Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

Study of the antimicrobial and antioxidant effects of propolis extracts, a type of Algarve Portuguese origin

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Master in Biomedical Sciences

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“I hear and I forget,
I see and I remember,
I do and I understand.”

Confucius

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List of symbols and abbreviations

ACF - Aberrant crypt foci

ALT - Alanine transaminase

ATCC - American type culture collection

BHI - Brain heart inffusion

BHT - Butylated hydroxytoluene

BSA - Bovine serum albumin

CAPE - Caffeic acid phenethyl ester

CAT - Catalase

CBA - Columbia blood agar base

CCl_4 - Carbon tetrachloride

COX - Cyclooxygenase

CREF - Cell line of Fischer rat embryo fibroblasts

CYP 2E1 – Cytochrom P450 2E1

D-GalN - D-galactosamine

DMH - 1,2-dimethylhydrazine

DNA - Deoxyribonucleic acid

DPPH - 1,1-diphenyl-2-picrylhydrazyl

DSMZ - German collection of microorganisms and cell culture

EC_{50} – Effective concentration

EDTA - Ethylenediaminetetraacetic acid

EEP - Ethanol extract of propolis

Eq. - Equation

FERN - Faculty of Natural Resources Engineering

FTC - Ferric thiocyanate

GC–MS - Gas chromatography-mass spectrometry

GP - Guaiacol peroxidase

GPX - Glutathione peroxidase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

H_2O_2 - Hydrogen peroxide

HeLa cells – Human epithelial cells

HIV-1 - Human immunodeficiency virus type 1

HPLC - High performance liquid chromatography

HSV - Herpes simplex virus

HuH13 cells - Human hepatocellular carcinoma cells

IC_{50} - Inhibitory concentration

INETI-DTIA - National Institute of Engineering, Technology and Inovation, Department of Food Industries Technology

KB cells - Human nasopharynx carcinoma cells

LB-1 - 1,1-dimethylallyl caffeic acid ester

LPS - Lipopolysaccharide

MBC - Minimum bactericidal concentration

MIC - Minimum inhibitory concentrations

MTT - 3-4,5-dimethylthiazol-2-il)2,5-diphenyltetrazoilium bromide

NADH - α-Nicotinamide adenine dinucleotide

ORL - Otorhinolaryngologic

PGE2 – Prostaglandin E2

PMS - Phenazyne

PMS-1 - Clerodane-type diterpene

PVP - polyvinylpyrrolidone

PWE - Propolis water extract

RNA - Ribonucleic acid

RNS - Reactive nitrogen specie

ROS - Reactive oxygen specie

Rpm - Rotations per minute

SOD - Superoxide dismutase

TLC - Thin layer chromatography

TNF-α - Tumour necrosis factor alpha

TPA - 12-O-tetradecanoylphorbol 13-acetate

TSA - Tryptone soya agar

TSB - Tryptone soya broth

XOD - Xanthine/ xanthine oxidase

WSD - Water-soluble derivative

Abstract

In this work, extracts (aqueous, ethanolic and methanolic) of propolis harvested at two different times (winter and spring) from several locations of the Algarve region (B. N. Arrodeios, B. N. Pé da Serra, B. S. Arneijoafra and T. N. Madeira) were tested for their biological activities and composition.

Results showed that propolis extracts were active against both Gram-positive and Gram-negative bacteria. All tested strains of bacteria showed susceptibility to the diluted propolis extracts (1:10) and in the majority of cases in a dose-dependent way. Most propolis samples collected at springtime showed higher antibacterial activity, in comparison with samples harvested at wintertime. There were also observed differences between collection sites and type of extract. These results correlate to HPLC results, where the same differences where observed.

Regarding propolis citotoxicity, results showed that aqueous propolis extracts have no effect and ethanolic propolis extracts causes a small decrease in cell viability.

Concerning antioxidant enzymatic activities, the superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GP) activities of samples of propolis were determined. Results showed that SOD activity was dependent on the collection time and decreased drastically from winter to spring in samples from B.N. Arrodeios and B.S. Arneijoafra. The opposite was observed in samples from B.N. Pé da Serra and T.N. Madeira. We expected that the samples with higher SOD activities would have higher CAT activities also. Such was not observed, which may suggest that there could be other antioxidant enzymes involved, different to the ones tested.

In respect with protein contents, major differences were not observed when comparing samples collected at different times, except for samples from B.S. Arneijoafra, where a decrease occurred from the sample collected at winter to sample collected at spring time.

This was the first study of the biological activities of Portuguese propolis from the Algarve region.

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Keywords: Portuguese propolis, antibacterial activity, antioxidant activity, citotoxicity, chemical composition

Resumo

O própolis é uma substância resinosa de origem natural produzida pelas abelhas (*Apis mellifera*) que recolhem e misturam resinas obtidas de várias plantas. Os extractos de própolis são usados na medicina tradicional desde a Antiguidade. Actualmente, descobriu-se que estes extractos possuem uma vasta gama de actividades biológicas, nomeadamente efeitos anti-bacterianos, anti-inflamatórios, antioxidantes, hepatoprotectores e actividade anti-tumoral. Estas acções farmacológicas devem-se provavelmente à presença de compostos antioxidantes, como por exemplo compostos fenólicos, especialmente flavonóides e ácidos fenólicos. Contudo, a composição química e actividade farmacológica podem variar bastante de região para região. Deste modo, para além das suas actividades farmacológicas, outro aspecto importante no estudo das características do própolis refere-se à sua origem botânica e consequente variação da composição química

Neste trabalho, extractos (aquosos, etanólicos e metanólicos) de própolis colhidos em duas épocas do ano (Inverno e Primavera) e em diferentes locais do Algarve (B. N. Arrodeios, B. N. Pé da Serra, B. S. Arneijoafra e T. N. Madeira) foram estudados e avaliados em relação à sua actividade anti-bacteriana, actividade enzimática antioxidante, citotoxicidade e composição química.

A actividade anti-bacteriana foi determinada através do método de difusão no agár utilizando vários volumes diferentes de extractos de própolis diluídos em *n*-propanol. Todas as estirpes de bactérias (*Salmonella enterica* subspecie *enterica* serovar *thyphimurium* ATCC 14028, *Staphylococcus aureus* CFSA2, *Haemophilus influenza* TD-4, *Streptococcus pneumonia* D39, *H.pylori* estirpe J99 e 26695) testadas mostraram susceptibilidade aos extractos diluídos (1:10) de própolis, e na maioria dos casos de modo dose-dependente. Os resultados mostraram que o própolis de origem portuguesa é activo contra bactérias Gram-positivas e Gram-negativas. As amostras de própolis recolhidas na Primavera mostraram maior actividade anti-bacteriana, em comparação com as amostras recolhidas no Inverno, excepto para *Haemophilus influenza* TD-4 e *Streptococcus pneumonia* D39. Também se observaram diferenças estatísticas entre as amostras de diferentes locais de recolha e entre os diferentes tipos de extracto.

Embora na maioria dos casos os três tipos de extractos de própolis testados tenham tido uma actividade anti-bacteriana semelhante a sua concentração em fenóis era muito diferente, com os extractos etanólico e metanólico tendo em alguns casos uma concentração cerca de 10 vezes superior à concentração dos extractos aquosos. O que mostra que apesar dos extractos aquosos terem uma concentração mais baixa em compostos fenólicos apresentam uma actividade anti-bacteriana semelhante à obtida com os outros extractos.

Em relação à citotoxicidade do própolis, pretendia-se determinar se os extractos de própolis quando usados nas mesmas quantidades que mostraram actividade anti-bacteriana teriam algum efeito na viabilidade celular, usando o ensaio colorimétrico do MTT (brometo de 3-4,5-dimetiltiazol-2-il)2,5-difeniltetrazoilio). Este ensaio permitiu estudar, utilizando células animais (Caco-2) a capacidade de resistência dessas células à toxicidade provocada pela presença dos compostos do própolis. Os nossos resultados mostram que os extractos aquosos de própolis não têm efeito na viabilidade celular e que os extractos etanólicos causam um pequeno decréscimo da viabilidade celular.

Considerando as actividades antioxidantes, determinou-se a actividade enzimática da superóxido dismutase (SOD), catalase (CAT) e guaiacol peroxidase (GP) de amostras de própolis através de ensaios espectrofotométricos. Os resultados mostram que a actividade da SOD é dependente do tempo de recolha e que decresce drasticamente do Inverno para a Primavera nas amostras recolhidas em B. N. Arrodeios e B. S. Arneijoafra. O oposto foi observado nas amostras recolhidas em B. N. Pé da Serra e T. N. Madeira. A actividade da SOD origina moléculas de peróxido de hidrogénio e oxigénio. Estas moléculas de peróxido de hidrogénio podem ser convertidas em moléculas de água e oxigénio através da acção de enzimas, como a CAT. Deste modo, era esperado que as amostras com maior actividade da SOD tivessem também maior actividade da CAT. Mas tal não foi observado, o que sugere a possibilidade de estarem envolvidas outras enzimas antioxidantes, diferentes das testadas.

Os resultados da determinação da actividade da CAT mostram diferenças estatísticas entre as amostras em relação local de recolha mas não em relação ao tempo de recolha, excepto para a amostra recolhida em B.N. Arrodeios. Para esta amostra a actividade da CAT quase que duplica quando se comparam os valores obtidos com a amostra colhida no Inverno com os valores obtidos com a amostra colhida na Primavera.

O conteúdo em proteínas solúveis dos extractos foi determinado de acordo com o método de Bradford, em que se utilizaram soluções de BSA (albumina de soro bovina) como amostras padrão de calibração. Em relação ao conteúdo proteico das amostras de própolis, não se observaram grandes diferenças estatísticas entre as amostras recolhidas entre o Inverno e a Primavera, excepto para as amostras recolhidas no B. S. Arneijoafra. Neste caso, ocorreu um decréscimo em relação à amostra recolhida no Inverno para a amostra recolhida na Primavera. Os resultados da determinação do conteúdo proteico não se correlacionam com os resultados das actividades enzimáticas, o que reforça a noção de que haverá outras enzimas presentes nos extractos de própolis diferentes das testadas neste estudo.

A análise realizada relativamente à composição foi feita através de HPLC (high performance liquid chromatography). Os cromatogramas obtidos mostraram diferenças entre as

amostras de própolis em relação aos locais e tempos de recolha e também entre os tipos de extracto. Estes resultados correlacionam-se com os resultados obtidos para a actividade anti-bacteriana, onde também foram observadas variações. A análise dos extractos por HPLC permitiu também identificar alguns dos compostos presentes nos extractos. Entre os compostos identificados estavam o ácido cafeíco, ácido ferúlico e galangina, compostos que já tinham sido antes descritos como compostos com actividade anti-bacteriana.

Deste modo, com este estudo mostrou-se que os extractos de própolis português apresentam actividade anti-bacteriana e antioxidante e que estas variam consoante o local e época de recolha. Os diferentes tipos de extractos mostraram ter composição diferente que também variava consoante o local e época de recolha, e também consoante tipo de solvente de extracção utilizado.

Por fim é de realçar que este é o primeiro estudo realizado sobre as actividades biológicas do própolis da região do Algarve.

Palavras-chave: Própolis português, actividade anti-bacteriana, actividade antioxidante, citotoxicidade, composição química.

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1. Introdution

1.1. Propolis: origin and composition

Propolis (sometimes also referred as bee glue) is a natural resinous substance collected by honeybees from various plant sources. The word propolis is derived from the Greek *pro*, for or in defence, and *polis*, the city, that means, defence of the city (or the hive) [1].

Propolis is a strongly adhesive, resinous substance collected, transformed and used by bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders. Honeybees (*Apis mellifera* L.) collect the resin from the cracks in the bark of trees and leaf buds of numerous tree species like birch, poplar, pine, alder, willow and palm. Bees may also use material actively secreted by plants, or exuded from wounds in plants (lipophylic material on leaves, mucilages, gums, resins, lattices, etc [2, 3]. Once collected this material is masticated, salivary enzymes added and the partially digested material is mixed with beeswax and used in the hive to seal holes, smooth out the internal walls and protect the entrance against intruders [1].

Figure 1.1 - Bee depositing propolis in the hive [4].

It is a resinous, sticky gum, the colour of which varies from yellow-green to dark brown depending on its source and age. It is difficult to remove from the human skin, since it seems to interact strongly with the oils and proteins of the skin [1]. It is hard and brittle when cold, but becomes soft and very sticky when warm hence the name bee glue [5].

In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen grains, which are a rich source of essential elements such

as, magnesium, nickel, calcium, iron and zinc and 5% various other substances, including organic debris [1,2]. The proportion of these types of substances and precise composition of propolis varies and depends on the place and time of collection [5].

1.2. Botanical sources of propolis

It was noted that the compounds in propolis resin originate from three sources: plant exudate collected by bees, secreted substances from bee metabolism, and materials which are introduced during propolis elaboration [5].

The source of the plant exudate was historically considered to be various indigenous poplar species, but this failed to explain why bees could produce propolis in the area of the equator where no poplars exist. Because the constituents of propolis directly reflects their source the introduction of more sophisticated chemical analysis allowed the identification of additional species of trees which could be used as a source of propolis for the foraging bees [3, 5]. In table 1.1 some of the identified propolis botanical sources are summarized.

