. resinifera* pollen dominated in P1 sample (58%) and *E. officinarum* pollen in P2 and P3 samples (44%). *Euphorbia* propolis volatiles were dominated by monoterpene hydrocarbons (35% in P1 and 31% in P2), α-pinene being the main component in both cases (16% in P1 and 15% in P2). Calcium (Ca) was the most important mineral element in both *E. resinifera* (P1) and *E. officinarum* (P2 and P3) propolis samples. Higher amounts of phenols, flavonoids, and dihydroflavonoids were found in *E. officinarum* P2 sample, which corresponded to a better antioxidant activity determined by the ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) free radicals, superoxide anion radicals, and to prevent lipid peroxidation through the thiobarbituric acid reactive substances (TBARS) assay. P1 and P3 showed the best ability for inhibiting the glucosidase activity, whereas the P2 sample showed the best ability for inhibiting acetylcholinesterase, lipoxygenase, tyrosinase, and xanthine oxidase activities. The minimum inhibitory concentration (MIC) value varied between 50-450 µL /mL determined against Gram positive and Gram-negative bacteria. *Euphorbia propolis* was able to inhibit the quorum sensing of the biosensor *Chromobacterium violaceum* CV026 and to impair the adherence and disrupt the bacterial biofilm including that of resistant bacterial pathogens.

Keywords: Propolis, *Euphorbia resinifera*, *Euphorbia officinarum*, Antioxidant, ROS, Volatiles, anti-diabetic, Anti-obesity, Hyperpigmentatin, Anti-Alzheimer, TBARS, Anti- Quorum sensing, Anti-Adherence, Anti-Biofilms, Chromobacterium violaceum, Staphylococcus aureus, Methicillin resistant S. aureus, Escherichia coli.

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1. Introduction

Nosocomial bacterial infections (healthcare institutions-associated infections) are a challenge to healthcare providers. These infections, either post-surgical, associated with medical implants, or with burns as well, are major causes of morbidity and mortality. In a healthcare setting, the infection risk by *Staphylococcus aureus* increases due to the weakened patients' immune systems (1). In Morocco, a study carried out in 2020 at the Mohamed V hospital center, in the Meknes region, showed that *S. aureus* only, is responsible for 86.6% bacterial infections in burns and all of them were identified as methicillin resistant *S. aureus* (MRSA) (2). The increase in antibiotic resistance by several human pathogen bacteria is a huge challenge to healthcare institutions. A particular group of bacteria is of special concern due to its extraordinary ability to develop multiresistant profile, which include, *Enterococcus faecium* vancomycin-resistant (VRE), *Staphylococcus aureus*, methicillin-resistant, vancomycin intermediate and resistant (MRSA/VRSA), *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* carbapenem-resistant and third-generation cephalosporin-resistant, abbreviated by the designation ESKAPE. These bacteria master antibiotic resistance mechanisms, namely i) enzymatic destruction or alteration of the agent, ii) modification or protection of the molecular target, iii) obstruction of the entry of the antibiotic into the cell, iii) use of efflux pumps (3). Other phenomena that help bacteria to fight the action of antibacterial agents and other stress conditions is their ability to form biofilm (adhere to biotic and abiotic surfaces and establish a cell-cell communication community) (4). Biofilm develops along determined phases, namely the bacterial cells adhere to a surface (the initial phase can be reversible), follows a permanent adhesion (irreversible), production of exopolysaccharides that allows the aggregation of the bacterial cells, following biofilm maturation, and by the action of fluids and natural processes inside the aggregate the sessile cells (adherent cells) can disaggregate (5). The bacterial colonization of medical devices (e.g., urinary catheters, central venous catheters, and prosthetic joint) in form of biofilms constitutes a serious risk to the health of patients (6). Some diseases are linked to the production of free radicals, such as Alzheimer's disease, Parkinson's disease, inflammatory disease, cardiovascular disease, cancer, stroke, and diabetes, despite they are an essential part of aerobic life and metabolism (7). Whenever there is an imbalance between the production and accumulation in cells and tissues of free radicals and their detoxification, oxidative stress arises (8). On the other hand, scientific evidence has shown that the inhibition of some enzymatic activities can be beneficial in the control/treatment of some diseases or health disorders. Lipoxygenase enzymes metabolize polyunsaturated fatty acids to produce hydro-peroxide fatty acids (9). Compounds that can inhibit lipoxygenase enzymes are considered as antioxidant as well as anti-inflammatory agents, since they can prevent the formation of lipid peroxides and eicosanoid inflammatory mediators, respectively (9).

Aging, obesity, diets of high purines, insulin resistance, hypertension, and some medications are factors that can trigger the hyperuricemia (high serum uric acid) (10). Allopurinol is used to manage gout, nevertheless, is effective for only 40% patients of hyperuricemia, and induces severe adverse effects such as Steven-Johnson syndrome, which may lead to death, and renal toxicity. Allopurinol acts by inhibiting xanthine oxidase, enzyme that converts hypoxanthine and xanthine into uric acid (10,11). The early treatment of diabetes can be managed through the control of postprandial blood sugar. Carbohydrates are hydrolysed into oligosaccharides by α-amylase, which in turn can be hydrolysed by α-glucosidase into monosaccharide's, such as glucose, easily absorbed by the intestinal border. The delay of this carbohydrates' absorption can be achieved by inhibiting those digestive enzymes to prevent higher levels of glucose in the blood (12). Owing to obesity increasing, even among the youngest, the number of patients undergoing various metabolic diseases, including adiposity and type 2 diabetes mellitus, is raising greatly (13). The inhibition of the pancreatic lipase enzyme will control obesity because there will not be the hydrolysis of triacylglycerols into monoacylglycerols and fatty acids, the sole way to be absorbed, preventing absorption of fats (14). Orlistat is a lipase inhibitor used to treat obesity-associated diseases nevertheless it presents side effects such as oily faecal spotting, abdominal pain, and flatus with discharge (13). The impaired cognitive function, observed in the Alzheimer's disease, has been attributed to an acetylcholine deficiency in the brain. Therefore, blocking acetylcholinesterase (AChE), which hydrolyses acetylcholine, might reduce the evolution of cognitive decline. Donepezil used in medicine in mild to moderate symptoms of Alzheimer's disease has side effects, such as urinary incontinence, nausea, diarrhoea, malaise, dizziness, and insomnia (15-17).

Natural products have been the target of study to find compounds with fewer adverse effects. Skin hyperpigmentation can be due to the tyrosinase action which is responsible for the oxidation of L-DOPA to dopachrome and further the production of melanin. Tyrosinase inhibitors can be useful for the treatment of many dermatological problems associated with melanin hyperpigmentation (18). Propolis also called bee glue is a natural substance produced by bees from plant resins, sap, and other botanical sources. It is used by bees to seal small gaps and cracks in their hives, as well as to sterilize and protect the hive from bacterial and fungal infections (19). The use of this natural substance throughout the years, either in traditional medicine or food supplements, had pushed researchers to go deeper to identify the compounds as well as the biological activities of propolis (20). So far, more than 500 chemical compounds have been identified and isolated from propolis (21,22). The capacity of scavenging free radicals and the power to protect several compounds from being oxidized make those compounds interesting as anti-inflammatories, antioxidants, and skin disease protectors, but also for preventing some diseases such as diabetes and obesity (20,22). Overall, the biological activities of propolis are

thought to be due to the presence of a wide range of bioactive compounds, including flavonoids, phenolic acids, terpenes, and volatiles (20).

The present work aimed both to chemically characterize and to evaluate the potential of two types of Moroccan propolis (*Euphorbia resinifera* and *E. officinarum*) as antioxidant, anti-inflammatory, antidiabetic, and anti-obesity, anti-tyrosinase, anti-acetylcholinesterase agents, by in vitro assays. In addition, it was also aimed to research the in vitro antibacterial activity against *S. aureus* strains, methicillin-resistant *S. aureus* MRSA, *E. coli*, and multiresistant *E. coli*, and their capacity to prevent bacterial biofilm formation.

2. Experimental

2.1. Propolis collection

Euphorbia resinifera (P1) and *E. officinarum* propolis (P2 and P3) were obtained in three apiaries located in the Morocco regions of Beni Mellal-Khénifra (P1), Souss-Massa-Tiznit (P2) and Guelmim- Oued noun (P3) (Figure 7.1).

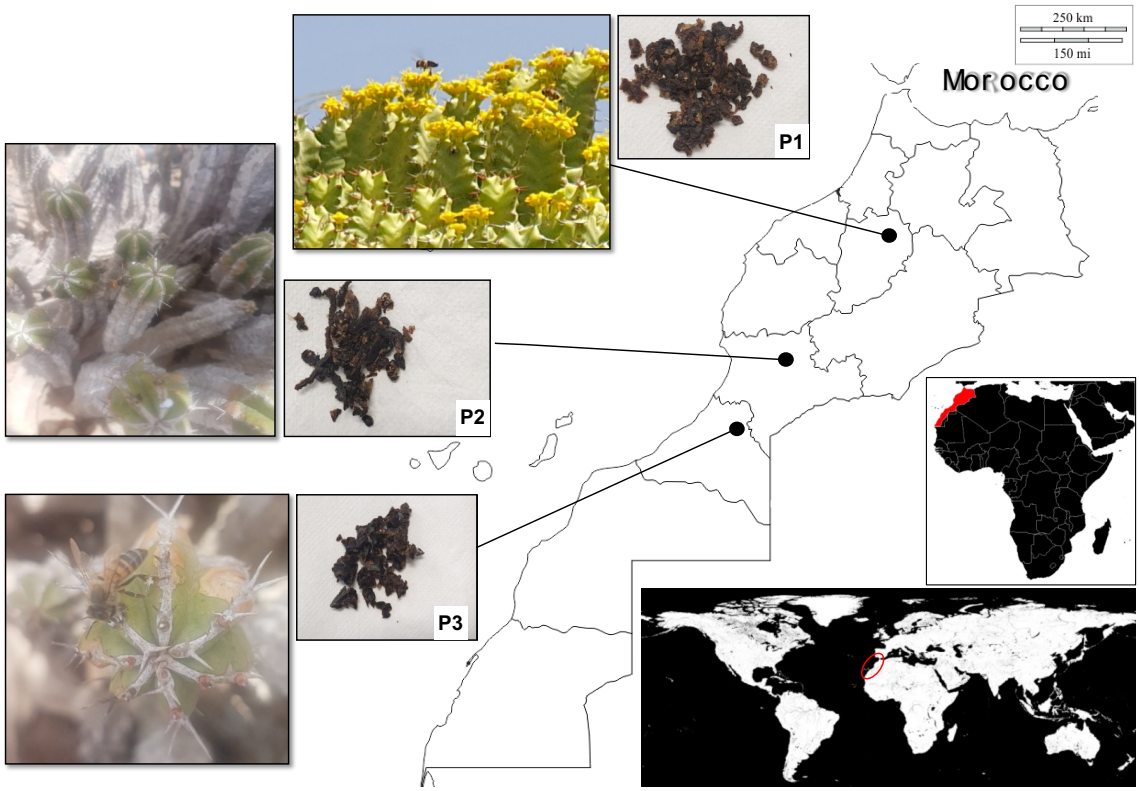

Figure 7.1. From right to left, geographical location of Morocco, and of the apiaries where the samples of *Euphorbia resinifera* (P1) and *Euphorbia officinarum* propolis (P2 and P3) were obtained. On the left, are the flowers and propolis of *Euphorbia resinifera* (P1) and *Euphorbia officinarum* (P2 and P3).

To harvest propolis, propolis traps (special grids) were placed inside the hives during the beginning of June. As the bees try to seal the grid holes, the trap gets filled with propolis. After collecting the trap by the end of July, the propolis was recovered and kept in dark at -4 °C.

2.2. Evaluation of pollen grains

The analysis of the propolis samples' pollen qualitative and quantitative spectrum was performed according to the International Commission for Bee Botany (ICBB), as previously detailed (23). Pollen identification and count were carried out using a light microscope (Leitz Messtechnik GmbH; Wetzlar, Germany) at 400× and 1000×.

2.3. Volatile organic compound extraction, analysis, and identification

Due to shortage of P3 propolis, the volatile profile was determined only in P1 and P2 samples. The volatile organic compounds (VOCs) were isolated by hydrodistillation in a Clevenger-type apparatus for 3 h at a distillation rate of 3 mL/min according to the European Pharmacopoeia protocol (2010) (24). At the end of the distillation procedure, the apparatus was cooled down for approximately 10 to 15 min and the VOCs recovered from the graduated tube by rinsing it with n-pentane distilled in the laboratory. The mixture of distilled n-pentane and volatiles was transferred to a clean glass vial and concentrated to approximately 10 µL using a blow-down evaporator system under a flux of nitrogen at room temperature. The concentrated samples were stored at -20 °C in the dark until further analysis. VOCs were quantified in a Clarus 400 Gas Chromatograph (PerkinElmer, Waltham, MA, USA) equipped with two flame ionization detectors (GC-FID). Two columns of different polarities were inserted into the injector port: a DB-1 fused-silica column (100% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Folsom, CA, USA) and a DB- 17HT fused-silica column ((50 % phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific). The oven temperature was programmed to rise from 45 to 175 °C at a rate of 3 °C/min, then to 300 °C at a rate of 15 °C/min and finally held isothermal for 10 min, for a total run time of 61.67 min. The split injector ratio was 1:40 and the injector and detector temperatures were 280 and 290 °C, respectively; the carrier gas was hydrogen, adjusted to a linear velocity of 30 cm/s. The percentage composition of the volatiles was computed by the normalization method from the GC peak areas, without the use of correction factors, calculated as mean values of two injections from each sample, in accordance with ISO 7609 (1985).

For the identification of compounds, the samples were analysed by GC-MS in a Perkin Elmer Clarus 690 gas chromatograph equipped with a DB-1 fused-silica column (100% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific) connected to a Perkin-Elmer SQ-8-T Mass Spectrometer (software version 6.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were set as previously described above with

the following additional settings: transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 m/z; scan time, 1 s. Compounds were annotated after the calculation of their retention index (RI) relative to a C7–C31 n-alkane series (Sigma) in accordance with the ISO 7609 (1985) protocol and followed by the comparison of spectra from a laboratory made library of essential oils, laboratory-synthesized compounds, and commercially available standards.

2.4. Quantification of mineral elements

Eleven mineral elements were quantified (Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, and Zn). The mineral analysis was performed as previously described by (25). Half a gram of raw propolis per sample was added to an acid mixture of 15 mL nitric acid (65%) + 5 mL hydrogen peroxide (30%) and digested in a pressurized system heated by microwave (Discover SP-D 80; CEM), using special 80 mL quartz tubes suitable for the equipment, following a gradual digestion program (Table 7.1). Afterwards, still in the quartz tube, about 20 mL of water was added (exothermic reaction) and then the sample was transferred to 50 mL volumetric flasks, and the volume was completed with water.

Table 7.1. Digestion program for mineral analysis

Step	Temperature (ºC)	Slope time (mn:s)	Step time (mn:s)	Pressure (PSI)	Magnetic stirring
1	Room temperature	-	00:15	<50	Mean
2	90	02:00	02:00	250	Mean
3	190	06:00	05:00	300	Mean
4	230	10:00	05:00	300	Mean

The measurements were done through flame atomic absorption spectroscopy with air-acetylene mixtures according to the manufacturer's programs using a novAA 350 system (Analytik Jena, Jena, Germany). The concentrations were expressed as mg/ kg propolis.

2.5. Hydro-alcoholic propolis extraction

The hydro-alcoholic extraction of the three samples of propolis was performed as previously described (26), with minor modifications. One gram of each *Euphorbia* propolis was extracted by maceration using 30 mL of 70% of ethanol for one week at 37 ºC under agitation (200 rpm). The final extract was centrifuged for 20 min at 5000 rpm. The supernatant was recovered and used in the various analyses carried out this study.

2.6. Quantification of total phenol, flavones, flavonol, flavanones and dihydrofavonols

2.6.1. Total Phenol Content

The total phenol content the samples was determined using the method previously described (26) in which 25 µL of propolis extract were mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) and 100 µL of 7.5% Na_2CO3. The solution was left for 1 h at room temperature and the absorbance was measured at 760 nm. The total polyphenol content was expressed as mg per g of gallic acid equivalents (GAE) using a calibration curve. The concentrations of gallic acid used ranged from 0.001 to 1 mg/mL. Tests were carried out in triplicate.

2.6.2. Total Flavones and Flavonol Content

The content of flavones and flavonol in the three hydro-alcoholic extracts was evaluated as previously reported (27). The extract (100 µL) mixed with 100 µL of $AlCl_3$ 20% was left incubated for 1 h at room temperature; after this period, the absorbance was read at 420 nm and total favones and favonols amounts were calculated as quercetin equivalents (QE) (mg per g) using a calibration curve. The concentrations of quercetin used ranged from 0.002 to 1 mg/mL. Tests were carried out in triplicate.

2.6.3. Total Flavanones and Dihydrofavonol Contents

The amounts of flavanones and dihydroflavonol in *Euphorbia* propolis extracts were determined using the method described by (26) in which 50 µL of the hydro-alcoholic extracts were mixed and heated at 50 ºC for 50 min with 2 mL of 2,4-dinitrophenylhydrazine (DNP) solution (1 g DNP in 2 mL of sulfuric acid diluted to 100 mL in methanol). Then the mixture was diluted to 10 mL with 10% KOH in methanol (w/v) after cooling at room temperature. A sample of 1 mL of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol. By the end, the absorbance was measured at 486 nm. Total flavanones and dihydroflavonol content are presented as naringenin equivalents (NE) (mg per g) using a calibration curve (0.002 – 1 mg/mL).

2.7. Antioxidant Activity

2.7.1. (1,1-Diphenyl-2-picrylhydrazyl) (DPPH) free radical scavenging activity

Determination of the DPPH radical scavenging activity was carried out as reported (25). Briefly, 25 µL of the hydro-alcoholic propolis extracts with different concentrations was added to 150 µL of DPPH solution (63.4 µ M) with 125 µL of ethanol 96%. After 30 min of incubation in room temperature, the absorbance was measured at 517 nm. The radical inhibition percentage was calculated with the equation:

Inhibition = $((A_0 - A_1) / A_0 \times 100)$; A_0: absorbance of the control and A_1: absorbance of the sample. The sample concentration providing 50% inhibition (IC_{50}) was achieved by plotting the inhibition % against the sample concentration. Tests were carried out in triplicate.

2.7.2. Nitric oxide free radical-scavenging activity

The NO scavenging activity was measured with a few modifications (25): 150 µL of propolis extract was mixed with 150 µL of (10 mM) sodium nitroprusside (SNP), after an incubation of 1 h and 30 min, 20 µL of the mixture N-(1-naphthyl) ethylenediamine dihydrochloride NED (0.1%) and sulfanilic acid PBS (1%) were added to 150 µL of samples mixture. After that, 130 µL of milliQ water was added and the reading was done at 540 nm. The concentration of the sample that allows 50% inhibition (IC*50*) was obtained by drawing the percentage of inhibition curve, as described above. Tests were carried out in triplicate.

2.7.3. Scavenging ability of superoxide anion radical

Scavenging ability of superoxide anion radical was assayed as reported by with a little modification (25): 25 µL of nitroblue tetrazolium NBT (514 µM) and 25 µL NADH solution (1989 µM) were added to the 25 µL of *Euphorbia* propolis extract and mixed well, subsequently 125 µL of distilled water was added. The phenazine methosulfate (PMS) solution (2.7 µM) is added before reading the absorbance at 560 nm. The inhibition determination was performed as aforementioned for the other assays. Tests were carried out in triplicate.

2.7.4. Inhibition of lipid peroxidation by thiobarbituric acid (TBARS)

The lipid peroxidation inhibition assay was evaluated as previously reported (28): 500 µL of egg yolk prepared in KCl solution (0.15 M) added to 200 µL of distilled water and 750 µL acetic acid, the solution was mixed with 750 µL of TBA + sodium dodecyl sulfate (SDS) solution (1.19 g of SDS in 100 mL distilled water). After that, the mixture was heated for 60 min in water bath at 95 ºC, cooled and completed to 5 mL with butanol, the supernatant was recovered after centrifugation (10 min, 3000 rpm). The reading was done at 532 nm. The sample concentration, providing 50% inhibition (IC_{50}), was achieved by plotting the inhibition percentage against sample concentrations. Tests were carried out in triplicate.

2.8. Inhibition of enzymatic activities

2.8.1. Inhibition of acetylcholinesterase

The acetylcholinesterase inhibition was carried out with few modifications (25). About 50 µL of propolis extract, 60 µL of enzyme (0.28 U/ mL) and 15 µL of buffer (0.1 M Tris-HCl, pH = 8) were mixed and incubated for 15 min at room temperature. After that, 50 µL of substrate (0.005 g of acetylcholine iodide in 10 mL of buffer) and 125 µL of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) (0.059 g in 50 mL of buffer) were added, the mixture was incubated for 1 h at room temperature and the absorbance was recorded at 405 nm. The following formula was used to determine the percentage of inhibition action: Inhibition = $((A_0 - A_1) / A_0) \times 100)$; where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. After the percentage of

inhibition of acetylcholinesterase activity was determined and the (IC_{50}) value was calculated. Tests were carried out in triplicate.

2.8.2. Inhibition of lipoxygenase

The lipoxygenase assay is used as an indicator of anti-inflammatory and antioxidant activity. The inhibition action of *Euphorbia* propolis extract was determined as reported with some modification (25). In short, 100 µL of hydro-alcoholic extract, 50 µL of linoleic acid (0.001 M) and 827 µL of borate buffer (0.1 M of boric acid, 0.005% tween, pH = 9) were well mixed and in the final 15 µL of lipoxygenase enzyme (0.054 g of enzyme in 1 mL borate buffer) was added.

The reaction mixture was followed spectrophotometrically at 234 nm. The following formula was used to determine the percentage of inhibition action: Inhibition = $((A_0 - A_1) / A_0) \times 100)$; where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Tests were carried out in triplicate.