Now, it is generally accepted and chemically demonstrated that in temperate zones the bud exudates of *Populus* species and their hybrids are the main source of bee glue. This is true for Europe, North America, and the non-tropical regions of Asia. Even in New Zealand, introduced poplar species are the source plants. In Russia however, and especially in its northern parts, birch buds (*Betula verrucosa*) supply bees with the necessary materials to produce propolis [3].

In tropical regions there are no poplars and birches, and bees have to find new plant sources of bee glue. By identifying the main propolis flavonoids in samples from “border areas” with almost tropical climate, where poplars are not always available, it was found that the leaf exudate of some *Cistus* spp. was a plant source of propolis in Tunisia [3]. Polyprenylated benzophenones have been isolated from propolis samples from tropical Venezuela. These compounds are main components of the resin exuded by the flowers of some *Clusia* species and it was demonstrated that *Clusia major* and *Clusia minor* (Guttiferae) were the main sources of propolis in the region concerned [3]. Chemical studies determined that the main source of Brazilian bee glue was the leaf resin of *Baccharis dracunculifolia*. Recently the chemistry of Cuban propolis has also been studied. Its main components are polyisoprenylated benzophenones, and this makes Cuban propolis different from both European and Brazilian bee glue. The plant source of this propolis type was detected to be the floral resin of *Clusia rosea*, from whence came the prenylated benzophenones [6].

Table 1.1- Botanical sources of propolis (adapted from ref. 3 and 5).

Genus and species	**Geographic location**
Populus nigra, P. italica	Bulgaria
Populus nigra	Albania
Populus tremula	Bulgaria
Populus suaveolens	Mongolia
Populus fremontii	USA (mainland)
Plumeria acuminata, Plumeria acutifolia	USA (Hawaiian islands)
Populus euramericana	United Kingdom
Betula, Populus, Pinus, Prunus and *Acacia* spp., *Aesculus hypocastane*	Hungary
Betula, Alnus spp.	Poland
Delchampia spp.	Equatorial regions
Clusia spp.	Equatorial regions
Clusia minor and *Clusia major*	Venezuela
Xanthorrhoea spp.	Australia
Poplar, birch, elm, alder, beech, conifer and Horsechestnut	``North temperate zone''
Populus species and their hybrids	Europe, North America , and the non-tropical regions of Asia
Betula verrucosa	Russia
Cistus spp.	Tunisia
Clusia rosea	Cuba
Araucaria spp. and *Baccharis* spp.	Brazil

1.3. Chemical composition

As mentioned before, the chemical composition of propolis is very complex and depends on the flora in the areas where it is collected. Also, due to its complex composition it is difficult to identify compounds with a simple fractionation of propolis. Usually, a propolis extract is prepared by extracting the soluble fraction in alcohol and leaving the alcohol-insoluble or wax fraction. Although ethanol extract of propolis (EEP) is the most common, extracts with other solvents have also been used allowing the identification of more than 300 constituents of propolis [1, 7].

Most of the identified compounds in propolis are polyphenols and the major polyphenols are flavonoids, accompanied by phenolic acids and esters, phenolic aldehydes, ketones [2]. For example, samples originating in the temperate region (Europe, Asia and North America), where bud exudates of different poplar buds are the main source of propolis, are characterized by similar chemical composition, the main constituents being phenolic compounds: flavonoid aglycones, aromatic acids and their esters [3]. In contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids and of acetophenone [3, 8]. Diterpenes, lignans and flavonoids (different from those in ‘poplar type’

propolis) have also been found [6]. Table 1.2 lists the most typical constituents of propolis samples from different geographic locations and their plant sources.

Table 1.2- Most typical constituents of propolis samples from different geographic locations and their plant sources (adapted from ref. 3).

Geographical origin	**Plant source**	**Typical constituents (main components)**
Europe, Asia, North America	*Populus* spp. (poplar)	Pinocembrin, pinobanksin, pinobansin-3-O-acetate, chrysin, galangin, caffeates (benzyl, phenylethyl, prenyl)
Northern Russia	*Betula verrucosa* (birch)	Acacetin, apigenin, enmanin, rhamnocitrin, kaemferid, α-acetoxybetulenol
Brazil	*Baccahris* spp.; *Araucaria* spp.	Prenylated p-coumaric acids; prenylated acetophenones, diterpenic acids
Cuba	*Clusia rosea*	Polyisoprenylated benzophenones
Canary Islands	Unknown	Furoruran lignans

Some of the recently identified compounds include: aromatic compounds, flavonoids, prenylated *p*-coumaric acids, acetophenone derivatives, caffeoylquinic acids, lignans, diterpenic acids, triterpenes, monoterpenes, sesquiterpenes, sugars and sugar alcohols. Vitamins B1, B2, B6, C, E, and mineral elements silver, cesium, mercury, lanthanum, antimony, copper, manganese, iron, calcium, aluminium, vanadium and silicon have all been identified in propolis samples [5].

By far, the largest group of compounds isolated are flavonoid pigments, which are ubiquitous in the plant kingdom [1]. It is not surprising, therefore, that the same flavones have been isolated from different samples of propolis and the series of flavonoids isolated from propolis correlate reasonably well with those present in the plants from which honeybees collect propolis. It has been suggested that some of the flavones are modified by secreted substances from the bee metabolism. If so, it seems likely that any transformation must occur in the presence of enzymes in the saliva of the bees during collection. Also, the simple aromatic compounds found in propolis also occur commonly in plants and their presence in propolis is therefore not unexpected [1].

It is important to note that most of the latest investigations on new propolis constituents are connected to their biological activity. Some examples are listed in Table 1.3. Some of the prenylated *p*-coumaric acids and diterpenic acids possess antibacterial and cytotoxic activities [3]. Caffeoylquinic acid derivatives showed immunomodulatory and hepatoprotective action [7, 9]. The furofuran lignans were shown to inhibit the growth of some bacteria and diterpenic acids isolated from Brazilian propolis showed cytotoxic and antibacterial activity [2, 3]. Caffeic acid

phenethyl ester (CAPE) is also cytotoxic towards tumour cells [2]. Anti-microbial properties of propolis seem attributable mainly to the flavonoids pinocembrin, galangin and pinobanksin. Pinocembrin also exhibits anti-fungal properties [2].

Table 1.3- Biological activity of new propolis constituents (adapted from ref. 3).

Type of activity	**Compound**
Antibacterial	*Flavonoids* Pinocembrin, galangin, pinobanksin; *Prenylated p-coumaric acids* 3,5-diprenyl-4-hydroxycinnamic acid; 3-prenyl-4-dihydro-cinnamoyloxycinnamic acid; 2,2,-dimethyl-6-carboxyethenyl-2H-1-benzopyran *Diterpenic acids* 15-oxo-3,13Z-kolavadiene-17-oic acid and its E-isomer, communic acid, imbricatoloic acid, isocupressic acid *Lignans* 3-acetoxymethyl-5-[(E)-2-formylethen-1-yl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran, sesamin, aschantin, sesartenin
Cytotoxic/Antitumoural	*Flavonoids* Aromadendrine-4'methyl ether; 3,5,7-trihydroxy-6,4'-dimethoxyflavon *Prenylated p-coumaric acids* 3,5-diprenyl-4-hydroxycinnamic acid; 9-E-,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran *Lignans* 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(E)-3-acetoxypropen-1 -yl]-2-methoxyphenoxy}propan-1,3-diol 3-acetate (erythro- and treo), yangambin *Caffeoylquinic acids* Caffeic acid phenethyl ester *Diterpenic acids* ent-17-hydroxy-3,13Z-clerodadien-15-oic acid
Immunomodulating	*Caffeoylquinic acids* 3-caffeoylquinic (chlorogenic) acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4,5-dicaffeoylquinic acid methyl Ester
Antihepatotoxic	*Caffeoylquinic acids* 4,5-dicaffeoylquinic acid; 3,4-dicaffeoylquinic acid; methyl 3,4-di-Ocaffeoylquinate; methyl 4,5-di-O-caffeoylquinate; 3,5-di-O-caffeoylquinic acid

As noted earlier, the largest group of compounds isolated from propolis are flavonoid pigments, which are ubiquitous in the plant kingdom and the series of flavonoids isolated from propolis correlate reasonably well with those present in the plants from which honeybees collect propolis. The substances identified in propolis are familiar constituents of food, food additives and/or generally recognized as safe substances. Conspicuous among the list of constituents are

hydroquinone, caffeic acid and its esters and quercetin, each of which have exhibited carcinogenic effects when administered to rodents. However, all three of these substances occur naturally in foods. Hydroquinone is present in beer and coffee (at levels of 1.25 to 40 ppm) and is approved as an indirect additive to food. While quercetin and caffeic acids (and esters of caffeic acid) are not approved for use in food, the contribution of these substances through consumption of propolis is minimal when compared with consumption from other natural sources. For example, a single apple (with peel) may contain 5.8 to 26 mg quercetin. Also, a single serving of lettuce may contain 27±56 mg caffeic acid. Therefore, propolis contributes an insignificant amount of these substances when compared with the daily diet [1].

1.4. Variability in propolis composition and importance of the knowledge of plant sources

To understand what causes the differences in chemical composition, it is necessary to keep in mind the plant origin of propolis. For propolis production, bees use materials resulting from a variety of botanical processes in different parts of plants. So, the plant origin of propolis determines its chemical diversity. Bee glue's chemical composition depends on the specificity of the local flora at the site of collection and thus on the geographic and climatic characteristics of this site. This fact results in the striking diversity of propolis chemical composition, especially of propolis originating from tropical regions (Table 1.2) [10].

The distinct chemistry of propolis from different origins suggests the possibility that the biological properties of different propolis types will be different. However, in most cases this is not true [10]. Actually, propolis is the defence of bees against infections, and the antibacterial and antifungal activity of all samples is not surprising. The similarity in many of the other types of activity is less obvious but it is a fact. Of course, the responsible compounds are different, as shown in Table 1.2: mainly flavanones, flavones, phenolic acid and their esters in poplar type (European) propolis, prenylated *p*-coumaric acis and diterpenes in *Baccharis* type (Brazilian) propolis; prenylated benzophenones in Cuban red propolis. The fact that different chemistry leads to the same type of activity and in some cases even to activity of the same order of magnitude is remarkable. Nonetheless, it is important to have detailed and reliable comparative data on every type of biological activity, combined with chemical data, in order to decide if some specific areas of application of a particular propolis type can be formulated as preferable [10].

In fact, more and more publications are appearing which combine antimicrobial and other biological studies with chemical analyses of the tested propolis samples [11-15]. The most often used techniques for chemical analyses are gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). Another recent trend is to combine qualitative

chemical characterization of the samples tested for biological activity with quantification of the major groups of biologically active substances of the corresponding samples [6]. And possibly the most interesting trend in propolis research is the comparative study of biological properties of propolis from different geographic locations and different chemical composition. The number of this type of studies is yet limited. For example, Kujumgiev and co-workers compared the antimicrobial (antibacterial, antifungal and antiviral) activity and chemical composition of propolis from diverse geographic origins [16]. The results presented unambiguous proof that in spite of the great differences in the chemical composition of propolis from different geographic locations, all samples exhibit significant antibacterial and antifungal (and most of them antiviral) activity. This study clearly demonstrated that in different samples, different combinations of substances are essential for the biological activity of bee glue [16].

In order for propolis to be accepted officially into the healthcare system, it needs chemical standardization that guarantees its quality, safety, and efficacy. Due to its plant origin, it is reasonable to approach the problem of propolis standardization in the same way as it is done for medicinal plants. If the active principles are known and accepted, they have to be quantified using an appropriate analytical method. If the active compounds are not known or still under discussion, the total extract is regarded as the "active principle" and in that case marker compounds must be use for quality control [10]. In the case of propolis, a lot of knowledge has already been gathered on active components and one of the most important active principles was found to be CAPE (caffeic acid phenethyl ester) [7]. However, CAPE is not present in most tropical samples not even in trace amounts, making it a bad candidate to be used for standardization. The same is true for many other active propolis constituents. In such case, it is necessary to formulate different propolis types according to their plant source and the corresponding chemical profile. Combined with the knowledge of active principles, it gives clues to standardization and quality control, allowing the specification of propolis types that have distinct chemical composition [10].

Therefore the knowledge about the botanical origin of propolis could be useful as a basis for the chemical standardization of propolis in addition to its importance to beekeepers to ensure that their bees have the proper plants in their flight range. It is known that colonies suffer when they cannot collect propolis, bees are even said to use "propolis substituents" like paints, asphalt and mineral oils which could severely threaten pharmaceutical uses of bee glue [3].

1.5. Propolis biological and pharmacological properties

1.5.1. Antioxidative activity

The antioxidant activity of propolis is one of its most studied biological effects with several papers on the subject, but first a small introduction to free radicals and antioxidant activity.

1.5.1.1. Free radicals and antioxidants

Free radicals are defined as any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons [17]. This broad definition encompasses a wide range of reactive species that include reactive oxygen species (ROSs) and reactive nitrogen species (RNSs) summarized in Table 1.4, as well as other reactive species.

Table 1.4- Some examples of free radicals.

$H\cdot$	Hydrogen	$RO\cdot$	Alcoxyl
$\cdot OH$	Hydroxyl	$HO_2\cdot$	Hydroperoxyl
$RO_2\cdot$	Peroxyl	$NO\cdot$	Nitric oxide
$O_2\cdot^-$	Superoxide anion	$NO_2\cdot$	Nitric dioxide or peroxynitrite

In biological conditions the majority of molecules aren't in the form of radicals, maintaining their electrons paired, but in certain conditions the free radicals, also called reactive species can be formed and can cause a physiological and pathological effect [18]. Low concentrations of ROS may be beneficial or even indispensable in processes such as intracellular signaling and defense against micro-organisms. Nevertheless, higher amounts of ROS play a role in the aging process as well as in a number of human disease states, including cancer, ischemia, and failures in immunity and endocrine functions. Some examples of diseases that have been associated with ROSs and antioxidant enzymes imbalance are given in table 1.5.

The continuos production of free radicals during metabolic process led to the development of antioxidant defence mechanisms in order to control intracellular levels and prevent cellular damage induction. Antioxidant agents are responsible for the inhibition and decrease of cellular damage caused by free radicals. So, antioxidants can be defined as substances that, present in a lower concentration than the oxidable substance, are capable of preventing or delaying the oxidation of another substance [20].

Table 1.5- Human diseases associated with ROSs and antioxidant enzymes imbalance (adapted from ref. 19)

Human diseases	
Allergy	Bronchial asthma, Intolerance to aspirin, Intolerance to foods, Response to mercury, Response to other drugs, Response to other oxidants
Cancer	Bladder, Bowel, Breast, Colorectal, Esophageal, Kidney, Leukemia, Liver, Lung, Prostate, Skin
Cardiac and vessels injuries	Atherosclerosis, Ischemia
Genetic and metabolic disorders	Chronic granulomatous disease, Diabetes, Down's syndrome
Infectious diseases	*Helicobacter pylori*, Hepatitis, HIV, *Influenza* virus, Pneumonia, Rheumatoid arthritis
Neurodegenerative diseases	Allergic encephalomyelitis, Alzheimer's disease, Amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, Prion disease
Ophthalmologic problems	Cataract, Glaucoma

1.5.1.2. Antioxidant defence system

Cells are capable of defending themselves against the deleterious effects of reactive species trough an antioxidant defence system. In healthy aerobic organisms, ROSs levels are in equilibrium with the antioxidant defence system. The metabolic imbalance between the ROSs production and the antioxidant defence system characterizes cellular oxidative stress [21]. This imbalance can be caused by several factors, for example: (i) mutations in the antioxidant defence enzymes; (ii) decrease of the intake of vitamins and other dietary constituents; (iii) rise in the ROSs production caused by environmental factors such as smoke and radiation; (iv) excessive physical activity; (v) fat intake; (vi) alcohol consumption; (vii) physical and mental stress; (viii) inflammations and infections, among others [18].

The antioxidant defence system can be divided as enzymatic and non-enzymatic. The most important agents in the antioxidant defence system are listed in Table 1.6. As part of the enzymatic antioxidant system are enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

Superoxide dismutase is an enzyme that is specific for superoxide radical, specie that is made by adding an extra electron onto the oxygen molecule. SOD catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 (Eq. 1) [19]. Hydrogen peroxide (H_2O_2) can later be removed from cells by two other enzymes: catalase and glutathione peroxidase. H_2O_2 can also be eliminated through a reaction where the bonds between the O_2 atoms break and there is formation of the hydroxyl radical ($^{\cdot}OH$), catalyzed by transitions

metals (Fenton reaction, Eq. 2), or by combination of $O_2^{\cdot -}$ with H_2O_2 (Haber-Weiss reaction, Eq. 3) [18].

Table 1.6- Most important agents in the antioxidant defence system (adapted from ref. 20).

Enzymatic	Superoxide dismutase, Catalase, Gluthatione peroxidase, NADPH-quinone oxidoredutase, Repair enzymes
Non-enzymatic	α-Tocopherol (vitamin E), β-Carotene, Ascorbic acid (vitamin C), Flavonoids, Plasma proteins, Selenium, Gluthatione,

In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). SOD destroys $O_2^{\cdot -}$ by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates. All types of SOD bind single charged anions such as azide and fluoride, but distinct differences have been noted in the susceptibilities of Fe^-, Mn^- or Cu/Zn-SODs. Cu/Zn-SOD is competitively inhibited by N_3^-, CN^- , and by F^- [19].

Equation 1. $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

Equation 2. $Fe^{2+} / Cu^+ + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+} / Cu^{2+}$

Equation 3. $H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe/Cu} OH^{\bullet} + OH^- + O_2$

Catalase reacts efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity (Eq. 4 and 5). Catalase is a haemprotein, present in most tissues in organelles denominated peroxisome, which protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells [18, 19].

Equation 4. $2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$

Equation 5. $ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$

Peroxidases belong to a large family of enzymes that are ubiquitous in fungi, plants, and vertebrates. These proteins usually contain a ferriprotoporphyrin IX prosthetic group and oxidize several substrates in the presence of hydrogen peroxide [22]. Glutathione peroxidase is a selenium-containing peroxidase; it contains a single selenocysteine (Sec) residue at its active site

in each of the four identical subunits, which is essential for enzyme activity [19]. Glutathione peroxidase removes H_20_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG), thereby protecting mammalian cells against oxidative damage (Eq. 6). In fact, glutathione peroxidase can be considered essential in the H_2O_2 and organic peroxides metabolism (Eq. 7), as well as ROSs metabolism.

Equation 6. $2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$

Equation 7. $2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$

This enzyme is present in animal cells, as well as, plants and bacteria, and its co-factor glutathione is synthesized intracelullarly from glutamic acid, cistein and glicine. Glutathione plays an important role in many biological process's, among them protein and DNA synthesis, as a cofactor of several enzymes, and cellular protection from iodizing agents and exogenous compounds, mainly ROSs [18]. Although GPX shares the substrate, H_2O_2, with CAT, it alone can react effectively with lipid and other organic hydroperoxides, being the major source of protection against low levels of oxidant stress [19].

Higher plants are known to possess a large set of peroxidases. These enzymes are usually expressed as several isoforms and their expression pattern is tissuespecific and developmentally regulated. Isoperoxidases are thought to participate in a wide range of physiological processes. These include H_2O_2 detoxification, cell elongation, cell wall construction and differentiation and the plant response to stress. On the basis of their function, as well as, subcellular localization, peroxidases are categorized into two different groups. Peroxidases which oxidize guaiacol (o-methoxyphenol), as a commonly used reducing substrate *in vitro,* are referred to as guaiacol peroxidases (GP) but for some time they were also called myeloperoxidases [23]. They are located in cytosol, vacuole, cell wall, apoplast and extracellular medium, but not in organelles and are assumed to be involved in a range of processes related to plant growth and development such as cell wall lignification, cell wall stiffening, auxin metabolism and root elongation. Ascorbate peroxidases belong to another group of plant peroxidases that show preference for ascorbic acid as reducing substrate. These enzymes are localized in chloroplast, microbody and cytosol and their main function is to scavenge H_2O_2 and defense against oxidative stress in plant cell [23]. In humans, guaiacol peroxidase has been investigated as a possible ovulation predictor and fertility indicator [24].

In addition to the protective effects of endogenous antioxidants, the inclusion of antioxidants in diet is of great importance and the consumption of fruits and vegetables has been related to a decrease in the risk of developing diseases associated with free radicals and oxidative

stress. Food, mainly fruits and vegetables contain antioxidants, like vitamins C, E and A, carotenoids, phenols and other that are capable of restraining the propagation of chain reactions and damage induced by free radicals [20].

Vitamins C, E and carotenoids are considered excellent antioxidants, capable of sequester free radicals very efficiently. The cooperative effect between vitamin C (ascorbic acid) and E (α-tocopherol) is frequently mentioned in literature, showing that the interaction of these vitamins is effective in lipid peroxidation inhibition and in DNA protection. Vitamin C acts in the aqueous phase as an excellent free radicals antioxidant but it can't act in lipophilic compartments [20]. Vitamin E major antioxidant action resides in its capability to suppress peroxyl radicals in the lipidic part of biological membranes and doing so disrupting the lipid peroxidation chain reaction. Several studies state that α-tocopherol can attenuate oxidative stress mainly because it protects membranes against lipid peroxidation. For this reason, vitamin E is the most commonly used oral supplement for disease prevention. Carotenoids like β-carotene and lycopene exert antioxidant functions in lipidic phases, through free radical suppression, for example superoxide anion, hydroperoxyl radical and hydroxyl radical. Carotenoids are known for this antioxidant action; however they can also reduce the formation of singlet oxygen by reacting directly with it and releasing energy in the form of heat [18].

Among the antioxidants present in vegetables, the most active and most frequently encountered are phenols, such as flavonoids, which act as chain-breaking antioxidants because their –OH group scavenges reactive radicals such as peroxyl radicals (RO_2·) (Eq. 8).

Equation 8. $-OH + RO_2^{\bullet} \longrightarrow R-O^{\bullet} + ROOH$

The resulting phenoxyl radical (R-O·) tends to be poorly reactive because of electron delocalization into the aromatic ring, so that the reactive $RO_2^{\cdot}$ radical is replaced by one of limited reactivity. Phenols sometimes have additional mechanisms of antioxidant action, *e.g.* by chelating transition metal ions [25] and can act in both lipophilic and hydrophilic compartments of the cell.

Amongst the most studied phenols and flavonoids are: caffeic acid, galic acid, elagic acid, quercetin, miricetin, rutin and narigenin [20]. Studies have also revealed that dietary flavonoids are potent radical scavengers, acting in a manner similar to ascorbate and α-tocopherol [26].

1.5.1.3. Propolis antioxidant effects

Propolis contains mostly flavonoids and phenolic compounds, which have been reported to have antioxidative properties. Due to the antioxidative effect, propolis may protect humans from deleterious oxidative processes. In consequence several groups of authors studied the antioxidative properties of propolis and their active constituents using various types of assays. For example, the antioxidative activity of five different propolis samples collected from Brazil were studied, towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and superoxide anion radical in the xanthine/xanthine oxidase (XOD) and α-nicotinamide adenine dinucleotide (NADH)/phenazyne (PMS) reactions. In comparison with methanol extracts, the water extracts of Brazilian propolis showed stronger antioxidative effects towards both DPPH free radical and superoxide anion radicals. Later, four dicaffeoylquinic acid derivatives were isolated from the propolis water extract (PWE), which showed a strong free radical scavenging activity in the DPPH free radical and xanthine XOD-generated superoxide anion radical assay systems. These compounds showed more potent free radical scavenging activity than the most commonly used antioxidants such as vitamin C, vitamin E and caffeic acid. Dicaffeoylquinic acid derivatives also showed an inhibitory activity on nitrite formation in LPS-induced murine macrophages. Similarly, CAPE, an interesting antitumour constituent, inhibited 5-lipoxygenase and soybean 15-lipoxygenase at micromolar concentrations [7].

The antioxidative properties of Sardinian propolis were also tested on polyunsaturated lipidic systems. Propolis extracts showed better antioxidative properties than a known antioxidant, vitamin E. Similarly, the antioxidative activity of Chinese, Australian, New Zealand and Japanese propolis extracts was also observed with positive activity. The author found that α-tocopherol is contained in almost all propolis samples and correlates with the antioxidative effect of propolis [7].

The antioxidative activity of 12 phenolic compounds isolated from Brazilian propolis was studied against peroxidation of linoleic acid in a micelle solution. The authors found that a new compound, 3-[3,4-dihydroxy-5-prenylphenyl]- 2-(E)-propenoic acid, possessed the highest potency (IC50, 0.17 μM) among them and was more effective than butylated hydroxytoluene (IC_{50}, 0.36 μM). They also reported that artepillin C (IC_{50}, 0.44 μM) is the most abundant of the isolated compounds in Brazilian propolis. Similarly, the DPPH free radical-scavenging activity of nine propolis samples collected from Brazil, Peru, the Netherlands and China was also tested [7]. The water extracts of Brazilian and Chinese propolis possessed higher DPPH free radical-scavenging activity than the corresponding methanolic extract. The ED_{50} values of these extracts ranged from 5.9 to 14.2μg/ml. On the other hand, the methanolic extracts of Peruvian and

Netherlands propolis exhibited stronger DPPH free radical-scavenging activity than the corresponding water extract. The water extracts of both CPI-type propolis and green propolis from Brazil, which are regarded as good quality propolis, showed the strongest scavenging activity with an ED_{50} value of 5.9μg/ml, whereas the water extract of Peruvian propolis showed an ED_{50} value of 94.9μg/ml. Caffeic acid was taken as a positive control with an ED_{50} value of 1.9μg/ml. The author also tested the DPPH free radical-scavenging activity of 27 compounds isolated from the methanolic extract of Brazilian propolis [7]. In a previous study a higher concentration of vitamin C was found in tissues such as kidney, stomach and small and large intestines, of vitamin E-deficient rats on treatment by propolis compared with the control group. These results would suggest that some components of propolis are absorbed into the blood, behave as hydrophilic antioxidants and conserve vitamin C [27].