2.8.3. Inhibition of tyrosinase

The tyrosinase activity was determined based on the protocol (27) with slight modification. The total assay mixture consisted of adding 50 µL of propolis extract, mixed with 170 µL of phosphate buffer (50 Mm; pH = 6.5) and 20 µL of tyrosinase enzyme (100 U/mL), the mixture was incubated for 40 min, after this period 60 µL of substrate (5 mM of L-Dopa) was added at the end. The reading was done at 492 nm, the inhibition percentage of the enzyme was calculated, and the IC_{50} values were compared. Tests were carried out in triplicate.

2.8.4. Inhibition of xanthine oxidase

The inhibitory activity of 3 $_{Euphorbia}$ propolis extracts was determined by minor modification of the method previously described by (27). For that, 50 µL of each propolis extract was mixed with 230 µL of phosphate buffer (50 mM, pH = 7.5) and 70 µL of xanthine oxidase enzyme (0.4 U/mL), after incubation at room temperature for 15 min, 330 µL xanthine solution (substrate) (0.150 mM) were added and the mixture was incubated or 30 min. In the final, 100 µL of HCl (1 M) was added to stop the reaction and the reading was done at 290 nm. The percentage of inhibition of xanthine activity was calculated and the IC_{50} was determined. Tests were carried out in triplicate.

2.8.5. Inhibition of α-glucosidase

α-Glucosidase inhibition capacity of $_{Euphorbia}$ propolis was determined as previously described (27). The total assay mixture consisted of adding 50 µL of propolis extract added to 50 µL of yeast α-glucosidase (2.4 U/mL) prepared in phosphate buffer (100 mM; pH = 6.8), the mixture was incubated for 10 min. After that, 100 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in phosphate buffer was added. The reaction solution was incubated at room temperature for 30 min, and by the end, 80 µL of sodium carbonate solution (0.4 mM) was added to stop the reaction. The reading was done at 405 nm, the assay was done in triplicate. The inhibition

percentage of the enzyme was calculated as follows: I% = ((A_0 −A_1) / A_0) × 100) and the IC_{50} was determined. Tests were carried out in triplicate.

2.8.6. Inhibition of lipase

The porcine pancreatic lipase inhibitory assay was adapted from (29): 50 µL of hydro-alcoholic propolis extract was mixed with 190 µL of Tris-HCl buffer (0.1 M; pH = 8) and 40 µL of lipase enzyme (200 U/mL) prepared directly before the assay. A first incubation was performed in 37 ºC at 20 min, after that 20 µL of 5 mM NPB (*p*-nitrophenyl butyrate) substrate was added to the mixture and incubated a second time for one hour and 20 min in 37ºC, and the reading was done at 400 nm. The following formula was used to determine the percentage of inhibition action: Inhibition = ((A_0 − A_1) / A_0) × 100); where A_0 is the absorbance of the control and A_1 is the absorbance of the sample and the IC_{50} was determined. Tests were carried out in triplicate.

2.9. Antimicrobial Activity

2.9.1. Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of the hydro-alcoholic extract of *E. resinifera* and *E. officinarum* was determined by the microdilution method previously described (26) with slight modifications. Three Gram positive bacteria, namely *S. aureus* ATCC 6538; methicillin resistant *S. aureus* 12 (MRSA12) and methicillin resistant S*. aureus* 15 (MRSA15) and the Gram-negative *Escherichia coli* DSM 1077 were tested. All bacterial strains were maintained at -80ºC and when required were grown in fresh Brain Heart Infusion agar plates (BHI agar). Inoculated plates were incubated at 37°C during 24 h. The tested concentrations of propolis extracts were 50, 100, 120, 150, 200, 250 and 300 µL/mL which were prepared in BHI broth. The bacterial strains were previously grown in 15 mL of BHI and incubated overnight in a water shaking bath at 37ºC. Afterwards, 100 µL of the BHI culture medium with the appropriate concentrations of propolis was distributed in the wells of the microplate containing 100 µL of the bacterial culture prepared the previous night. The inoculated microplates were incubated at 37 ºC. The optical density readings were done at 600 nm ($O.D_{600}$) using a microplate reader (Tecan Infinite, M200, Männedorf, Switzerland). The antibiotic chloramphenicol (30 µg/mL) was used as control. The concentration of propolis that inhibited 95-100% of growth was considered the MIC value, and the lowest concentration of propolis that did not allow the recovery of the bacterial cells in culture plates was considered the Minimum Bactericidal Concentration (MBC).

2.9.2. Evaluation of anti-adherence activity

The anti-adherence activity of *Euphorbia* propolis extracts was evaluated as described by (30) with slight modifications, namely each bacterial suspension, *S. aureus* ATCC 6538, MRSA12, *E. coli* DSM 1077 and the multiresistant strain *E. coli* I73194 were exposed to propolis extracts (100, 200 and 250 µL/ mL). For this, 200 µL of the culture was distributed across a flat-bottom 96-well

microplate and maintained at room temperature inside a flow cabinet during 30 min. Afterwards the bacterial suspension was collected, and the wells were washed with phosphate-buffered saline (PBS). Then, the microplate was dried at 80ºC for 30 min to heat-fixation of the bacterial cells. After cooling the adherent cells were stained for 1 min with 220 µL of crystal violet (0.1%). The stain was removed, and the wells were washed twice with PBS followed by dissolution of the stain with 220 µL of ethanol–acetone (80:20), and after 15 min the $O.D_{595}$ was determined using a microplate reader (Tecan Infinite, M200, Männedorf, Switzerland).

2.9.3. Determination of anti-biofilm activity

The evaluation of the anti-biofilm activity of the propolis samples was done according to the method reported by (31) with slight modifications. The bacterial suspensions of MRSA12 and the multiresistant *E. coli* I73194 were previously grown in BHI. Each bacterial culture was diluted 1:10 with fresh BHI and further grown at 37ºC until an OD_{600} = 0.2 (2.0 × 107 CFU/mL). Six sterile non-breakable coverslips (22 x 22 mm x 0.25 mm) were distributed across a 6 well plate, and each coverslip was covered with 3 mL of a diluted bacterial suspension (1:10). The 6 well plate was incubated for 24 h at 37ºC to allow the production of a mature biofilm. Afterwards the bacterial suspension was removed, and each well was washed thrice with PBS. Then the formed biofilm was exposed to 2.5 mL of propolis samples. The solvent (ethanol at 70%) was used as control. The viability of sessile cells was determined after 6 and 24 h of exposure. The experiment was done using three biological and two technical replicates. The count of sessile cells was determined according to the method described by (1). Briefly, the coverslips were transferred into 10 mL of BHI, and the tubes were then sonicated for 7 min. After sonication each coverslip was rapidly removed, and serial decimal dilutions were prepared and inoculated in BHI agar. The inoculated plates were incubated at 37ºC for 24-48 h and the colonies counted.

2.9.4. Visualization of the biofilm cells by Fluorescence Staining

The LIVE/DEAD Baclight (Invitrogen Molecular Probes, Eugene, OR, USA) was used to visualize the biofilm cells. For this, each coverslip was inverted mounted in a microscope slide with 25 µL LIVE/DEAD fluorescent dye and stained for 15 min before observation. The observation was performed using the microscope Axio Imager Z2 (Zeiss, Oberkochen, Germany).

2.10. Statistical analysis

The results are reported as mean ± standard deviation (SD) of three independent replicates. Statistical analysis of data was carried out by computer using SPSS (Version 23.0, Inc., and Chicago, IL, USA). One-way ANOVA and Tukey post hoc multiple comparison tests were used to analyse data using Graph Pad Prism 9 statistical software. P-values less than 0.05 were considered significant.

3. Results and discussion

3.1. Pollen grains

Propolis sample P1, collected in Beni Mellal-Khénifra, showed predominantly pollen grains of *E. resinifera* (59%) (Table 7.2), followed by *Genista hirsuta* (9%). P2 and P3 propolis simples were dominated by *E. officinarum* pollen grains (47% and 45%, respectively), followed by *Hypericum elodes* (14%) and *Smilax aspera* (21%), respectively.

Table 7.2. Propolis samples, place, year of production and the most predominant pollen of three *Euphorbia* propolis samples from Morocco

Sample	Propolis type	Pollen species	(%)	Region / year	Coordinates
P1	*Euphorbia resinifera*	*Euphorbia resinifera*	58.6 ±1.1	Beni Mellal-Khénifra	32° 22′ 06″ N,
		Genista hirsuta	9.0±0.4	(2019)	6° 22′ 09″ W
		Asparagus albus	8.0±1.4		
		Populus nigra	7.1±1.0		
		Kleinia anteuphorbium	5.9±0.4		
		Caesalpinia spinosa	4.0±0.7		
		Pinus pinaster	3.9±0.3		
		Eucalyptus cinerea	1.6±0.4		
		Convolvulus arvensis	1.6±0.4		
P2	*Euphorbia officinarum*	*Euphorbia officinarum*	46.6±0.5	Souss-Massa- Tiznit	29° 43′ N,
		Hypericum elodes	13.9±1.5	(2019)	8° 58′ W
		Quercus rotindifolia	10.3±1.0		
		Populus nigra	7.1±1.0		
		Eucalyptus cinerea	6.7±0.5		
		Pinus pinaster	6.4±0.2		
		Kleinia anteuphorbium	3.4±0.8		
		Ilex aquifolium	3.3±0.3		
		Asparagus albus	1.9±0.6		
P3	*Euphorbia officinarum*	*Euphorbia officinarum*	44.8±0.5	Guelmim-Oued noun	28° 27′ N,
		Smilax aspera	21.0±1.4	(2019)	10° 07′ W
		Cistus crepis	14.6±1.0		
		Caesalpinia spinosa	9.9±0.8		
		Campanula rotundifolia	5.7±0.3		
		Hypericum elodes	3.9±0.6		
		Populus nigra	3.8±0.8		
		Convolvulus arvensis	3.0±0.17		
		Ilex aquifolium	2.9±0.2		
		Quercus rotindifolia	1.8±0.3		

As far as we know, this is the first time that palynological data has been provided for *Euphorbia* propolis. These differences can be attributed to the different regions where the samples were collected (Souss-Massa- Tiznit and Guelmim-Oued noun). Pollen grains from anemophilous or entomophilous flowers can adhere to the resins, when collected by the honeybees, or may come from harvested pollen inside the hives (32). This fact may provide indication about the vegetation around the beehives as well as the geographical origin of propolis (33,34). In this context, and some decades ago, (35) examined 56 propolis samples from several countries to establish the geographical origin of propolis based on palynological studies. For example, the author reported that a very high percentage of pollen grains of *Eucalyptus* and a considerable percentage of *Daphne* pollen grains characterized the Moroccan propolis. To the best of our knowledge, this is the first time that palynological data has been provided for *Euphorbia* propolis from Morocco, and according to the results obtained it should be considered that other types of pollen grains can be found in Moroccan propolis. Overall, many more palynological studies are needed to better respond to the acceptable medicinal quality of propolis, more and more, required by the consumers. Such as for honey, monofloral honey is more appreciated, not only for its characteristic flavour and aroma but also for its intrinsic biological properties, the same can be considered for propolis.

3.2. Propolis volatiles profile

As stated in the materials and method section, only *E. resinifera* P1 and *E. officinarum* P2 propolis samples were available for volatile determination, and their profile is depicted in Table 7.3, in accordance with their elution order on the DB-1 column. In total, ninety-nine components were identified, accounting for 85% and 88%, respectively, of the total volatiles. Monoterpenes constituted the major fraction, with hydrocarbons (35% in *E. resinifera* P1 and 31% in *E. officinarum* P2) and oxygen-containing compounds in high percentages (15% in both cases).