Different extraction methods and extracts of propolis have been recently investigated with respect to their antioxidant activity. It is well known that the aqueous and ethanolic extracts from propolis possess different chemical composition and biological activities. For example, the antioxidant activities of ethanol and petroleum ether extracts, whose polarity is much weaker than that of water and ethanol, from Brazilian propolis were determined by DPPH radical-scavenging and ferric thiocyanate (FTC) methods, using α-tocopherol and butylated hydroxytoluene (BHT) as references. The DPPH assay showed that ethanol extract possessed significantly higher activity compared with BHT and petroleum ether extract but lower than that of α-tocopherol. Results from the FTC assay indicated that the activity of ethanol extract was higher than that of α-tocopherol and petroleum ether extract but lower than BHT. Basically, this antioxidant activity was dose-dependent and ethanol extract exhibited higher activity than that of petroleum ether extract at the same concentration [28]. As to extraction methods, the antioxidant activities of ethanolic extracts of propolis obtained by different extraction methods (high hydrostatic pressure extraction, leaching at room temperature and heat reflux extraction) was examined in relationship to their total polyphenol and flavonoid contents by two different assays, namely, the β-carotene bleaching and DPPH free radical scavenging assay systems. The results showed that the ethanolic extracts of propolis obtained by high hydrostatic pressure extraction and leaching at room temperature had relatively strong antioxidant activities, which may be correlated with the total polyphenol and flavonoid contents. Antioxidant activities of ethanolic extracts of propolis obtained by high hydrostatic pressure extraction were the same as those of ethanolic extracts of propolis obtained by leaching at room temperature. Leaching at room temperature usually needs a few days, and can take even more than 7 days, while high hydrostatic pressure extraction needs only 1 minute. These findings further illustrate that the

high hydrostatic pressure extraction has a bright prospect for extracting flavonoids from propolis [29].

Several studies examine both antioxidant activity and chemical composition, which can vary with time and geographical area of collection. For example, the antioxidant activity of propolis from various areas of China was examined recently. All propolis samples except that from Yunnan had relatively strong antioxidant activity accompanied by high total polyphenol contents [8]. Propolis with strong antioxidant activity contained large amounts of antioxidative compounds, such as caffeic acid, ferulic acid and caffeic acid phenethyl ester. On the other hand, propolis from Yunnan and Hainan had compounds not present in propolis from other areas [8]. Similarly, the antioxidant activities of propolis of various geographic origins, *i.e.*, Argentina, Australia, Brazil, Bulgaria, Chile, China, Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States, and Uzbekistan was compared [30]. EEP from Argentina, Australia, China, Hungary and New Zealand had relatively strong antioxidant activities, and were also correlated with the total polyphenol and flavonoid contents. Propolis with strong antioxidant activity contained antioxidative compounds such as kaempferol and phenethyl caffeate [30].

Recently pollen analysis, total phenols content and antioxidant activity were studied for the first time in Portuguese propolis samples. The antioxidant capacity of propolis extracts was assessed through the scavenging effects on DPPH and reducing power of iron (III)/ ferricyanide complex assays. A concentration- dependent antioxidative capacity was verified in DPPH and reducing power assays. Low values of EC_{50} on DPPH scavenging assay were obtained for Bornes and Fundão propolis (of 6.22μg/ml and 52.00μg/ml, respectively). The high activity of propolis from Bornes could be related with their different pollen composition. The results obtained indicate that Portuguese propolis is an important source of total phenols showing antioxidant properties [31].

1.5.2. Antimicrobial activity

Propolis has been widely used in folk medicine due to its biological and therapeutic activities such as, microbicidal action. In its native application, a primary function of propolis in the hive is to act as a biocide, and may act against invasive bacteria, fungi and even invading larvae [1, 5].

There is increasing scientific and commercial interests, a better understanding of its action will provide a scientific basis for its better therapeutic application in human or veterinary medicine whether it is associated or not with conventional treatments [32, 33]. From the beginning of propolis research, several groups of authors have studied its antimicrobial properties. There are a

number of studies documenting the *in vitro* antimicrobial activity of propolis, its extracts and constituents. The activity is fairly broad spectrum with activities against Gram-positive and Gram-negative bacteria, protozoa, fungi and viruses [1, 2].

1.5.2.1. Antibacterial activity

Due to propolis use in popular medicine, it is necessary to test and prove the efficacy of propolis against pathogenic bacteria that are easily spread like *S. aureus, E.coli* and *Salmonella* [34]. For example, *S. aureus* is widely used in susceptibility tests because it's widely disseminated in the environment. It's usually part of the normal microbial flora in humans and can be found on nasal passages, skin and mucous membranes. Besides, it can cause several infections ranging from localized purulent lesions to systemic infections [34, 35].

Several studies demonstrate that EEP inhibited the growth of various bacteria including strains of *Streptococcus*, *Bacillus*, *Pseudomonas aeruginosa* and *E. coli*. Fuentes and Hernandez (1990) showed that EEP had a pronounced activity against Gram-positive bacteria, including *S. aureus, E. coli, P. aeruginosa, B. subtilis, S. epidermidis* and *Streptococcus* sp (B haemolytic). These results were confirmed later on with the same *E. coli* strain [5]. In table 1.7 are listed some of the results obtained with different propolis extracts on different microganisms.

The effect of propolis against Gram-positive bacteria and yeasts is much greater than that against Gram-negative bacteria [36, 37]. And, although the reported degree and scope of activity among the general categories of susceptible organisms is variable it is, in a sense, markedly similar, with activities generally below 10 mg/ml [1].

Synergism between propolis and antibacterial agents has also been observed. Studies demonstrate a marked synergistic effect of propolis on the anti-bacterial activity of streptomycin and cloxacillin, and a moderate synergistic effect on the anti-bacterial activity of chloramphenicol, cefradine and polymyxis B in culture medium containing a fixed amount of a standard strain of *S.aureus* [2]. For example, the effect of propolis extracts and *Zingiber. officinale* extracts when combined with clarithromycin were evaluated on 25 clinical *H. pylori* isolates. The results showed that the combinations of propolis extract with clarithromycin and *Z. officinale* extract with clarithromycin exhibited improved inhibition of *H. pylori* with synergistic or additive activity [38]. Similarly, a possible synergistic effect between ethanolic extracts of propolis from Brazil and Bulgaria and some antibiotics (Amoxicillin, Ampicillin and Cefalexin) was investigated against *Salmonella Typhi*. Brazilian and Bulgarian propolis showed an antibacterial action, but the sample from Bulgaria was shown to be more efficient. Both samples showed a similar synergistic effect with the antibiotics. Propolis samples showed an important

antibacterial action, as well as, a synergistic effect with antibiotics against *Salmonella Typhi* [33]. Extracts of propolis have been shown to potentiate the effect of certain antibiotics. The antibiotic action against *S. aureus* (various strains) and *E. coli* was increased by the addition of propolis to nutrient medium. The presence of propolis prevented or reduced any gradual build-up in tolerance of *Staphylococci* to antibiotics [5].

Table 1.7 – Propolis extracts and propolis constituents' antibacterial activity.

Substance	**Tested organism**	**Comments/results**	**Ref.**
EEP from two regions of Brazil	*Salmonella enteritidis a*nd *S.typhimurium*	Both propolis samples showed a bactericidal activity against *S. enteritidis* and *S. typhimurium*, showing a remarkable inhibitory effect after 14 hours and bactericidal effect after 24 hours incubation. *Salmonella* growth was only inhibited by higher propolis concentration (10.0% v/v). It was also observed that EEP from Mossoró was more effective than that from Urubici.	32
EEP from Brazil and Bulgaria	*Salmonella* Serovar-*typhimurium* and Serovar *enteritidis*, *S. typhi* (00238) and S. *typhimurium* (13311)	Brazilian and Bulgarian propolis showed an antibacterial action against all *Salmonella* serovars. The minimal inhibitory concentrations (MIC) of propolis were similar, although they were collected in different geographic regions. *Salmonella typhimurium*, isolated from human infection, was more resistant to propolis than *Salmonella enteritidis*.	39
EEP from Bulgaria	*94 H. pylori* strains (clinical isolates)	By the agar-well diffusion method, only 13.8% of the strains exhibited no inhibition by 30µl propolis extract and all isolates were inhibited to some extent by 90µl of the extract per well. The mean diameters of growth inhibition by 30, 60 or 90µl propolis extract or 30µl 96%ethanol per well were 16.8, 19.2, 27.5 and 8.3 mm, respectively. With 90µl propolis extract per well, 69.4% of the strains exhibited large diameters of growth inhibition (>20 mm) versus 26.6% with 30µl per well. With moist propolis discs, inhibition was detected in more strains (92.1%) than with dried discs (78.2%), with mean inhibitory diameters of 18.7 and 13.8 mm, respectively. Bulgarian propolis had a strong and dose-dependent activity against most of the *H. pylori* strains tested.	36
Aqueous and ethanolic extract of propolis and plants	11 *H. pylori* strains (clinical isolates) and *H. pylori* ATCC 43629	The results show a significant *in vitro* effect of plant extracts against *H. pylori.* An inhibitory activity against H. pylori strains was recorded in a large percentage of tested samples. The inhibitory zone diameter obtained by the disc diffusion test for propolis was 12 and 20mm for aqueous and ethanolic extract, respectively. MIC values of ethanolic extracts were from two to four concentration steps lower than the aqueous ones.	38
Methanol extract of *Eucalyptus* propolis and *Castanea* propolis	*S.aureus* ATCC 29213, *Listeria monocytogenes* F 1483 and F 1462, *E.coli* ATCC 25922, *S.typhimurium* ATCC 14028, and yeasts	There was no significant difference between propolis samples in antibacterial activity; however the yeasts were shown to be more sensitive to eucalyptus-propolis. Gram negative bacteria were susceptible to none of the samples tested.	40

(Continuation of Table 1.7)

Substance	Tested organism	Comments/results	Ref.
EEP from Brazil and Bulgaria	*Neisseria meningitidis* BH92/02, *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 25923 and 16504	EEP from Bulgaria was more effective than EEP from Brazil against bacteria, particularly *N. meningitidis* and *S. pneumoniae*. Although with different classes of components, both propolis extracts showed bactericidal activity and it was possible to establish a positive correlation with the high content of flavonoids of the Bulgarian extract.	41
EEP from different regions of Brazil	*S. aureus* ATCC 25923 and *E. coli* ATCC 35218	EEP inhibited the growth of *S.aureus*, with inhibition diameters from 8 to 13mm, but not that of *E. coli*. Flavonoids content was variable, depending on the propolis sample. According to the results, it may be concluded that EEP showed effective action against Gram-positive bacteria, independently on their geographic origin, and a positive correlation between antibacterial activity and flavonoids content.	37
EEP from different regions of Bulgaria	*S.aureus 209 and E.coli WF+, C. albicans 562,* Avian influenza virus A (H7N7)	All samples were active against the fungal and Gram-positive bacterial test strains (with diameters of the inhibitory zone ranging from 11 to 29mm), and most showed antiviral activity. The activities of all samples were similar in spite of the differences in their chemical composition.	16
Constituents of Brazilian propolis	*S. aureus* 209, *E. coli* WF+ and *C.albicans* 562	Three of the major components demonstrated significant antimicrobial activity, with inhibition diameters from 14 to 23mm, 0 to 14mm and 0 to 26mm *for S. aureus, E. coli* and *Candica albicans*, respectively.	12

The difference in antimicrobial activity observed in different studies might be attributed to a difference in virulence of the tested organisms and to a difference in the flavanoids content, in a similar manner to what's described for the antioxidant activity. For example, a study correlated the flavonoid content with activity against *Bacillus subtilis* and that flavonoid content varied considerably with the 38 samples gathered in parts of Croatia with differing climate and vegetation [2]. The efficacy of the flavanoids was succinctly demonstrated by previous studies, showing a difference in efficacy between propolis and a constituent flavonoid (i.e. pinocembrin) somewhere between one- and 10-fold [1]. The antibacterial activity of propolis is reportedly due to flavonoids and aromatic acids and esters present in resin. Galangin, pinocembrin and pinostrobin have been recognized as the most effective flavonoid agents against bacteria. Ferulic and caffeic acid also contributes to bactericidal action of propolis [5]. It was reported that the mechanism of antimicrobial activity was complicated and could be attributed to a synergism between flavonoids, hydroxyacids and sesquiterpenes [5]. Later, the synergistic effect of propolis compounds was studied and the EEP and its fractions were tested for inhibitory activity against periodontitis-causing bacteria. All of the assayed bacterium species were susceptible to propolis extract. The two fractionation methodologies yielded fractions which were active against bacteria, with minimum inhibitory concentrations (MIC) ranging from 64 to 1024 mg/ml. TLC

(thin layer chromatography) and HPLC analyses of the extract and of active fractions showed the presence of phenolic compounds of varied polarity. None of the assayed fractions was more active than the extract, suggesting that the antibacterial activity is probably due to the synergistic effect of several compounds [42].

The antibacterial activity of different fractions of Brazilian propolis was examined towards *S.aureus*, and observed that the antibacterial activity is mainly due to polar phenolic compounds. Later, antibacterial compounds from Brazilian propolis were isolated including four labdane-type diterpenic acids, *i.e*. isocupressic acid, acetylisocupressic acid, imbricatoloic acid and communic acid, together with syringaldehyde [7].

The effects of crude propolis and fractions against *Helicobacter pylori* (the intestinal bacteria known to be associated with gastric ulcer) were studied. The authors found that the ethanol extract had anti-*H. pylori* activity, and from its active fractions p-coumaric acid, 3-prenyl-4-dihydrocinnamoyloxycinnamic acid and artepillin C were isolated as active compounds. Other authors also reported similar results, including effects of some isolated compounds, including labdane-type diterpenes and phenolic compounds that have some degree of anti-*H. pylori activity* [43].

In addition, the method of extraction and the solvent employed for the extraction can also produce variability of results as noted in a study that compared propolis extracts made with water or 40% or 96% ethanol [1]. The same was observed in a study that showed a wide range of anti-microbial activities with an oil preparation; the glycerine solutions show little inhibition of Gram-positive bacteria, whereas the ethanol and propylene glycol solutions show good activity against yeasts [2]. Similarly, it was noted that the ethanolic extracts of propolis were more active than the aqueous extracts; probably the alcohol solubilizes significantly higher amounts of bioactive compounds than the aqueous extraction [44].

The antibacterial, antifungal and antiviral activity of propolis from different geographical origins was examined. All the propolis samples were active against the fungal and gram-positive bacterial test strains, and most showed antiviral activity (against avian influenza virus). The antibacterial activities of these samples were similar, in spite of differences in their chemical composition [16]. In a similar study the antimicrobial activities of German, French and Austrian propolis were evaluated against *S. aureus, E. coli* and *Candida albicans*. German propolis possessed the highest antimicrobial activity against *S. aureus* and *E. coli*, which contain phenylethyl transcaffeate, benzyl ferulate and galangin as major components. Austrian propolis with high contents of pinocembrin, on the other hand, showed the highest activity against *Candida albicans* [7]. The effect of a new variety of propolis from Northeastern Brazil was studied on the growth of mutans *Streptococci* and the antibacterial activity towards oral

pathogens. The results showed that the new variety of propolis was exceptionally effective in all *in vitro* parameters tested against mutans *Streptococci*; biological effects of propolis are likely not to be due solely to flavonoids and (hydroxy) cinnamic acid derivatives [45]. Later, the influence of the seasonal effect on the Brazilian EEP, collected during 6 months was evaluated in terms of antibacterial activity and phenolic composition. The antimicrobial properties were evaluated by MIC and MBC on *S. mutans* Ingbritt 1600 and the profile of chemical composition by UV-visible spectrophotometry, HPLC-RF and GC-MS. Their results demonstrated that the season in which propolis is collected influences its chemical composition, resulting in modifications in its antibacterial activity [46].

Recently, an action mechanism for propolis antibacterial activity has been suggested: it affects the cytoplasmic membrane and inhibits bacterial motility and enzyme activity. Also, propolis exhibits bacteriostatic activity against different bacterial genera and can be bactericidal in high concentrations [36].

1.5.2.2. Antiprotozoan activity

The antiprotozoan activity of propolis was verified in experimental animals infected with *Eimeria magna*, *E. media* and *E. perforans* treated with 3% EEP and other antiprotozoan drugs. The coccidiostatic effect of propolis was higher than other drugs. Propolis preparations were classified as a good coccidiostat against *Chilomonas paramecium* [5]. The effect of EEP on the growth of the protozoan parasite *Giardia lamblia* was also tested *in vitro*. At an EEP concentration of 11.6 mg/ml there was a 98% inhibition effect [5].

Studies also demonstrate failures in pathogens control by propolis with non-efficacious or marginal activity against the parasites *Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis* or *Trypanosoma cruzi, in situ*. For example, the effects of different formulations of propolis were studied on *T.cruzi*-infected mice. They administered up to 5000 mg/kg/day to mice and monitored the parasitaemia kinetics and survival rate but could not find any effect on parasitaemia, survival time and mortality. Activity against *Toxoplasma gondii* and *Trichomonas vaginalis* was evident only after 24hr of incubation with propolis extracts at concentrations of 150mg/ml [1, 7]. Later, the antibacterial activity of four phenolic compounds isolated from Brazilian propolis, i.e. 3-(2,2-dimethyl-8- prenylbenzopyran-6-yl)propenoic acid, 3-prenyl-4-hydroxycinnamic acid, 3,5-diprenyl-4-hydroxycinnamic acid and 2,2-dimethyl-6-carboxyethenyl-2H- 1-benzopyran, was reported against *T.cruzi*[47].

Recently, the effect of Brazilian EEP on *T. cruzi* and its effect on experimental infection of mice were further investigated. The IC_{50}/4 days for inhibition of amastigote proliferation was

8.5±1.8µg/ml, with no damage to the host cells. In epimastigotes EEP induced alterations in reservosomes, Golgi complex and mitochondrion. In trypomastigotes, EEP led to the loss of plasma membrane integrity. The *in vitro* studies indicate that EEP interferes in the functionality of the plasma membrane in trypomastigotes and of reservosomes and mitochondrion in epimastigotes. Acutely infected mice were treated orally with EEP. The extract reduced the parasitemia, although not at significant levels; increased the survival of the animals and did not induce any hepatic, muscular lesion or renal toxicity. Since EEP was not toxic to the animals, it could be assayed in combination with other drugs. They concluded that EEP could be a potential metacyclogenesis blocker, considering its effect on reservosomes, which are an important energy source during parasite differentiation [48].

1.5.2.3. Antifungal activity

It was reported that propolis exhibited an important antifungal activity against *Trichophyton* and *Mycrosporum* in the presence of propylene glycol, which interacts synergistically at a 5% concentration. Furthermore, it was also reported that combinations of some antimycotic drugs with propolis (10%) increased their activity on *Candida albicans* yeasts. The greatest synergistic effect against most strains was obtained when propolis was added to antifungal drugs [5]. Extracts have also been shown to inhibit the elaboration of toxins, *e.g.* ochratoxin A by *Aspergillus sulphureus* [1].

Studies tested 30 propolis samples produced in Cuba against 2 strains of *C.albicans* and later, the antifungal activity of propolis extracts (10% in ethanol) against 17 fungal pathogens was verified. The EEP inhibited *Candida* and all tested dermatophytes [5]. Another study evaluated the antifungal activity of EEP against *C. albicans, C. parapsilosis, C. tropicalis* and *C. guilliermondii*; 98% of fungi samples were sensitive to EEP concentrations of less than 5.0%. It was also observed that in *in vitro* tests, propolis concentrations of 5 or 10% prevented growth of *Trichophyton verrucosum*. The antifungal activity of propolis was also observed in some plant fungi *in vitro* [5].

Similarly, the seasonal effect of Brazilian propolis was tested for antibacterial activity against *Candida tropicalis* and *C.albicans*. There was no significant difference observed between the propolis of different seasons in terms of the antibacterial activity, but all possessed stronger antibacterial activity towards gram-positive bacteria than to gram-negative bacteria; however the constituents were not described [49].

The antifungal effect of ethanolic extract of Turkish propolis treatments in four nonpasteurized fruit juices was evaluated against 6 different yeasts isolated from the

corresponding spoiled juices. These isolated yeasts included: *Candida famata, C. glabrata, C. kefyr, C. pelliculosa, C. parapsilosis* and *Pichia ohmeri*. In this study, the adition of propolis in apple, orange, white grape and mandarin juices ranging from 0.01 to 0.375 mg/ml inhibited the growth of all spoilage yeasts at 25 °C. MIC ranges of propolis were 0.02-0.375, 0.04-0.375, 0.01-0.185 and 0.02-0.185mg/ml in mandarin, apple, orange and white grape juices, respectively. In terms of MIC ranges, propolis showed greater antifungal activity than Na benzoate (positive control). As a result, propolis had significant antimicrobial activity against the yeast isolates from spoiled fruit juices. It was concluded that propolis is worthy to study further as a natural preservative for foods prone to fungal spoilage [50].

1.5.3. Antitumour activity and citotoxicity

Both propolis extracts and its constituents have been studied in recent years for *in vitro* cytotoxic and antitumoural activity by different methods of tissue culture.

The cytostatic activity of propolis ether and butyl alcohol extracts on HeLa cells and human nasopharynx carcinoma cells (KB cells) was examined. The ethereal propolis fraction exhibited the strongest cytostatic activity [1, 5]. Shortly after, an inhibitory concentration (IC_{50}) of 10mg extract/ml was demonstrated against HeLa cells using an alcoholic extract of propolis. In the same study, propolis flavonoids were also tested and HeLa cells were found to be more sensitive to quercetin and rhamnetin, but less sensitive to galangin [5]. Similar work has been reported by others, including experiments with water and ethanolic extracts of propolis [1]. Recently, it was reported that the ethanolic extract of Brazilian red propolis showed cytotoxic activity for HeLa tumour cells with an IC_{50} of 7.45μg/ml [51].

The antitumoural activity of caffeic acid derivatives, *e.g.*, methyl ferulate, methyl acetyl ferulate, methyl acetyl isoferulate and methyl diacetyl caffeate, has also been reported [5]. Caffeic acid phenethyl ester (CAPE), one of the active component propolis has showed significant cytotoxicity towards various tumour cell lines and antitumour activity in several studies [7]. In one of those studies the investigators explored the differential effect on normal and transformed cells with a cell line of Fischer rat embryo fibroblasts (CREF) and transformed by adenovirus serotype 5 (wt3A). After 72 hr and at CAPE concentrations as high as 8 mg/ml, approximately 75% of the CREF cells remained unaffected, yet under the same conditions, the wt3A cells were nearly 90% inhibited. Although the authors did not speculate on a specific mechanism for this difference, they did find that a dose of 10μg/ml of CAPE completely inhibited the incorporation of [^{3}H]thymidine into the DNA of breast carcinoma. Similar effects were observed in the melanoma, colon (HT 29) and renal carcinoma cell lines, but the CAPE

effect on normal fibroblasts and melanocytes was significantly less. They concluded that human tumour cell lines displayed a significantly greater sensitivity to the action of CAPE than analogous normal lines [1, 5].

Because of its simplicity of structure and interesting cytotoxic property several authors further studied the antitumour activity of CAPE and the possible mechanism of its antitumour property. A study reported that CAPE had potent inhibitory effects of CAPE on TPA-induced tumour promotion in mouse skin, together with its effects on the synthesis of DNA, RNA and protein in HeLa cells. The direct relationship between the cytotoxic effects of CAPE and the induction of DNA fragmentation and apoptosis was established through an examination of the toxicity of CAPE towards oncogene-transformed rat embryo fibroblast cells [7]. It has also been demonstrated that CAPE can modulate the redox state of the cells and therefore the sensitivity of the cells to CAPE-induced cell death may be determined by the loss of normal redox state regulation in transformed cells [1].

In vitro tests of extracts of Brazilian propolis on human hepatocellular carcinoma, KB and HeLa cell lines showed that the cytotoxic effects were caused by quercetin, caffeic acid and phenyl ester constituents of propolis [1]. Later, a new clerodane-type diterpene PMS-1 was isolated from propolis also possessing cytotoxicity towards human hepatocellular carcinoma HuH13 cells. HuH13 cells growth was inhibited at 10mg/ml and lethality at 20mg/ml while human. PMS-1 cytotoxicity was also reported against HeLa, KB and rat W3Y cells [1, 7].

A similar compound, artepillin C, also isolated from Brazilian propolis has showed cytotoxic activity. Artepillin C exhibited preferential cytotoxicity against tumour cells in an *in vitro* system. The observed cytotoxicity seemed to be partly attributable to the induction of apoptosis-like DNA fragmentation. The compound showed antitumour activity more effective than 5-fluorouracil (a known anticancer drug) against transplantable human tumour cell lines, in a histoculture drug response assay system. When xenografts of human tumour cells were transplanted into nude mice, the cytotoxic effects of artepillin C were most noticeable towards carcinoma and malignant melanoma. In addition to suppression of tumour growth, an increase in the ratio of CD4/CD8 T cells and in the total number of helper cells was observed. On the basis of these findings, the author concluded that artepillin C activates the immune system and reveals direct antitumour activity [7].

A study further examined the cytotoxicity of nine different propolis samples collected from Brazil, Peru, the Netherlands and China, towards a highly livermetastatic murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells. The methanol extracts of propolis from the Netherlands and China possessed higher cytotoxicity with an ED_{50} value of 3.5 and 3.9μg/ml, respectively, towards murine colon 26-L5 carcinoma cells, while the methanol extract of

propolis from Brazil had varying cytotoxicity. Moreover, 27 compounds, isolated from a methanol extract of Brazilian propolis, were further tested for their cytotoxicity against human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma cells. Some of the phenolic compounds possessed potent cytotoxicity having ED_{50} values less than 10μg/ml [7]. Aditionaly, 13 compounds were isolated from methanolic extract of the Netherlands propolis and were tested for their antiproliferative activity against murine colon 26-L5, murine B16-BL6 melanoma, human HT-1080 fibrosarcoma and human lung A549 adenocarcinoma cell lines. From the isolated compounds the benzyl, phenethyl and cinnamyl caffeates possessed potent antiproliferative activities with EC_{50} values of 0.288, 1.76 and 0.114 mM, respectively, toward colon 26-L5 carcinoma. These caffeates were considered to be active constituents of the Netherlands propolis in their antiproliferative activity, which may be derived from their antioxidative activity [52].