Table 7.3. Percentage composition of the essential oils isolated by hydrodistillation from P1 and P2

Components	RI	P1	P2
3-Methyl-2-butenol	726	t	0.3
Hexanal	739	t	
n-Octane	800	t	
Hexanol	883	t	
Heptanal	897	t	
n-Nonane	900	0.3	0.6
Tricyclene	921	0.4	0.2
α-Thujene	924	0.7	0.3
α-Pinene	930	15.9	14.7
Camphene	938	1.4	1.0
Thuja-2,4(10)-diene*	940	1.5	1.0
Sabinene	958	3.0	2.1
β-Pinene	963	6.8	6.5
n-Octanal	973	1.0	1.2
1,2,4-Trimethyl benzene	975		t
β-Myrcene	975	0.5	t
Hexyl acetate	995	0.5	0.8
α-Terpinene	1002	0.7	0.3
p-Cymene	1003	1.3	1.3
1,8-Cineole	1005	0.2	1.2
β-Phellandrene	1005	t	t
Limonene	1009	0.7	1.3
2-Methyl butyric acid butyl ester	1017		t
trans-β-Ocimene	1027	t	t
γ-Terpinene	1035	1.6	1.2
2,5-Dimethyl styrene	1059	0.4	0.4
Terpinolene	1064	0.4	0.4
n-Nonanal	1073	2.4	3.8
α-Campholenal	1092	1.9	2.0
n-Undecane	1100	t	t
trans-Pinocarveol	1106	1.9	1.4
cis-Verbenol	1114	t	t
trans-Verbenol	1114	0.5	1.1
Pinocarvone	1121	1.2	0.9
Terpinen-4-ol	1148	0.9	0.4
Myrtenal	1153	1.3	1.0
Verbenone	1164	0.4	0.3
Myrtenol	1168	1.9	2.3
Hexyl butanoate (= Hexyl butyrate)	1173	t	t
n-Decanal	1180	4.3	7.8
trans-Carveol	1189	0.2	0.1
n-Dodecane (C12)	1200		t
Cuminaldehyde	1200		t
Carvone	1210		t
Hexyl 2-methyl butyrate	1220	0.6	1.1
2-*trans*-Decenal	1236	0.7	0.4

Components	RI	P1	P2
Nonanoic acid	1263	t	
Bornyl acetate (= Borneol acetate)	1265	3.3	3.0
n-Undecanal	1288	0.2	0.2
trans-Theaspirane	1300		
n-Tridecane	1300	t	0.2
α-Terpenyl acetate	1334	1.3	1.2
trans-2-Undecenal	1334	0.3	0.2
α-Cubebene	1345	0.3	0.2
α-Copaene	1375	t	0.2
Hexyl hexanoate	1375	0.3	t
β-Bourbonene	1379	2.3	2.0
n-Dodecanal	1397	0.7	1.0
n-Tetradecane	1400	t	0.1
β-Caryophyllene	1414	0.8	0.6
β-Copaene	1426	0.2	0.1
Aromadendrene	1428		0.2
α-Humulene	1447	0.4	0.2
allo-Aromadendrene	1456	0.7	1.5
Germacrene D	1474	0.4	0.2
Valencene	1484		0.3
α-Muurolene	1494	0.2	
trans,trans-α-Farnesene	1500	0.9	1.1
trans-Calamenene	1505	t	t
δ-Cadinene	1505	0.6	0.4
α-Calacorene	1525	0.2	0.2
Elemol	1530	t	0.1
Spathulenol	1551	6.4	3.0
β-Caryophyllene oxide	1561	0.3	0.5
Cedrol	1574	1.5	1.0
γ-Eudesmol	1609		0.2
T-Cadinol	1616	0.3	
δ-Cadinol	1621	0.4	
β-Eudesmol	1622	t	0.4
α-Eudesmol	1634	0.3	0.7
Cadalene	1640		0.5
α-Bisabolol	1656	2.0	0.7
n-Heptadecane	1700	0.3	0.6
n-Octadecane	1800		t
n-Nonadecane	1900	0.7	1.7
Hexadecanoic acid (= Palmitic acid)	1908		0.1
n-Eicosane	2000	t	t
Abietatriene	2045	0.1	0.2
n-Heneicosane	2100	0.7	1.7
n-Docosane	2200		0.2
n-Tricosane	2300	1.0	2.0
n-Tetracosane	2400	t	t
n-Pentacosane	2500	0.7	1.4
n-Hexacosane	2600	t	0.1

Components	RI	P1	P2
n-Octacosane	2800	t	0.1
n-Heptacosane	2700	0.8	t
n-Nonacosane	2900	0.4	0.8
n-Triacontane	3000	t	0.1
n-Hentriacontane	3100	0.4	1.0
% Identification		84.9	87.6
Grouped components			
Monoterpene hydrocarbons		35.3	30.7
Oxygen-containing monoterpenes		15.0	14.9
Sesquiterpene hydrocarbons		7.0	7.7
Oxygen-containing sesquiterpenes		11.2	6.6
Diterpene hydrocarbons		0.1	0.2
Oxygen-containing diterpenes		t	t
Phenylpropanoids		t	t
Fatty acids		t	0.1
Alkanes		5.0	10.0
Others		11.3	17.4

RI: In lab calculated Retention Index relative to C7-C31 *n*-alkanes on the DB-1column. * Identification based on mass spectra only. **t: trace (< 0.1%).**

The main difference between P1 and P2 was the percentages of sesquiterpenes, alkanes and other compounds (Table 7.3). P2 had higher percentage of alkanes (10%) and others (non-terpene aldehydes, alcohols, and esters) (17%) and lower percentages of oxygen-containing sesquiterpenes (7%) than P1 (5%, and 11% in both cases, respectively) (Table 7.3). The percentages of spathulenol, α-bisabolol, and cedrol were relatively higher in P1 than in P2, which may partially explain the highest percentage of the oxygen-containing sesquiterpenes in P1 sample (Table 7.3). Higher percentages of the alkane's heneicosane, tricosane, pentacosane, heptacosane, nonacosane and hentriacontane in P2 contributed to the highest percentage of alkanes than in P1. Nonanal (4%), decanal (8%) and hexyl 2-methyl butyrate (1%) were present in higher percentages in P2 samples than in P1 ones (2%, 4%, and 1%, respectively (Table 3). Almost of these compounds, with exception of hexyl 2-methyl butyrate, were also reported as constituents of several Moroccan propolis volatiles. Their percentages also varied which allowed to distinguish two clusters (36). Seemingly, this is the first time that the chemical composition of the volatiles isolated from propolis where *E. resinifera* and *E. officinarum* pollen grains dominate, from Morocco, was studied. Getting in mind that the volatile chemical composition of propolis is strongly dependent on the local flora at the harvesting location (37), differences are expected to occur in the volatile profile of propolis samples.

3.3. Mineral elements compounds

P1 sample (*E, resinifera*) revealed higher contents of analysed minerals except zinc compared with samples P2 and P3 (both *E. officinarum*), yet calcium (Ca) was the most abundant element in the three propolis samples (Table 7.4). Therefore, the samples presented a similar profile to other previously reported Moroccan propolis in which Ca or Na generally dominated (38-40), although (39) had reported as main elements present in the seven samples Ca, Na, K, and Mg. The mineral content in these samples can be informative of the geographical region where propolis was gathered but can also be an indicator of environmental pollution (41). The levels of Fe, Zn, and Ni were generally higher than those reported by (38) for Moroccan propolis, or Poland propolis (42). We do not consider that pollution can be present in beehive surroundings; nevertheless, it is something that must be enlightened, mainly if propolis is taken directly as a powder because ethanolic extracts generally present lower amounts of mineral elements. Metals are not well extracted by ethanolic solutions (43). So, this matter must be clarified in the future. The utilization of this bee product as a food supplement must guarantee safety and quality (44).

Table 7.4. Element content (mg/g) in Moroccan *Euphorbia* propolis

	Element content (mg/g)		
Sample	**P1**	**P2**	**P3**
Ca	16.61±0.20^{a}	1.35±0.16^{a}	1.13±0.2^{a}
Co	< LOD^{1}	< LOD^{1}	< LOD^{1}
Cr	< LOD^{2}	< LOD^{2}	< LOD^{2}
Cu	< LOD^{3}	< LOD^{3}	< LOD^{3}
Fe	1.20±0.02^{d}	0.75±0.12^{b}	0.19±0.009^{c}
K	1.46±0.03^{c}	0.65±0.005^{c}	0.43±0.01^{b}
Mg	2.39±0.02^{b}	0.53±0.05^{d}	0.19±0.02^{c}
Mn	0.04±0.01^{f}	0.017±0.01^{g}	0.0047±0.0005^{g}
Na	0.49±0.01^{e}	0.09±0.001^{e}	0.08±0.01^{e}
Ni	0.0037±0.0006^{h}	0.0026±0.0001^{h}	0.0042±0.0004^{g}
Zn	0.033±0.001^{g}	0.04±0.03^{f}	0.005±0.001^{f}

LOD1: 0.0089 mg/g. LOD2: 0.0081 mg/g. LOD3: 0.0126 mg/g. The values in the same column followed by the same letter are not significantly different ($p < 0.05$) by Tukey's multiple range test.

3.4. Total phenol, flavones, flavonol, flavanones and dihydroflavonol content

The hydro-alcoholic extracts of *E. resinifera* P1 and *E. officinarum* P2 had the highest levels of total phenolic compounds (39.7 and 21.7 mg GAE/g, respectively) (Table 7.5). The same happened for the total flavonoids (flavonols/flavones and dihydroflavonols). Nevertheless, P2 always showed the highest amounts of all groups of phenols, followed by P1 and P3. Despite P2 and P3 had predominantly *E, officinarum* pollen grains, they were collected in different regions,

and the dissimilarity was higher than for P1 and P2, in which the former has higher *E. resinifera* pollen grains than the remaining P2 and P3. Therefore, the amounts of total phenols or flavonoids did not provide information about the collection region or the type of pollen present in the samples. The values of total phenols are within the range of samples reported by other authors for propolis collected in Morocco and expressed as mg GAE/g: 0.74 – 92.22 mg/g GAE (38), 12.02 – 134.04 mg GAE/g (58), 19.91 mg GAE/g (46). For flavonols/flavones, the amounts were generally lower than those previously reported and which values were expressed as mg QE/g: 0.16 – 129.60 mg QE/g (26), 0.20 – 34.27 mg QE/g (45), and 3.28 mg QE/g (46), 1.19 – 108.11 mg QE/g (38). Regarding the dihydroflavonol amounts, they are within the range of those previously reported for Moroccan samples (0.92 – 15.95 mg eriodictyol/g) (26), although the reference used has been different in both cases (naringenin in the present work and eriodictyol by those authors.