Cytotoxic activity of propolis extracts was also reported in mice bearing Ehrlich carcinoma *in vivo*. Survival rate after EEP treatment was compared with that of bleomycin, each given alone or in combination. The survival rate of the mice at 50 days was 55% after EEP and 40% after bleomycin, while all the mice treated with the EEP + bleomycin combination demonstrated shorter survival than the controls. The authors concluded that the antitumour effect of propolis was due to the flavonoids inhibiting the incorporation of thymidine, uridine and leucine into the carcinoma cells, thus leading to an inhibition of DNA synthesis. The reduced activity of bleomycin and EEP administered simultaneously is attributed to reduced activity of bleomycin in the presence of EEP-containing cytochrome C reductase inhibitors [1, 5]. Similar studies examined the antitumour effects of water-soluble parts of Brazilian propolis in combination with anticancer drugs in mice bearing Ehrlich carcinoma and observed significant inhibitory effects on Ehrlich carcinoma when the water-soluble parts of propolis and its fractions were injected; in some cases disappearance of the tumour was observed [7].

Propolis potential in carcinogenesis and mutagenesis assays was also investigated. The aberrant crypt foci (ACF) assay has been used to evaluate the initiation and promotion steps in chemical carcinogenesis. For example, the effect of EEP on the process of colon carcinogenesis and DNA damage were evaluated in the male Wistar rats using the ACF assay and the comet assay, respectively. For both tests, animals were treated with the colon carcinogen 1,2-dimethylhydrazine (DMH) for 2 weeks in order to induce both DNA damage and ACF. The animals were divided into groups that received EEP at three different doses, either simultaneously or after DMH treatment. The results show that only the intermediate dose (30mg/kg) of propolis, administered after DMH initiation, was significantly associated to a smaller number of aberrant crypts in the distal colon. No effect on DNA damage in peripheral

blood cells, however, was verified by the comet assay. These data suggest that propolis has a protective influence on the process of colon carcinogenesis, suppressing the development of preneoplastic lesions, and probably exerts no protection against the initiation of carcinogenesis [53].

1.5.4. Other biological activities

1.5.4.1. Antiviral activity

In addition to other biocidal properties, propolis and its extracts clearly have viricidal properties as well. The *in vitro* effect of propolis was investigated on several DNA and RNA viruses including herpes simplex type 1 (an acyclovir resistant mutant), herpes simplex type 2, adenovirus type 2, vesicular stomatitis virus and poliovirus type 2. The inhibition of poliovirus propagation was clearly observed through a plaque reduction test and a multistep virus replication assay with a selectivity index equal to 5. At the concentration of 30mg/ml, propolis reduced the titre of herpes viruses by 1000, whereas vesicular stomatitis virus and adenovirus were less susceptible. In addition to its effect on virus multiplication, propolis was also found to exert a virucidal action on the enveloped viruses herpes simplex (HSV) and vesicular stomatitis virus [54].

In virological studies carried out with extracts obtained with various solvents, some fractions of propolis effected the replication of influenza viruses A and B, vaccinia virus and Newcastle disease virus in different biological testing systems. The action of these active fractions was similar both in strain spectrum and in the degree of anti-influenza activity of propolis concentrations from 0.2-3.0 mg/ml [1,5].

A variety of natural products or their derivatives have been considered as potential candidates for the treatment of human immunodeficiency virus type 1 (HIV-1) infection including propolis extracts. The anti-HIV-1 activity of EEP in CD4+ lymphocytes and microglial cell cultures was investigated. Propolis inhibited viral expression in a concentration-dependent manner (maximal suppression of 85 and 98% was observed at 66.6μg/ml propolis in CD4+ and microglial cell cultures, respectively). Similar anti-HIV-1 activity was observed with propolis samples from several geographic regions. The mechanism of propolis antiviral property in CD4+ lymphocytes appeared to involve, in part, inhibition of viral entry. While propolis had an additive antiviral effect on the reverse transcriptase inhibitor zidovudine, it had no noticeable effect on the protease inhibitor indinavir. The results of this *in vitro* study support the need for

clinical trials of propolis or one or more of its components in the treatment of HIV-1 infection [55].

Substances isolated from propolis have also been examined for antiviral activity. For example, the effect of propolis flavonoids (acacetin, kaempferol, chrysine, quercetin and galangin) on the infectivity and replication of some herpes virus, adenovirus, coronavirus and rotavirus strains was analysed. Two of the flavonoids studied, chrysine and kaempferol, were highly active in inhibiting the replications of several herpes viruses, adenoviruses and a rotavirus. The flavonoids acacetin and galangin were not active in the viruses studied even at concentrations 100 times greater than chrysine and kaempferol; and quercetin was the least effective of all [1, 5]. It was also verified that luteolin was more active than quercetin, but remarkably less than caffeic acid, in the inhibition of Amazon parrot herpes virus (strain KS144/70) at range concentration of 12.5-200.0mg/ml. Phenolics such as, caffeic acid were found to have a weak activity against influenza although *vaccinia* and adenovirus were more sensitive than polio and parainfluenza virus [5].

The antiviral activities of constituents of propolis, such as esters of substituted cinnamic acids, have also been studied *in vitro*. One of them, isopentyl ferulate, significantly inhibited the infectious activity of influenza virus A/Hong Kong (H3N2) at 50mg/ml [1, 5]. Similar results were found when the in vitro activity of 3-methylbut-2-enyl caffeate identified in propolis samples was tested against HSV-1. The same synthetic compound showed strong inhibition of HSV-1 growth at a concentration of 25mg/ml. Some authors suggested that the antiviral activity of propolis is due to both the main constituents and the minor components like 3-methylbut-2-enyl caffeate and 3-methylbutyl ferulate [5].

1.5.4.2. Hepatoprotective effect

Several studies have been made on the hepatoprotective effects of propolis on induced liver damage. Propolis exhibits hepatoprotective effects in acute liver damage induced in rats by carbontetrachloride and in mice by paracetamol and allyl alcohol [2, 7]. It is known that hepatic GSH has a protective role against chemically-induced cellular injury. GSH is one of the most important anti-oxidant molecules of the liver and at physiological concentrations contributes to the maintenance of the normal redox state of cells. Propolis is able to reverse the depletion of GSH induced by paracetamol in mice and thereby prevent cell death [2].

Liver injury induced by carbon tetrachloride (CCl_4) is the best characterized system of xenobiotics induced hepatotoxicity. The protective effect of EEP on hepatic CYP 2E1 activity, oxidative stress and ultrastructure was examined against CCl_4 induced toxicity. Intraperitoneal

injection of CCl_4 (1,5ml/kg) induced hepatotoxicity after 24h of its administration that was associated with elevated malonyldialdehyde (index of lipid peroxidation), lactate dehydrogenase and c-glutamyl transpeptidase release (index of a cytotoxic effect). Hepatic microsomal drug metabolizing enzymes of CYP 2E1 showed sharp depletion after CCl_4 exposure. The toxic effect of CCl_4 was evident on CYP 2E1 activity by increased hexobarbitone induced sleep time and bromosulphalein retention. EEP showed significant improvement in the activity of both enzymes and suppressed toxicant induced increase in sleep time and bromosulphalein retention. Choleretic activity of liver did not show any sign of toxicity after propolis treatment at a dose of 200mg/kg. Histopathological evaluation of the liver revealed that propolis reduced the incidence of liver lesions including hepatocyte swelling and lymphocytic infiltrations induced by CCl_4. Electron microscopic observations also showed improvement in ultrastructure of liver and substantiated recovery in biochemical parameters. Protective activity of propolis at 200mg/kg dose was statistically compared with positive control silymarin (50mg/kg), a known hepatoprotective drug. So, they concluded that propolis may play hepatoprotective role via improved CYP 2E1 activity and reduced oxidative stress in living system [56].

The hepatoprotective effect of the oral administration of propolis water extract (PWE) was also evaluated in the D-galactosamine (D-GalN)/Lipopolysaccharide (LPS)-induced liver injury model in mice. In the control group, it was observed that the alanine transaminase (ALT) level rapidly increased 8h after administration of D-GalN/LPS, while ALT levels in the 200 and 100mg/kg, p.o. PWE-treated groups were only slightly increased. PWE also showed significant hepatoprotective activity against CCl_4-induced liver cell injury in cultured rat hepatocytes. The fractionation and chemical analysis, guided by *in vitro* hepatoprotective activity (CCl_4-induced liver cell injury in cultured rat hepatocytes), led to the isolation of four dicaffeoylquinic acid derivatives from PWE, i.e. methyl 3,4-di-Ocaffeoylquinate, 3,4-di-O-caffeoylquinic acid, methyl 4,5-di-O-caffeoylquinate and 3,5-di-O-caffeoylquinicacid. All these dicaffeoylquinic acid derivatives possessed a significant hepatocyte protective activity against CCl_4-induced cell injury. Similarly, Cuban propolis was also reported to have a hepatoprotective effect against D-GalN-induced hepatitis in rats [7].

Besides the *in vivo* model, it has also been observed a dose-dependent hepatoprotective effect of aqueous propolis extract on isolated rat hepatocytes against CCl_4-toxicity in vitro. Later, the hepatocyte protective effect of 24 constituents of a methanol extract of Brazilian propolis was examined on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes to find the active principle of the alcohol extract. Of the tested compounds the flavonoids 3,5,7-trihydroxy-4'-methoxyflavanol, betuletol , kaempferide and ermanin were found to possess a potent inhibitory effect with IC_{50} values less than 25μM. Most of the tested compounds

possessed stronger hepatocyte protective effects than silibinin (IC_{50}, 39.6μM), a clinically used drug, on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes [43].

These findings suggest that both aqueous and alcohol extracts of propolis possess hepatoprotective effects on both chemically (CCl_4, paracetamol and D-GalN) as well as, immunologically (D-GalN/LPS)-induced liver injury models. The chemically induced liver injuries result from plasma membrane perturbation due to the generation of cellular radicals or the impairment of membrane component synthesis. Thus, free radical scavengers should be probable candidates as antihepatotoxic agents in chemically induced liver injury models. The antioxidative properties of propolis and its phenolic compounds including flavonoids may play an important role in their potent hepatoprotective activity in chemically induced liver injury models [43].

1.5.4.3. Anti-inflamatory effect

One of the traditional medicinal properties of propolis is an anti-inflammatory effect, and propolis is commonly used for the treatment of some skin inflammatory diseases. A significant reduction of acute inflammation provoked by zymosan in mice after the oral application of water soluble derivatives of propolis was observed at a dose of 150mg/kg. Later, the anti-inflammatory activity of propolis and 19 phenolic compounds was also studied. The author evaluated the luminol-enhanced chemiluminescence produced through scavenging of free radicals. It was observed that the EEP showed maximal inhibition (92%) at a concentration of 25μg/ml. CAPE abolished the chemiluminescence completely at a concentration of 10μM, while three flavone derivatives kaempferide, kaempferol and galangin diminished this chemiluminescence by 73%–93% at the same concentration. The remaining phenolic components showed varying degrees of inhibition of 5%–98% at a 100μM concentration. The anti-inflammatory activity of flavonoids and CAPE is mainly due to their antioxidative activity [7].

The anti-inflammatory activity of Cuban red propolis together with antipsoriatic and analgesic effects was evaluated. The ethanol extract at 50mg/kg showed significant anti-inflammatory activity in the cotton-pellet granuloma assay in rats. The propolis extract significantly inhibited the development of hind paw oedema induced by carrageenin in rats at 100mg/kg oral dose and reduced the Croton oilinduced ear oedema in mice, an effect equal to that of indomethacin [7]. The *in vivo* effect of dietary propolis and propolis components, *i.e.* CAPE, caffeic acid, quercetin and naringenin, was studied on arachidonic acid metabolism. The ethanol extract of propolis was found to suppress prostaglandin and leukotriene generation by murine peritoneal macrophages *in vitro* and during zymosan-induced acute inflammation *in vivo*.

Moreover, propolis significantly suppressed the lipoxygenase pathway of arachidonic acid metabolism during inflammation and CAPE was the most potent modulator of the arachidonic acid cascade among the tested propolis constituents [57].

The anti-inflammatory activity of 14 commercial ethanol extracts of propolis was also reported; using a mouse ear inflammation model induced by arachidonic acid and observed varying degrees of anti-inflammatory activity. The 20% and 40% propolis (95% ethanol) solution, which was prepared by the authors showed dose-dependent antiinflammatory activity. Similarly, the anti-inflammatory effect of propolis in alkali-injured rabbit eyes was studied and found that propolis lowered the inflammatory cell infiltration as potently as dexamethasone [7].

Propolis also exhibits anti-inflammatory effects against acute and chronic models of inflammation (formaldehyde and adjuvant-induced arthritis, carrageenan- and PGE2-induced paw oedema). Recently, it was demonstrated that propolis inhibits in a concentration-dependent manner COX activity from lung homogenates of saline or LPS-treated rats. Among the compounds tested, only CAPE and galangin contributed to the anti-inflammatory activity of propolis; however, the contribution of CAPE was greater [2].