Table 7.5. Phenol, flavonol/flavones and dihyrodroflavonols contents of three hydro-alcoholic propolis extracts from tree different region of Morocco

Sample	Phenol (mg GAE/ g propolis)	Flavonol/Flavones (mg QE/ g propolis)	Dihydroflavonol (mg Naringenin Eq/ g propolis)
P1	21.7±1.2 [b]	0.4±0.0 [b]	6.1±0.1 [b]
P2	39.7±1.0 [a]	0.7±0.1 [a]	7.6±0.2 [a]
P3	1.3±0.1 [c]	0.1±0.0 [c]	3.1±0.1 [c]

3.5. Antioxidant activity

Table 7.6 depicts the IC_{50} values determined in all assays for the three hydro-alcoholic extracts of propolis. In all cases, the sample P2 had the lowest IC_{50} values, that is, the best activity, not only for scavenging free radicals but also for preventing lipid peroxidation. This is expected since the P2 sample had the highest amount of all phenols groups and generally there is a correlation between phenols and antioxidant activity, although such activity is widely influenced by the method used as well as by the chemical structure of the polyphenol (47). The inverse correlation between phenols, flavonoids, and antioxidant activity is depicted in Table 7.7. The inverse correlation is also expected since the values of the antioxidant activity are provided as IC_{50}. Regarding the DPPH method, the IC_{50} values obtained in the present work are within the range found for other Moroccan propolis samples, although nearest to the highest values: 0.019 – 1.813 mg/mL (48); 0.08 – 2.90 mg/mL (48); 0.007 – 1.94 mg/mL (26); and 0.021 – 1.190 mg/mL (49), but higher (poorer activity) than those reported by other authors (0.012 – 0.054 mg/mL (39); 0.021 – 0.035 mg/mL (40); and 0.0331 mg/mL. For superoxide anion radical scavenging activity, the IC_{50} values were within the range of those already reported for Moroccan propolis extracts, but in this case, the values found were closer to the lowest IC50 values found by the authors: 0.15 – 2.31

mg/mL (48), 0.215 mg/mL – not detected (26). The capacity for scavenging NO free radicals was also within the range found for (48) and (26) (0.08 – 2.90 mg/mL and 0.025 – 15.072 mg/mL, respectively.

Table 7.6. IC_{50} values (mg/mL) for the three hydro-alcoholic Euphorbia propolis extracts

	IC_{50} values (mg/mL)			
Sample	**DPPH**	**NO**	**Superoxyde**	**TBARS**
P1	0.3±0.0 [b]	0.9±0.0 [b]	0.4±0.0 [b]	2.3±0.0 [b]
P2	0.2±0.0 [c]	0.4±0.0 [c]	0.3±0.0 [c]	1.7±0.0 [c]
P3	2.1±0.0 [a]	1.9±0.0 [a]	0.5±0.0[a]	3.9±0.1 [a]

The results are shown as the mean ± standard error (n = 3). The values in the same column followed by the same superscript letter are not significantly different ($p > 0.05$) by Tukey's multiple range test.

The antioxidant activity of the hydro-alcoholic extracts *Euphorbia* propolis was evaluated using four methods: three methods based on the ability of samples to scavenge free radicals (DPPH, $O2^{\bullet-}$ and $NO^{\bullet}$), and one method based on the capacity for preventing lipid peroxidation. The method of DPPH is widely used because DPPH is a very stable radical, does not require complex preparations; the analysis procedure is very rapid and is of low cost. Nevertheless, this radical has low biological relevance because it does not exist in biological systems and therefore is unable to reflect *in vivo* action (47), in contrast to the nitric oxide radical and superoxide anion radical.

Reactive oxygen species and other free radicals may attack carbon-carbon double bonds in lipids, taking out one hydrogen from one carbon and inserting an oxygen molecule. This radical reaction originates a mixture of lipid peroxyl radicals, and hydroperoxides as the primary products, and malondialdehyde (MDA) and 4-hydroxynonenal as the end product of lipid peroxidation (50). The propolis samples also prevented the formation of the MDA in which P2 was revealed to be better than the remaining samples. A similar trend was observed in the free radical scavenging activity (Table 7.5). Once again, the activity found was determined by the amounts of polyphenols. Overall, the propolis samples were able to act in different steps of the oxidation process: quenching the free radicals and in the case, and still an excess of these reactive substances remains and production of peroxyl radicals occurs, propolis samples will be able to prevent the passage of these radicals to MDA, an indicator of lipid peroxidation (50). This capability was already reported by (45) for Moroccan propolis which activities ranging from 0.051 mg/mL to very little activity which did not permit the determination of an IC50 value.

Table 7.7. Pearson correlation coefficients for compounds/antioxidant activities, and compounds/enzyme inhibitions.

	Phenol	Flavonoid	Dihydroflavonol	DPPH	NO	Superoxide	TBARS	ACTI	Lipoxygenase	Tirosynase	Xanthine oxidase	Glucosidase	Lipase
Phenol	1	0.977**	0.982**	-0.902**	-0.983**	-0.937**	-0.975**	-0.998**	-0.943**	-0.952**	-0.919**	0.022	0.053
Flavonoid	0.977**	1	0.960**	-0.851**	-0.950**	-0.893**	-0.941**	-0.984**	-0.899**	-0.946**	-0.871**	-0.063	-0.034
Dihydroflavonol	0.982**	0.960**	1	-0.961**	-0.998**	-0.978**	-0.996**	-0.985**	-0.985**	-0.910**	-0.971**	0.190	0.219
DPPH	-0.902**	-0.851**	-0.961**	1	0.964**	0.991**	0.970**	0.902**	0.994**	0.793*	0.999**	-0.450	-0.476
NO	-0.983**	-0.950**	-0.998**	0.964**	1	0.980**	0.999**	0.983**	0.987**	0.917**	0.975**	-0.199	-0.229
Superoxide	-0.937**	-0.893**	-0.978**	0.991**	0.980**	1	0.982**	0.107	0.995**	0.832**	0.994**	-0.355	-0.383
TBARS	-0.975**	-0.941**	-0.996**	0.970**	0.999**	0.982**	1	0.976**	0.990**	0.913**	0.979**	-0.229	-0.258
ACTI	-0.998**	-0.984**	-0.985**	0.902**	0.983**	0.935**	0.976**	1	0.944**	0.951**	0.920**	-0.022	-0.053
Lipoxygenase	-0.943**	-0.899**	-0.985**	0.994**	0.987**	0.995**	0.990**	0.944**	1	0.848**	0.998**	-0.351	-0.379
Tirosynase	-0.952**	-0.946**	-0.910**	0.793*	0.917**	0.832**	0.913**	0.951**	0.848**	1	0.816**	0.130	0.100
Xanthine oxidase	-0.919**	-0.871**	-0.971**	0.999**	0.975**	0.994**	0.979**	0.920**	0.998**	0.816**	1	-0.412	-0.439
Glucosidase	0.22	-0.063	0.190	-0.450	-0.199	-0,355	-0.229	-0.022	-0.351	0.130	-0.412	1	0.999**
Lipase	0.053	-0.034	0.219	-0.476	-0.229	-0,383	-0.258	-0.053	-0.379	0.100	-0.439	0.999**	1

Pearson correlation significance levels: ** significant at $p < 0.01$; *significant at $p < 0.05$

3.6. Inhibition of enzymatic activities

The capacity trend of the Moroccan propolis extracts for inhibiting the activity of acetylcholinesterase, lipoxygenase, tyrosinase and xanthine oxidase (Table 7.8) was like that verified for the antioxidant activity, which may indicate the importance of the phenols' content on these in vitro activities. This can be confirmed through Table 7.6, in which the inverse correlation was clear between the amounts of any group of polyphenols (total phenols, flavonol/flavones and dihydroflavonol) and the inhibitory activities expressed as IC_{50} values. In this set of activities, the IC_{50} values were lower, meaning higher activity, for lipoxygenase and xanthine oxidase activities, which may show higher inhibitory action on these enzymes than on acetylcholinesterase and tyrosinase ones (Table 7.8). These results also indicate there is higher inhibitory activity on those enzymes in which also originate ROS. After the action of lipoxygenase on the substrate there is the release of hydroperoxides fatty acids (9), and with the action of xanthine oxidase on the respective substrate, superoxide radical anions are delivered (11) that is, it seems that the propolis extracts are better inhibitors of those enzymes in which oxidative stress is also involved. The correlation between the phenols' amounts, regardless of the group, and the inhibitory activities of Moroccan propolis collected at diverse places on those enzymes were also reported by other authors (45,51). For similar concentrations of phenols described by (26) and those found in the present work, similar anti-lipoxygenase and anti-tyrosinase activities were found (51), nevertheless the anti-xanthine oxidase and anti-acetylcholinesterase activities were poorer in our work when compared to those previously reported (51). In the last two activities beyond the phenols' amounts other factors can explain these differences.

Table 7.8. Enzyme's inhibitory activities of *Euphorbia* three hydro-alcoholic propolis extract

	IC_{50} (mg/mL)					
Samples	**ACTI**	**Lipoxygenase**	**Tyrosinase**	**Xanthine oxidase**	**Glucosidase**	**Lipase**
P1	4.7±0.0^{b}	0.7±0.0^{b}	2.4±0.0^{b}	0.8± 0.007^{b}	0.5± 0.0^{a}	7.2±0.1^{a}
P2	2.8±0.1^{c}	0.6±0.0^{c}	1.0±0.0^{c}	0.7± 0.003^{c}	0.1± 0.0^{b}	1.0±0.0^{b}
P3	6.9±0. 1^{a}	1.0±0.0^{a}	3.0±0.0^{a}	2.0± 0.010^{a}	0.1± 0.0^{b}	0.8±0.1^{b}

The results are shown as the mean ± standard error (n = 3). The values in the same column followed by the same letter are not significantly different ($p < 0.05$) by Tukey's multiple range test.

No correlation between the amounts of phenols or flavonoids and the anti-glucosidase and anti-lipase activities was found (Table 7.7). The highest amounts of phenols did not mean better activity (Table 7.8). The inhibition of glucosidase activity by other Moroccan propolis was correlated with the amounts of phenols (51), and particularly flavonoids' amounts (48). In bee propolis from Australia, (13) found that the introduction of geranyl and prenyl groups to both the

flavonol and flavanones dramatically increased inhibitory activity, and according to the authors the hydrophobicity of the flavonoids might have an important role on the lipase inhibition.

3.7. Antimicrobial Activity

3.7.1. Antimicrobial Properties

The antibacterial activity of the hydro-alcoholic extract of two types of *Euphorbia* propolis, *E. resinifera* (P1) and *E. officinarum* (P2 and P3), was examined by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (Table 7.9).

Table 7.9. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of Euphorbia propolis extracts.

	Bacteria	**MIC (µL/mL)**	**MBC (µL/mL)**
Euphorbia resinifera (P1)	*S. aureus* ATCC 6538	50	150
	MRSA12	100	300
	MRSA15	120	250
	E. coli DSM 1077	150	250
Euphorbia officinarum (P2)	*S. aureus* ATCC 6538 (C48)	150	200
	MRSA12	100	200
	MRSA15	250	300
	E. coli DSM 1077	200	350
Euphorbia officinarum (P3)	S. *aureus* ATCC 6538 (C48)	250	300
	MRSA12	200	400
	MRSA15	300	450
	E. coli DSM 1077	350	400

Regarding the MIC values the *E. resinifera* propolis (P1) showed the lowest values followed by *E. officinarum* (P2) evidencing the highest susceptibility of the tested bacteria to P1 propolis, namely *S. aureus* ATCC 6530 showed a MIC value of 50 µL/mL in contrast to P2 and P3 that reached 150 and 250 µL/mL, respectively. *S. aureus* MRSA 12 showed a similar MIC value for P1 and P2 (100 µL/mL), contrary to S. aureus MRSA 15 that showed increased MIC values, namely the lowest was achieved using P1 propolis, 120 µL/mL following 250 µL/mL for P2 and 300 µL/mL for P3. A similar behaviour was observed in *E. coli* DSM 1077, a MIC value of 150 µL/mL for P1 propolis and 200 µL/mL for P2 and 350 µL/mL for P3. All tested bacterial strains were less susceptible to P3. Regarding the MBC value, which is the lowest concentration of an agent that kills the target bacterium it was observed that the strain S. aureus ATCC 6530 showed the lowest MBC value, 150 µL/mL for P1 propolis, whereas the highest was reached by S. aureus MRSA 15, 450 µL/mL with P3 propolis (Table 7.8). These results evidence the best antibacterial activity of P1 and P2 propolis. The MIC and MBC values of propolis can vary depending on the type and origin of propolis, as well as the strain of the tested bacteria. From several studies, it has

been observed that propolis is more effective against Gram positive bacteria than Gram negative bacteria (52; 53; 26), which differ in the cell wall composition, particularly Gram positive bacteria have a thick layer of peptidoglycan in their cell wall, in turn Gram negative bacteria have an outer membrane rich in lipopolysaccharides and thinner layer of peptidoglycan in their cell wall. Therefore, the powerful effect of propolis against Gram positive bacteria in comparison with Gram-negative could be explained by the protection given by the outer membrane structure of Gram-negative bacteria and the production of hydrolytic enzymes that block and break down the active ingredients of propolis (53). However, besides the accumulated knowledge about the composition and content of volatiles, phenols, and flavonoids of propolis the exact contribution of these compounds on the antibacterial capacity of propolis, is still very limited (54). In the study conducted by (55) it was reported that the antibacterial capacity may be due to the nature and grouping of those chemical compounds, in other words, the chemical compounds having electronegative carbonyl, amine, imine, sulphide, thiol, methoxyl and hydroxyl groups are highly polar and lipophilic, and having these features, in contact with the bacterial cells, can injury the cellular membrane structure allowing the escape of the cellular contents and therefore arresting the bacterial growth and ultimately causing the cell death. The characterization of our propolis samples evidenced that the propolis of *E. resinifera* (P1) and *E. officinarum* (P2) were the two samples enriched in phenol, flavonoid and dihydroflavonol (Table 7.5). Moreover, the grouping of monoterpenes is the main grouping of volatile compounds for the two propolis extracts (15% and 14, 9 % for P1 and P2, respectively) (Table 7.3). One example of a monoterpenes observed that was found in our *Euphorbia* propolis with the best antibacterial activity is α-pinene (15.9 % in P1 and 14.7 % in P2). α-Pinene has been shown to have antimicrobial activity against a variety of bacteria, including *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa* (56). Sesquiterpenes, the second most dominant group in *E. resinifera* and *E. officinarum* propolis (P1 and P2) achieving 11.2 % in P1 and 6.6 % in P2 (Table 7.3), have been responsible for the growth inhibition of both Gram positive and Gram-negative bacteria (57; 58).

3.7.2. Anti-quorum sensing activity

Quorum sensing (QS) is a process by which bacteria communicate with each other and coordinate their behaviour through the production and sensing of small signalling molecules called auto-inducers (59). Inhibition of quorum sensing (quorum quenching, QQ) has emerged as a promising strategy for combating bacterial infections, as it can disrupt bacterial biofilm formation and impair other bacterial virulence traits (58). In the current study, the anti-quorum sensing activity of *E. resinifera* and *E. officinarum* extract was determined using the *C. violaceum* CV026 biosensor, in which the production of the violacein is regulated by the QS system (26). The results on the anti-QS ability are illustrated in Figure 7.2.

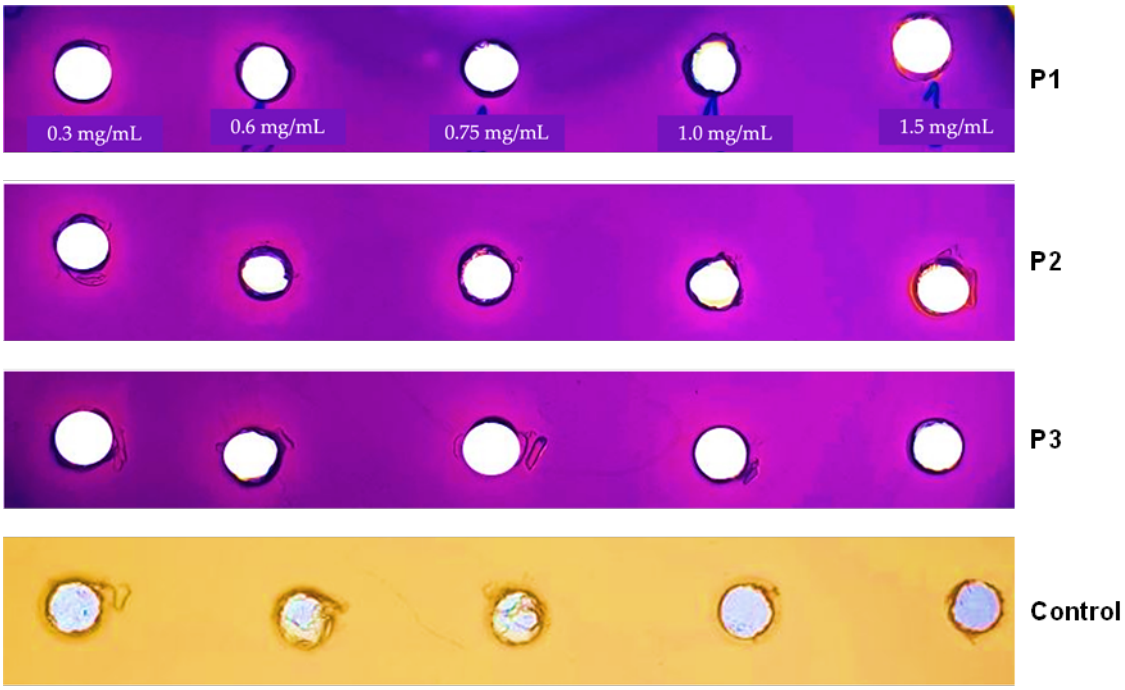

Figure 7.2. Anti-QS properties of propolis. Control: No addition of C6-HSL to the culture medium. P1: *Euphorbia resinifera* propolis. P2 and P3: *Euphorbia officinarum* propolis. N-hexanoylhomoserine lactone (C6-HSL) at 0.12 µg/mL was added to the culture medium. The assay was conducted using three independent triplicates.

The *Euphorbia* propolis (P1, P2 and P3) were tested using five different concentrations (0.3; 0.6; 0.75; 1 and 1.5 mg/mL). The highest inhibition of violacein production was observed at the lowest concentrations, namely 0.3 and 0.6 mg/ mL for *E. resinifera* (P1), following *E. officinarum* (P2) at concentration (0.75 mg/ mL). In contrast, the (P3) sample did not record a remarkable inhibition at all tested concentrations. The growth of the *C. violaceum* CV026 biosensor (Control) was not affect by the exposure to the different concentrations of the tested propolis samples (Figure 7.2). The inhibition of QS by Moroccan propolis was reported previously (26), where the tested Moroccan propolis showed the ability to inhibit the QS system of the *C. violaceum* CV026 at 1.22 (mg/ mL). The *Euphorbia* propolis showed higher anti-QS activity since the QS system was inhibited at much lower concentration (0.3 mg/mL). The different results between the two studies may be explained by the chemical composition and the geographical origin of the tested propolis samples.

3.7.3. Anti-adherence and anti-biofilm activity

Inhibition of the adherence to surfaces (biotic or abiotic) can prevent the establishment of bacterial communities that will form a mature biofilm (sessile cells) that is very difficult to eliminate. The results of the impact of *E. resinifera* and *E. officinarum* propolis on the bacterial adherence ability are illustrated in Figure 7.3. Our results, evidence that the *E. resinifera* (P1) and *E. officinarum*

samples at the concentration of 250 µL/mL were able to reduce significantly ($p < 0.0001$) the adherence of *S. aureus* ATCC 6538 in comparison with chlorohexidine (0.2 %, v/v). In contrast, P1 stimulated the adherence of both the *S. aureus* MRSA 12 and MRSA 15 in a concentration dependent manner in opposition to the P2 and P3 samples that were able to efficiently inhibit the adherence of the two MRSA strains. Interestingly, the P1 sample also induced the adherence of the *E. coli* DSM 1077 in a concentration dependent manner, but such effect was not observed on the multiresistant strain of *E. coli* I73194. The adherence of *E. coli* DSM 1077 and the multiresistant strain of *E. coli* I73194 was better inhibited by the propolis sample P3 (Figure 7.3). The propolis sample P3, as mentioned above showed the lower content of phenol, flavonol/flavones and dihydroflavonol in comparison with the propolis samples P1 and P3 (Table 7.3), and we can anticipate that this lower content is not beneficial for the control of the bacterial growth but can act to provide the attachment of the bacterial cells. Contrarily, P1 and P2 showed a better antibacterial activity and a respective higher content on those components, and maybe as a response to a stress condition the *S. aureus* ATCC 6538, MRSA and *E. coli* DSM 1077 cells in the presence of P1 propolis their adherence was induced (4).

In virtue of the chemical composition, the good antioxidant, and the content of phenols and flavonoids the propolis sample P1 was selected for testing its ability to disrupt the biofilm formed by the *S. aureus* MRSA 12 and the multiresistant strain *E. coli* I73194. The results evidenced the ability of this propolis sample P1 to disturb the biofilm produced by these two resistant bacteria (Figure 7.4 A; B, D, E). In contrast, the exposure of the bacterial biofilm to the 70 % ethanol (control) did not cause any injury to the sessile cells (Figure 7.4 C, F). Our findings are in accordance with other studies that reported the ability of propolis to damage bacterial biofilms, and this capacity is particularly beneficial in cases of chronic infections that are difficult to treat (60-62).