1.5.4.4. Effects on the immune response and propolis allergies

The list of applications of propolis and its extracts is nearly endless and many are described above. As a result of this wide utilization of propolis, reports of allergic reactions have been identified for nearly all occupations and all parts of the body [1]. For example, reports of affected occupations include beekeepers, artists, housewives, honey extractors, a tailor, a physician and an engineer. Reported affected parts of the body include, but are not limited to, the hands, forearms, face, neck, perioral region, feet, eyelids, external ear, vulva and penis. There are also reports of pets affected as the result of owners using propolis-containing home remedies [1].

Although poplar bud constituents are probably responsible for allergy to propolis, there was reports of subjects who, although responsive to patch testing with propolis, beeswax and balsam of Peru, were negative to cinnamic acid, grass pollens and trees including poplar and others known to be sources of propolis [1].

It has been reported that the primary allergen in propolis is LB-1; wich consists of a mixture of 3-methyl-2-butenyl caffeate (54%), 3-methyl-3-butenyl caffeate (28%), 2-methyl-2-butenyl caffeate (4%), phenylethyl caffeate (8%), caffeic acid (1%) and benzyl caffeate (1%). The majority-held opinion is, however, that LB-1 is 1,1-dimethylallyl caffeic acid ester. Attemptes to identify the specific allergen and to determine whether there was a true cross-reaction or a pseudo cross-reaction have been made. The investigators first isolated 1,1-

dimethylallylcaffeic acid (LB-1) ester from the buds of *Populus nigra* L. They determined the threshold of irritation in guinea pigs via open epicutaneous application of three different dilutions of propolis (20%, 10%, 1%), and (LB-1) (10%, 3%, 1%) dissolved in acetone onto the clipped and shaved flank of guinea pigs. They report the threshold of irritation for propolis was found to be higher than 20% and for LB-1 to between 3 and 10%. To determine sensitivity and specificity, the animals were given propolis or LB-1 in Freund's Complete Adjuvant. Challenge was 11 days after induction using open epicutaneous elicitation by application of 0,05ml of subirritant doses of propolis and LB-1 on the clipped and shaved flanks of the sensitized animals. The reactions were read at 24, 48 and 72h. The results clearly demonstrated that propolis and its constituent LB-1 are both strong contact sensitizers. At a 1% concentration, the mean response of propolis was 2.6 and of LB-1 3.0 at the 72h reading. Challenge with LB-1 and poplar bud extracts on propolis-sensitive guinea pigs produced reactions as well and were as strong as propolis itself. The authors reasoned that these responses could not be regarded as cross-reactions as the responsible sensitizer, LB-1, found in propolis, is a constituent derived from the poplar buds. Thus, in most cases of propolis allergy, the poplar bud constituent 1,1-dimethylallyl caffeic acid ester, must be considered as the responsible agent [1].

To determine the effect in humans, nine patients who were sensitive to propolis were patch tested with propolis (10% in white petrolatum), poplar bud extracts (1%), and LB-1 (1%). In some cases the flavonoid tectochrysin was tested (1% in petrolatum) as well. Application was carried out on the backs of the patients and read after 24 and 72hr. In eight out of nine patients, LB-1 was positive at 2+ or greater at 72hr. Balsam of Peru, included in the standard series, and only gave a positive response in two out of nine. Positive reaction to tectochrysin (from poplar buds) was seen in three out of five patients [1].

At least a part of the key to the question of pseudo cross-reactivity to propolis may lie in the immunostimulatory effects of propolis reported by a number of investigators. For example, the seasonal effect of the immunomodulatory action of propolis on antibody production in bovine serum albumin (BSA)-immunized rats was studied. They compared the effect of Brazilian and Bulgarian propolis, some isolated compounds and *Baccharis* extract on anti-BSA antibody levels and concluded that propolis stimulates antibody production, independently of the season and geographic origin. Caffeic acid, quercetin and *Baccharis* extract had no effect on antibody production, although the importance of isolated compounds is well reported in other biological assays. Propolis action is a consequence of plant-derived products with synergic effects, while isolated compounds or extracts from its plant sources had no effect in this assay [58]. Propolis also exhibits immunostimulatory and immunomodulatory effects *in vitro* on macrophages, while *in vivo* it increases the ratio of CD4/CD8T cells in mice [2, 59].

Enhancement of the immune response does not appear, however, to be through an enhanced activation of complement. That is, when human or guinea pig complement is treated with a water-soluble derivative (WSD) of propolis in vitro, C3 functional activity is impaired, as indicated by suppression of complement-mediated haemolysis. This suppression of immune (anti-inflammatory response) was also seen in vivo with mice when administered 150mg/kg of WSD, intravenously or intraperitoneally. The WSD also influenced the process of acute inflammation provoked by zymosan in mice, regardless of route, although there was a delay in onset of difference when given by the ip route [1].

Many other biological and pharmacological properties of propolis have been described by various authors, including regeneration of cartilaginous tissue, bone tissue and dental pulp, anaesthetic activity, increasing the number of plaque-forming cells in the spleen of populations of immunized males, choleretic and antiulcer action *in vitro*, anticaries in rats, protection agent against gamma irradiation in mice, anti-leishmaniosis in hamster, and inhibition of dihydrofolate reductase activity [2,5]. In addition, CAPE, one of the active compounds in propolis, has been found effective in suppressing posterior capsule opacification in pigmented rabbits, protection of the spinal cord from ischaemia-reperfusion injury in rabbits and prevented reperfusion injuries in rats by eliminating oxygen radicals and inhibiting polymorphonuclear leukocyte infiltration [7, 60].

1.6. Propolis applications: Historical and current uses

There is a long history of use of propolis since the ancient times: Egyptians made use of the anti-putrefactive properties of propolis and used it to embalm cadavers, Greek and Roman physicians, Aristoteles, Dioscorides, Pliny and Galen also recognized propolis medicinal properties and in the seventeenth century the London pharmacopoeias listed propolis as an official drug. Between the seventeenth and twentieth century the drug became very popular in Europe on account of its anti-bacterial activity [2].

Because of propolis reputed properties (antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, anti-inflammatory, anaesthetic and antioxidant) the list of preparations and uses is nearly endless. Propolis preparations can be found in the form of capsules (either in pure form or combined with aloe gel and *rosa canina* or pollen), as an extract (hydroalcholic or glycolic), as a mouthwash (combined with melissa, sage, mallow and/or rosemary), in throat lozenges, creams, and in powder form (to be used in gargles or for internal use once dissolved in

water). It is also available commercially as purified product in which the wax has been removed [1, 2].

The current applications of propolis include:

- Treatment of dermatological diseases where propolis is used as an antiseptic, antimycotic, bacteriostatic, antiviral and fungistatic agent. It has been used in wound healing, tissue regeneration, treatment of burns, neurodermatitis, leg ulcers, psoriasis, herpes simplex and genitalis, activity against dermatophytes, pulp gangrene and as an astringent [1,5];
- Treatment for rheumatism and sprains [1];
- Treatment of otorhinolaryngologic (ORL) diseases with propolis extracts were described for subjects suffering of external otitis, chronic mesotympanic otitis and tympan perforation with positive therapeutic results in most cases. Propolis effects in other ORL diseases were also reported: acute inflammations of the ear, treatment of mesotympanitis, pharyngitis, tuberculosis, chronic bronchitis, rhinopharyngolaryngitis, pharyngolaryngitis, vasomotor catarrh treatment and rhinitis [5];
- Treatment of stomatological diseases where propolis is used in the therapies of acute colitis, chronic colitis, acute gastric ulcers, and acute duodenal ulcers [5];
- Treatment in odontology where propolis propoperties as an anaesthetic and in tissue regeneration are used. It is used in toothpaste and mouthwash preparations treating gingivitis, cheilitis, periodontitis, plaque, stomatitis and buccal affections [1,5].

A B C

Figure 1.2 - Examples of products containing propolis, A) candy, B) tooth paste and C) gelatinous capsules, propolis extract and spray [61-63].

Additionaly, it has also found in pharmaceutical and cosmetic products such as face creams, ointments, lotions and solutions. It is also a constituent of health foods and is marketed in tablets, powder and chewing gum [1, 5].

Non-personal product or medicinal applications include propolis use as an ingredient in the varnish of stringed musical instruments, such as violins, and in the repair of accordions. It

has been proposed as a chemical preservative in meat products and has been tested for bioactivity against larvae of the greater wax moth (*Galleria mellonella* L.), a common apiary pest, although little effect was noted [1].

1.7. Objectives

The objective of this work was to evaluate the influence of collection time and region, and extraction method on the biological activites (antimicrobial, antioxidant and citotoxicity) of propolis from Algarve.

The biological activities analysed in this study were the antimicrobial activity against Gram-positive (*Staphylococcus aureus* CFSA2, *Listeria monocytogenes* EGD, *Listeria monocytogenes* strains C882 and T8, *Streptococcus pneumonia* D39) and Gram-negative (*Salmonella enterica* subspecie *enterica* serovar *thyphimurium* ATCC 14028*, Helicobacter pylori* strains J99 and 26695, and *Haemophilus influenza* TD-4) microorganisms, the enzymatic antioxidant activity (SOD, CAT and GP activity) and the citotoxicity of propolis extracts in Caco-2 cell lines.

2. Materials and Methods

2.1. Reagents

2.1.1. Biological materials

2.1.1.1. Propolis

In this study we used propolis samples obtained from four different locations: Barrocal Norte (B.N.) Arrodeios, B.N. Pé da Serra, Barrocal Sul (B.S.) Arneijoafra and Transição Norte (T. N.) Madeira in the Salir region (see Fig. 2.1) and samples were collected at two different times, winter and spring. The samples were collected manually and stored in a dark cabinet at room temperature or frozen at -80ºC for later enzyme extraction.

Figure 2.1 – Approximate samples collection locations in the Salir region: 1- Transição Norte Madeira, 2- B. S. Arneijoafra, 3- B. N. Pé da Serra and 4- B. S. Arrodeios (adapted from ref. 64).

2.1.1.2. Microorganisms

The bacteria used in this study included five Gram-positive namely *Staphylococcus aureus* CFSA2, *Listeria monocytogenes* EGD, *Listeria monocytogenes* strains C882 and T8, *Streptococcus pneumoniae* D39, and four Gram-negative, *Salmonella enterica* subspecie *enterica* serovar *thyphimurium* ATCC 14028*, Helicobacter pylori* strains J99 and 26695, and *Haemophilus influenza* TD-4. In table 2.1 are listed the provenance of these bacteria.

Table 2.1 - List of the microorganisms used.

Microorganism	Origin	Source
Staphylococcus aureus CFSA2	An environmental isolate	Microbiology Laboratory, Faculdade de Engenharia de Recursos Naturais (FERN), University of Algarve
Listeria monocytogenes EGD	A clinical isolate	Trudeau Institute, USA
Listeria monocytogenes C882	Isolated from cheese	Microbiology Laboratory, FERN, University of Algarve
Listeria monocytogenes T8	Isolated from cheese-making dairy	Microbiology Laboratory, FERN, University of Algarve
Streptococcus pneumoniae D39	Strain from serotype 2, cps2	University of Leicester, Dept. Infection, Immunity and Inflammation, UK
Salmonella enterica subspecie *enterica* serovar *thyphimurium* ATCC 14028	Isolated from animal tissue (chicken heart and liver 4 weeks old)	INETI-DTIA, Amadora, Portugal
Helicobacter pylori J99	Isolated from the intestine of a patient with a duodenal ulcer	National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal
Helicobacter pylori 26695	Isolated from the stomach of a patient with gastritis	National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal
Haemophilus influenza TD-4	A clinical isolate from the sputum of a patient	German collection of microorganisms and cell culture (DSMZ)

2.1.1.3. Cell lines

In this study, Caco-2 cell line was used. The Caco-2 cell line is an immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells that is often used a cell monolayer absorption model.

2.1.2. Other reagents

The rest of the reagents used were all appropriate for microbiology, cell culture and/or enzymatic assays, and all solutions were prepared in distilled water, except when indicated otherwise. All chemicals used were pro analysis grade.

All culture media, solutions and materials used in the microbial and cell culture assays were sterilized by autoclaving at 121ºC for 20 minutes or by filtration with a 0.2µm diameter pore filter (polyethersulfone membrane, VWR International). The biological residues and materials used were all inactivated by autoclaving at 121ºC for 30 minutes or by washing with a freshly prepared 10% (v/v) bleach solution.

2.2. Methods

2.2.1. Preparation of propolis extracts

In this study we tested three types of propolis extractions: aqueous, ethanolic and ethanolic, gently given by S. Nunes. The aqueous and methanolic extracts were prepared as described previously [65]. Briefly, for the aqueous extract 1g of propolis was cut into small pieces and extracted with 10ml of water at 80ºC for 3h, with agitation at 140rpm. Following this extraction, this mixture was then centrifuged 5000g for 10min (Beckman J2-MC centrifuge) and the supernatant was collected to give the water extract. The pellet was further extracted with 10ml of methanol under reflux for 3h. Following this extraction, this mixture was then centrifuged as described above and the supernatant was collected to give the methanolic extract [65].

The ethanolic extract was prepared as described previously [66]. So, for the ethanolic extract 1g of propolis was cut and dissolved in 10ml of 70% ethanol in a 50ml flask and left for 96h at 37ºC in an orbital agitator at 200rpm. It was than filtered (Scheicher&Schuell MicroScience GmbH, 20µm diameter pore filter) [66].

The propolis extracts were then diluted in *n*-propanol and were used for the antimicrobial and cytotoxicity assays.