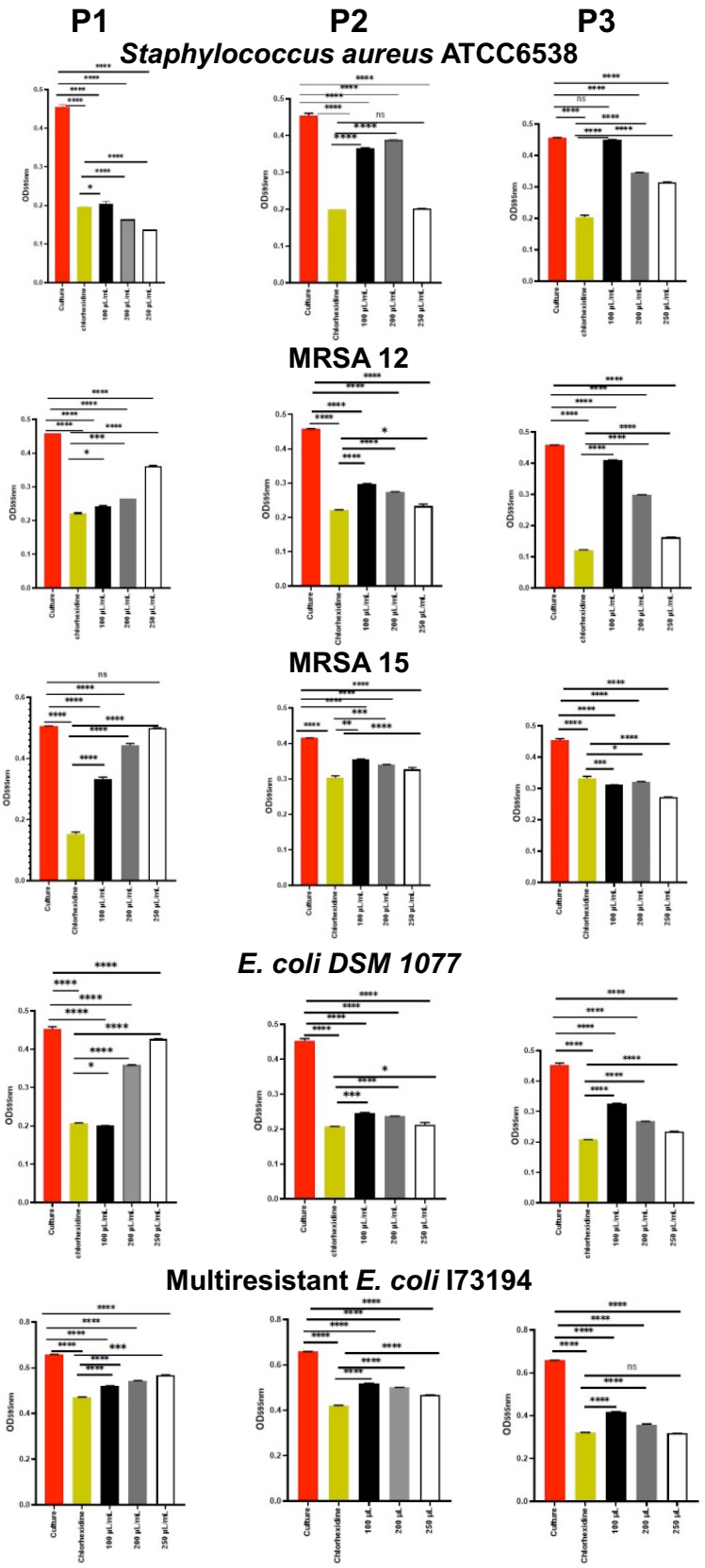

Figure 7.3. Inhibition of the bacterial adherence by the propolis samples P1, P2 and P3. Data represent the mean of three biological replicates. Error bars represent the standard deviation. *p < 0.05. ** p < 0.01. **** p < 0.0001. ns: not significant.

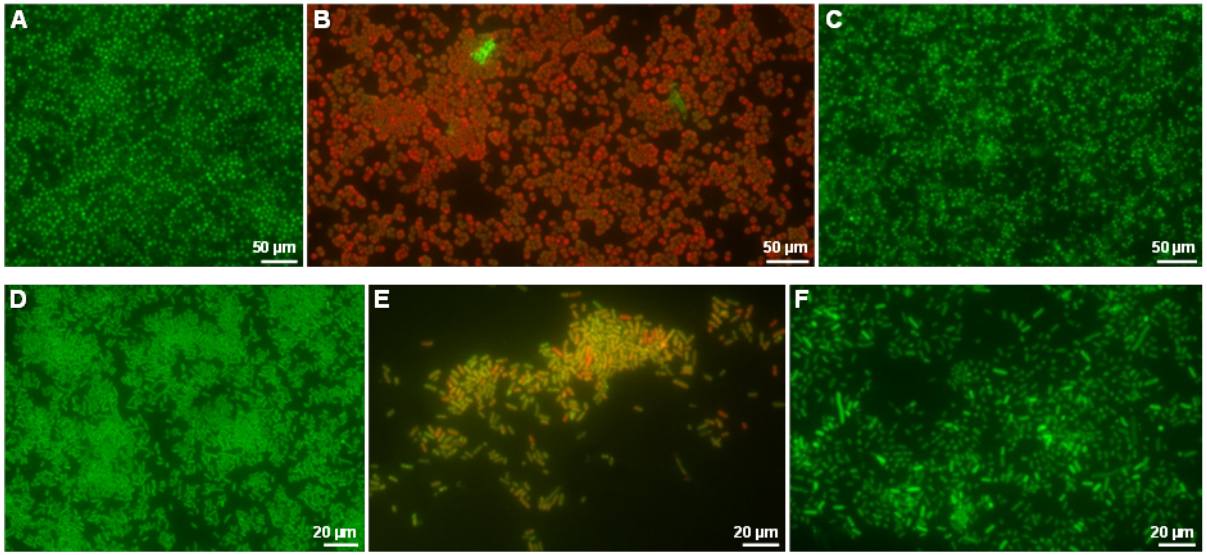

Figure 7.4. The impact of the *Euphorbia resinifera* (P1) propolis hydro-alcoholic extract on the disruption of biofilm formed by MRSA12, A) control no exposure to antibiofilm agent, B) after exposure to propolis P1, C) after exposure to 70% ethanol, and multiresistant *E. coli* I73194 D) control no exposure to antibiofilm agent, E) after exposure to propolis P1, F) after exposure to 70% ethanol. The bacterial cells formed the biofilm during 24 h and were visualized after staining with LIVE/DEAD Baclight.

4. Conclusion

In summary, propolis is a natural resinous substance made from various plant sources by bees. The studies described in this article highlight multiple beneficial properties of propolis, including antioxidant, anti-inflammatory, antibacterial, antiquorum-sensing, and anti-biofilm effects. These bioactivities are believed to be due to the presence of bioactive compounds such as polyphenols, flavonoids, phenolic acids, and some volatiles, including terpenoids, in propolis. This result suggests that propolis is promising as a valuable natural product for the development of therapeutic drugs and functional foods. However, further studies are needed to elucidate its mechanism of action, optimize its dosage form, and evaluate its safety and efficacy in different populations. Propolis could be used as a natural product or supplement to potentially support the human body's health due to its diverse biological activities.

Funding

This research was supported by funding from the Fundação para a Ciência e a Tecnologia (FCT/MCTES) Grant No. MED UIDB/05183/2020 to MGM and Grant No. CESAM UIDP/50017/2020, UIDB/50017/2020, and LA/P/0094/2020 to ACF, and Grant No. UIDB/04326/2020, UIDP/04326/2020 and LA/0101/2020 to MCC and JC.

Authors contributions:

MGM conceived the research with inputs from LME, LEG, MCC, JC, MLF and ACF. LME performed the palynological assays. OB and ACF extracted and analysed metabolites. MCC, JC, OB and SEG performed the mineral analyses. OB and SLG performed the antioxidant and inhibitory enzyme assays. OB, IM and MLF performed microbial assays, MGM, MLF, LME, MCC, LEG, ACF, provided analytical support and supervision. MGM wrote the manuscript with inputs from OB, MLF and ACF. All authors edited and approved the final version of the manuscript.

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General Conclusions and Future perspective

Euphorbia resinifera

Euphorbia officinarum

General Conclusions and future perspectives

The present work was intended to make a global characterization for the aerial parts (without latex and flowers) of *Euphorbia resinifera* and *Euphorbia officinarum*, two endemic species in Morocco as well as of the respective honey and propolis. The global characterization of the *E. resinifera* cyathia was also performed.

The scientific review made about these two Moroccan species showed that there is much more research on their latices than the other parts of the plants. More recently, research about bee products derived from *Euphorbia* species has aroused the interest of several researchers.

Regarding the aerial part of the plant, and more precisely in what concerns the aqueous extracts obtained by maceration, it turned out that the ratio of plant and volume of water, time, and temperature of the extraction are very important factors to have extracts with higher amounts of phenols and better biological properties (antioxidant capacity, measured through different methods, and enzyme inhibitory properties, mainly inhibition of α-glucosidase). The evaluation of the inhibition of this enzyme activity was done because, in some regions of Morocco, these species are used for the management of type 2 diabetes mellitus. When the decoction was used for the extraction of phenols (temperature around 100 ºC), the ratio of plant and volume of water, and time extraction also influence the amounts of phenols extracted and the biological activities. The best conditions of temperature, extraction time, and plant/volume of water ratio also depend on the *Euphorbia* species.

E. resinifera cyathia produces a plethora of different classes of specialized metabolites, including carotenoids, flavonoid glycosides, and polyamines which confer antioxidant properties to water decoctions as we measured with *in vitro* antioxidant assays. Moreover, high levels of benzaldehyde and nonanal, volatile compounds emitted by flowers of species pollinated by bees, were also present. The primary metabolites hexoses, amino acids, and vitamins were also measured and they were present in relatively high amounts. The capacity for scavenging free radicals of the floral cyathia floral extracts corresponded to two-thirds and half the quenching capacity of 2,6-di *terc*-butil-*p*-cresol (BHT) and ascorbic acid pure compounds used as positive control, respectively.

The *E. officinarum* and *E. resinifera* honey generally fulfill the quality requirements in what concern the physicochemical properties (moisture, acidity, pH, levels of HMF, proline, diastase activity, ash, and electrical conductivity), although in one case, high levels of Al, Cu, and Fe could be detected, which may be related to some environmental contamination by these elements in the area surrounding beehives or even the inadequate use of metallic containers for honey storage.

The reducing sugars were significantly higher in *E. resinifera* samples than in the monofloral *E. officinarum* honey. The potassium (K) was the most important mineral element with an average content of 409 mg/kg. The phenolic profile of the *Euphorbia* honey extracts was quite different:

gallic acid, 4 hydroxybenzoic acid, and p-coumaric acid were detected in all samples, although in different ratios, whereas naringenin was identified in all but one sample, and kaempferol was only present in two samples. Abscisic acid was detected in five of the seven studied honey samples, being the major compound detected in one *E. resinifera* honey.

The antioxidant properties of *E. officinarum* and *E. resinifera* as well as their ability to inhibit acetylcholinesterase, lipoxygenase, tyrosinase, and xanthine oxidase enzymes were evaluated and compared. *E. resinifera* honeys had a better ability to inhibit the lipoxygenase activity than *E. officinarum* honey, while in the remaining biological activities it was not possible to observe differences between the two monofloral honey types. Generally, the methanolic extracts of the honey recorded better activities in all the biological activities, than the corresponding entire honey, nevertheless in some cases an inverse correlation between the total phenol of the honey extract and the activities was found, meaning these results beyond the total phenolic content, the type of compound is also important. Moreover, the *Euphorbia* aqueous extracts have generally better biological properties (capacity for inhibiting lipoxygenase, acetylcholinesterase, tyrosinase, and xanthine oxidase activities; and capacity for scavenging the DPPH, superoxide, and nitric oxide free radicals) than the honey from the same floral origin. In what concerns the anti-xanthine oxidase, there was not any correlation between phenol content and anti-xanthine oxidase activity, therefore non-phenolic compounds can be responsible for the xanthine oxidase inhibitory activities.

The *E. officinarum* and *E. propolis* propolis have never been studied before. Calcium (Ca) was the predominant mineral element in all propolis samples. *Euphorbia propolis* volatiles were evaluated in one sample of *E. officnarum* and one sample of *E. resinifera*. In both cases, monoterpene hydrocarbons dominated, being α-pinene the main component. Higher levels of phenols, flavonoids, and dihydroflavonoids were detected in one sample of *E. officinarum*, which corresponded to superior antioxidant activity, due to its ability to scavenge DPPH and nitric oxide (NO) free radicals, superoxide anion radicals, and to prevent lipid peroxidation.The capacity to inhibit α-glucosidase, acetylcholinesterase, lipoxygenase, tyrosinase, and xanthine oxidase activities was not specific for *E. officinarum* or *E. resinifera*, that is, one sample of *E. officinarum* may present the best inhibitory α-glucosidase activity along with one sample of *E. resinifera*, but there is other *E. officinarum* propolis samples that presents the worst activity. The minimum inhibitory concentration (MIC) value ranged from 50 to 450 μL/mL against Gram-positive and Gram-negative bacteria. *Euphorbia* propolis displayed the ability to inhibit quorum sensing in the biosensor *Chromobacterium violaceum* CV026 and disrupted bacterial biofilm formation, including that of resistant bacterial pathogens.

Future perspectives

Aware that the present research work is a starting point for *E. officinarum* and *E. resinifera*, several aspects should be deeply explored in the near future:

A limitation in the use of natural products for therapeutic purposes is the variability of biological activities due to the changeability of concentrations of active ingredients. In this way, it will be necessary to confirm the results obtained in the present research work using many more plants collected in diverse places. The same can be considered for honey and propolis samples.

A detailed chemical composition of the plant, honey, and propolis samples will be necessary to go deeper and, more importantly, be able to relate possible biological and/or pharmacological activities with already fractionated extracts or even with isolated compounds.

The evaluation of antioxidant activity and other biological activities relies on chemical reactions—chemical test-tube assays, as done in the present research work, and human and murine cell lines, generally without physiological relevance. Shortly, these assays should be based on using relevant human cell cultures.

It will be interesting to investigate whether there is a relationship between the chemical composition and biological properties of the aerial parts and cyathia of *Euphorbia* with those of honey and propolis harvested in areas where these species predominate. Moreover, in the case of propolis presenting better activity than the respective honey, it would be important to inquire about the possibility of adding propolis or its fractions to honey samples without loss of honey quality.

The monoflorability identification of *E. officinarum* and *E. resinifera* honey should be facilitated in which the pollen analysis could be replaced by using physical methods that would be faster, cheaper, and can be used by beekeepers.

The presence of some mineral elements in relatively high amounts in one honey sample suggests a more detailed investigation into this finding and determines whether it was an isolated case or whether there are polluting factors, either environmentally or during production or storage.

Conclusion Générale et perspectives d'avenir

Le présent travail avait pour objectif de faire une caractérisation globale des parties aériennes (sans latex et fleurs) d'*Euphorbia resinifera* et d'*Euphorbia officinarum*, deux espèces endémiques au Maroc ainsi que du miel et de la propolis respectifs. La caractérisation globale de la cyathia d'*E. resinifera* a également été réalisée.

L'examen scientifique effectué sur ces deux espèces marocaines a montré qu'il y a beaucoup plus de recherches sur leurs latex que sur les autres parties des plantes. Plus récemment, les recherches sur les produits apicoles dérivés des espèces d'*Euphorbia* ont suscité l'intérêt de plusieurs chercheurs.

En ce qui concerne la partie aérienne de la plante, et plus précisément en ce qui concerne les extraits aqueux obtenus par macération, il s'est avéré que le rapport plante/volume d'eau, le temps et la température de l'extraction sont des facteurs très importants pour obtenir des extraits avec une teneur plus élevée de quantités de phénols et de meilleures propriétés biologiques (capacité antioxydante, mesurée par différentes méthodes, et propriétés inhibitrices d'enzymes, principalement inhibition de la α-glucosidase). L'évaluation de l'inhibition de cette activité enzymatique a été réalisée car, dans certaines régions du Maroc, ces espèces sont utilisées pour la prise en charge du diabète sucré de type 2. Lorsque la décoction est utilisée pour l'extraction de phénols (température autour de 100 ºC), le rapport plante/volume d'eau, ainsi que le temps d'extraction influencent également les quantités de phénols extraites et les activités biologiques. Les meilleures conditions de température, de temps d'extraction et de rapport plante/volume d'eau dépendent également de l'espèce *Euphorbia*.

E. resinifera cyathia produit une pléthore de différentes classes de métabolites spécialisés, notamment des caroténoïdes, des glycosides flavonoïdes et des polyamines qui confèrent des propriétés antioxydantes aux décoctions d'eau, comme nous l'avons mesuré avec des tests antioxydants *in vitro*. De plus, des niveaux élevés de benzaldéhyde et de nonanal, composés volatils émis par les fleurs des espèces pollinisées par les abeilles, étaient également présents. Les principaux métabolites, les hexoses, les acides aminés et les vitamines, ont également été mesurés et étaient présents en quantités relativement élevées. La capacité d'élimination des radicaux libres des extraits floraux de cyathia correspondait aux deux tiers et à la moitié de la capacité d'extinction du 2,6-di tercbutil-p-crésol (BHT) et des composés purs d'acide ascorbique utilisés comme contrôle positif, respectivement.

Les miels d'*E. officinarum* et d'*E. resinifera* remplissent généralement les exigences de qualité en ce qui concerne les propriétés physico-chimiques (humidité, acidité, pH, niveaux de HMF, proline, activité diastase, cendres et conductivité électrique), bien que dans un cas, des niveaux élevés d'Al, Cu et Fe ont pu être détectés, ce qui peut être lié à une certaine contamination de l'environnement par ces éléments dans les environs des ruches ou encore à l'utilisation inadéquate de conteneurs métalliques pour le stockage du miel.

Les propriétés antioxydantes d'*E. officinarum* et d'*E. resinifera* ainsi que leur capacité à inhiber les enzymes acétylcholinestérase, lipoxygénase, tyrosinase et xanthine oxydase ont été évaluées et comparées. Les miels d'*E. resinifera* avaient une meilleure capacité à inhiber l'activité

lipoxygénase que le miel d'*E. officinarum*, tandis que dans les autres activités biologiques, il n'était pas possible d'observer de différences entre les deux types de miel monofloral. Généralement, les extraits méthanoliques du miel ont enregistré de meilleures activités dans toutes les activités biologiques, que le miel entier correspondant, néanmoins dans certains cas on a trouvé une corrélation inverse entre le phénol total de l'extrait de miel et les activités, ce qui signifie que ces résultats vont au-delà du total contenu phénolique, le type de composé est également important. De plus, les extraits aqueux d'Euphorbia ont généralement de meilleures propriétés biologiques (capacité à inhiber les activités lipoxygénase, acétylcholinestérase, tyrosinase et xanthine oxydase; et capacité à piéger les radicaux libres DPPH, superoxyde et oxyde nitrique) que le miel de même origine florale. En ce qui concerne l'anti-xanthine oxydase, il n'y avait aucune corrélation entre la teneur en phénol et l'activité de l'anti-xanthine oxydase, donc des composés non phénoliques peuvent être responsables des activités inhibitrices de la xanthine oxydase.

Les propolis *E. officinarum* et *E. resinifera* propolis n'ont jamais été étudiées auparavant. Le calcium (Ca) était l'élément minéral prédominant dans tous les échantillons de propolis. Les substances volatiles de la propolis d'Euphorbia ont été évaluées dans un échantillon d'*E. officinarum* et un échantillon d'*E. resinifera*. Dans les deux cas, les hydrocarbures mono terpéniques dominaient, l'α-pinène étant le composant principal. Des niveaux plus élevés de phénols, de flavonoïdes et de dihydroflavonoids ont été détectés dans un échantillon d'*E. officinarum*, ce qui correspondait à une activité antioxydante supérieure, en raison de sa capacité à éliminer les radicaux libres DPPH et d'oxyde nitrique (NO), les radicaux anions superoxydes et à prévenir les lipides peroxydation. La capacité à inhiber les activités de la α-glucosidase, de l'acétylcholinestérase, de la lipoxygénase, de la tyrosinase et de la xanthine oxydase n'était pas spécifique d'*E. officinarum* ou d'*E. resinifera*, c'est-à-dire qu'un échantillon d'*E. officinarum* peut présenter la meilleure activité inhibitrice de la α-glucosidase avec un échantillon d'*E. resinifera*, mais il existe d'autres échantillons de propolis d'*E. officinarum* qui présentent la pire activité. La valeur de la concentration minimale inhibitrice (CMI) variait de 50 à 450 µL/mL contre les bactéries Gram-positives et Gram-négatives. La propolis d'Euphorbia a montré la capacité d'inhiber la détection du quorum dans le biocapteur *Chromobacterium violaceum CV026* et a perturbé la formation de biofilm bactérien, y compris celle d'agents pathogènes bactériens résistants.

Perspectives d'avenir

Conscients que les présents travaux de recherche constituent un point de départ pour *E. officinarum* et *E. resinifera*, plusieurs aspects devraient être approfondis dans un avenir proche:

Une limitation dans l'utilisation de produits naturels à des fins thérapeutiques est la variabilité des activités biologiques due à la variabilité des concentrations d'ingrédients actifs. De cette façon, il sera nécessaire de confirmer les résultats obtenus dans le présent travail de recherche en utilisant beaucoup plus de plantes collectées dans divers endroits. La même chose peut être envisagée pour les échantillons de miel et de propolis.

Une composition chimique détaillée des échantillons de plantes, de miel et de propolis sera nécessaire pour approfondir et surtout pouvoir relier d'éventuelles activités biologiques et/ou pharmacologiques avec des extraits déjà fractionnés ou même avec des composés isolés.

L'évaluation de l'activité antioxydante et d'autres activités biologiques repose sur des réactions chimiques – des tests chimiques en éprouvette, comme ceux réalisés dans le présent travail de recherche, et des lignées cellulaires humaines et murines, généralement sans pertinence physiologique. À court terme, ces tests devraient être basés sur l'utilisation de cultures de cellules humaines pertinentes.

Il sera intéressant d'étudier s'il existe une relation entre la composition chimique et les propriétés biologiques des parties aériennes et des cyathies d'Euphorbia avec celles du miel et de la propolis récoltés dans les zones où ces espèces prédominent. De plus, dans le cas de propolis présentant une meilleure activité que le miel concerné, il serait important de s'enquérir de la possibilité d'ajouter de la propolis ou ses fractions aux échantillons de miel sans perte de qualité du miel.

L'identification de la monoflorabilité du miel d'*E. officinarum* et d'*E. resinifera* devrait être facilitée, l'analyse du pollen pouvant être remplacée par l'utilisation de méthodes physiques qui seraient plus rapides, moins chères et pourraient être utilisées par les apiculteurs.

La présence de certains éléments minéraux en quantités relativement élevées dans un échantillon de miel suggère une enquête plus approfondie sur ce résultat et détermine s'il s'agit d'un cas isolé ou s'il existe des facteurs polluants, soit environnementaux, soit lors de la production ou du stockage.