2.2.2. Preparation of propolis extracts for enzymatic assays

For the enzyme extraction, 500mg of propolis were macerated with liquid nitrogen. Then, we added 2ml of 100mM sodium phosphate buffer, pH 7.8 supplemented with 1% (w/v) PVP,

0.1mM EDTA and 0,2% (v/v) Triton X-100 to the macerated propolis and centrifuged it for 10min at 10,000rpm, under 4ºC (Mikro 200R Hettich centrifuge).

After the centrifugation the supernatant was collected and centrifuged for a second time. The supernatant from this second centrifugation constituted our extract and was used for enzyme activity and protein determinations.

2.2.3. Bacterial cultures

2.2.3.1. Culture media

Bacterial cultures were kept at -80ºC and were recovered prior to each assay. The *H. pylori* strains were recovered from preservation at -80ºC in Columbia blood agar (CBA) medium supplemented with 10% blood and vancomycin (10µl/ml). For the bacteria S. *pneumoniae* and *H. influenza* the same culture medium was used but supplemented with 5% instead of 10% blood. The *Listeria* and *Salmonella* strains were recovered in tryptone soya agar (TSA) and *S. aureus* were recovered in brain heart infusion (BHI) medium. In table 2.2 the different culture media' composition are summarized and in table 2.3 the media used for each microorganism are listed.

Incubation of *H. pylori*, *S. pneumoniae* and *H. influenza* cultures was done at 37ºC, 5% CO_2 for 24 to 48h and *S. aureus* and *Salmonella* cultures was done 37ºC for 24h. Incubation of *Listeria* cultures was done at 30ºC for 24h.

Table 2.2 – Bacterial media and their composition.

Medium[1]	**Used concentration**	**Composition**
Brain heart infusion (BHI)	37.0g/ml	Calf brain infusion solids 12.5g/l; Beef heart infusion solids 5.0g/l; Proteose peptone 10.0g/l; Glucose 2.0g/l; Sodium chloride 5.0g/l; Di-sodium phosphate 2.5g/l
Tryptone soya broth (TSB)	30.0g/l	Pancreatic digest of casein 17g/l; Papaic digest of soybean meal 3.0g/l; Sodium chloride 5.0g/l; Di-basic potassium phosphate 2.5g/l; Glucose 2.5g/l
Tryptone soya agar (TSA)	30.0g/l	Pancreatic digest of casein 17g/l; Papaic digest of soybean meal 3.0g/l; Sodium chloride 5.0g/l; Di-basic potassium phosphate 2.5g/l; Glucose 2.5g/l; Agar 15g/l
Columbia blood agar base (CBA)	39.0g/l	Special peptone 23.0g/l; Starch 1.0g/l; Sodium chloride 5.0g/l; Agar 10g/l

[1]All media were purchased from Oxoid.

Table 2.3 – Growth media.

Microrganism	Solid medium	Liquid medium
Staphylococcus aureus CFSA2	BHI + agar	BHI
Salmonella enterica serovar *thyphimurium* ATCC 14028	TSA	TSB
Listeria monocytogenes EGD	TSA	BHI
Listeria monocytogenes T8	TSA	BHI
Listeria monocytogenes C882	TSA	BHI
Helicobacter pylori J99	CBA + 10% blood	BHI
Helicobacter pylori 26695	CBA + 10 % blood	BHI
Streptococcus pneumoniae D9	CBA + 5% blood	BHI
Heamophilus influenza TD-4	CBA + 5% blood	BHI

2.2.3.2. Antibiotics

When appropriate, the antibiotics used were cloranphenicol, penicillin G, optochin and vancomycin. The first three were used as positive controls in discs with concentrations of 30µg, 10µg and 5µg, respectively, from Oxoid.

Vancomycin was used in the selection medium for *H. pylori* growth at a concentration of 10µl/ml and the stock solution was prepared in MilliQ water at concentration 1000 times higher than the final concentration in the medium (10mg/ml).

2.2.4. Determination of the antimicrobial potential of propolis extracts through agar diffusion method

The antimicrobial activity was determined by the agar diffusion method as previously described [67, 68].

For *Listeria* strains, *Salmonella, S. aureus* and *S. pneumoniae* a loop full of bacterial culture from each of the triplicate plates was used to inoculate 10ml of the appropriate liquid medium (see table 2.3). This pre-inoculum was left to grow overnight (approximately 14-16h), at 37ºC (except for *Listeria* that was incubated at 30ºC). In the next morning, small volumes of each one of these inocula were used to inoculate another 10ml of appropriate broth and incubated for a further 2-4h at 37ºC (or 30ºC), in order for the culture growth to reach the exponential

phase, which was verified through the measurement of the optical density of the culture at 600nm using a spectrophotometer Pharmacia Biotech, Novaspec II.

For the *H. pylori* strains and *H. influenza* two Petri plates with the appropriate solid medium (see Table 2.3) were inoculated using a sterile swab and these were incubated for 24h at 37ºC, 5% CO_2 (except for *H. pylori* that was incubated for 48h). One of the plates was used to replace the stock cultures and the other one was used to inoculate three other plates with the appropriate solid medium, constituting the triplicates. After the incubation time, each of the triplicate Petri plates was washed with BHI and the all the biomass at the Petri plate's surface was removed and transferred to a sterile *eppendorff* tube. Then it was centrifuged at 3500rpm for 5min (centrifuge Mikro 22R, Hettich) and the supernatant removed. Then, the bacterial pellet was washed twice with Ringer solution and the bacterial suspensions were adjusted to the appropriate turbidity (McFarland turbidity standard 0.5-1, BioMérieux). These were prepared with Ringer solution (sodium chloride 2.25g/L. potassium choride 0.105g/L. calcium chloride 0.12g/L. sodium hydrogen carbonate 0.05g/L, BioKar Diagnostics)

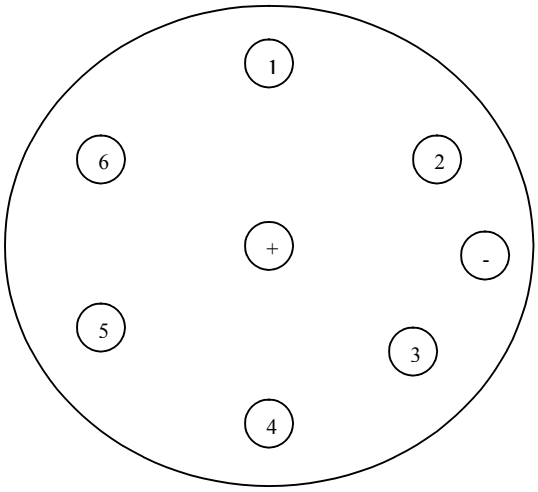

Figure 2.2 – Schematic representation of the plates with the discs, (+) positive control, (-) negative control and (1-6) samples.

Next, Petri plates containing the appropriate solid medium (see Table 2.3) were inoculated with 100µl of the previously prepared bacterial suspensions. Sterile filter paper discs (6mm, Oxoid) containing 3µl of diluted propolis extract (1:50) in *n*-propanol were applied or 5, 10, 15 and 20µl of diluted propolis extract (1:10) in *n*-propanol, sterile *n*-propanol (used as negative control) and 30µg of the antibiotic chloramphenicol or 10µg of penicylin G per disc (used as positive control) were distributed across the inoculated plates (see Fig. 2.2 and 2.3). The

diameter of the inhibition zone (mm) was measured after incubation for 24-48h at 37ºC (except for *Listeria* that was incubated at 30ºC).

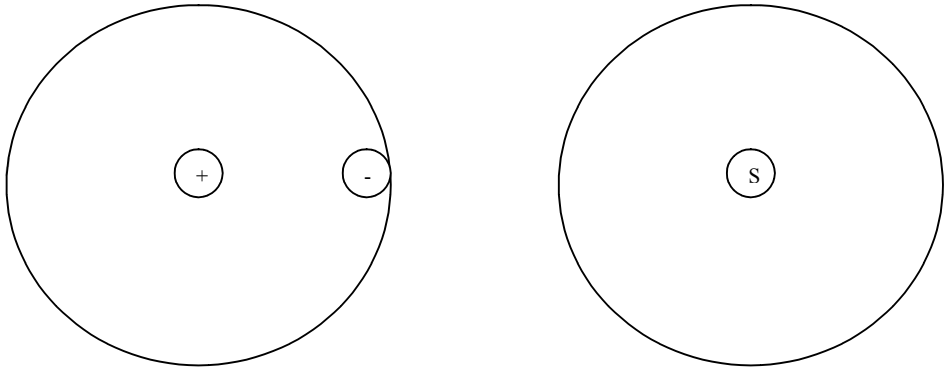

Figure 2.3 – Schematic representation of the plates with the discs, (+) positive control, (-) negative control and (S) samples.

2.2.5. Animal cell cultures

2.2.5.1. Maintenance and propagation of the cell line

The cell line was grown in the appropriate medium (see table 2.3) in T-flasks at 37ºC with 5% CO_2 in a humid atmosphere (Sanyo CO_2 incubator MCO-18AIC).

After the cells had reached a confluence degree of 70-80% they were subcloned. For the cell line subclonning the culture medium was removed and the cell monolayer was washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2mM potassium phosphate monobasic and a pH of 7.4). Next the cells were covered with a 0.25% trypsin/EDTA solution (Invitrogen) for 5min. After verifying the cell detachment complete culture medium was added and the cell culture was split into new T-flasks, and the volume was completed with culture medium.

Table 2.4 – Culture medium used in for animal cell cultures.

Cell line	Culture medium
Caco-2	Dulbecco's modified essential medium (DMEM, Invitrogen), 15% fetal bovine serum (FBS, Invitrogen), 1% L-gluthamine (200mM, Invitrogen), streptomycin (100µl/ml) and penicillin G (100U/ml)

2.2.5.2. Recovery and criopreservation of the cell lines

It is important to keep a stock of cell lines to avoid the occurrence of senescence or mutation, and for that we used criopreservation.

Prior to the criopreservation the cell lines were grown to a confluence degree of 80-90% and were detached from the T-flask as described previously. The obtained cell cultures were then centrifuged at 300×g for 5min (centrifuge Heraerus instruments, Megafuge 1.0R) and cellular pellet was resuspended in cold criopreservation solution (Cell culture freezing medium, Gibco, Invitrogen) that consisted in completed culture medium supplemented with 10% dimethyl sulphoxide (DMSO). The cell line suspension was then distributed to previously labelled criopreservation tubes that were gradually frozen overnight. Afterwards, the tubes containing the cells were kept at -80ºC.

The cell defrost was done very quickly by placing the criopreservation tubes in a water bath at 37ºC for 3 to 5min. Next, complete medium also at 37ºC was added and it was centrifuged at 300×g for 5min. After removing the supernatant more complete medium was added and the cells were transferred into a T-flask that was kept in the incubator a 37ºC with 5% CO_2. Cellular growth was verified every 24h using an inverted phase microscope and the culture medium was substituted after 24h of the recovery process.

2.2.6. Determination of citotoxicity of propolis extracts through the colorimetric MTT assay

The effect of propolis extracts in the *in vitro* growth of the cell lines was determined by the colorimetric MTT (3-4,5-dimethylthiazol-2-il)2,5-diphenyltetrazoilium bromide) assay. This method allows to evaluate indirectly the cellular viability by measuring the mitochondrial desidrogenase activity. It is based on the capability of live cells to reduce the yellow MTT salt into a purple product, formazan. The observed colour change is correlated to the degree of metabolic activity of the cells allowing us to estimate the cellular viability.

The activity of the selected propolis extracts in the cell lines was analysed regarding viability parameters. The propolis extracts dilutions were done using culture medium without phenol red, because it can seriously affect the obtained results. The volumes used were 1, 5, 10, 15 and 20µl for 200µl of medium and the incubation periods were 1, 4 and 24 hours. As controls, culture medium, culture medium with *n*-propanol and water, culture medium with *n*-propanol and 70% ethanol and culture medium with 5% hydrogen peroxide were used.

For this assay the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen) was used. The procedure was done according to the manufacturer instructions.

After growing the cell cultures to confluence, the cells were trypsinized and then complete culture medium without phenol red was added. The cell numbers were counted and the cell suspension was diluted till a concentration of 5-10×10^3 cells/ml. The cell suspension was then distributed to 96 well microplates and was incubated at 37ºC with 5% CO_2 for 24 to 48h. Following this incubation time, the culture medium was replaced with medium containing the propolis extracts and the cells were incubated for 1, 4 and 24h.

After the defined incubation times in the presence of the propolis extracts, the culture medium was removed and 100µl of fresh medium and 10µl of MTT dissolved in PBS were added. The cells were incubated for 4h at 37ºC with 5% CO_2. Then, 85µl of the medium were removed and 50µl of DMSO were added; it was mixed with the remaining medium using the pipette and it incubated for 10min at 37ºC with 5% CO_2. The optical density was measured at 540nm in a microplate reader (Infinite 200, Tecan).

2.2.7. Enzymatic antioxidant activity study

2.2.7.1. Catalase activity determination

Catalase activity was determined by monitoring the decrease in absorbance at 240nm due to H_2O_2 (ε=39.4$M^{-1}cm^{-1}$) consumption at 25ºC [69]. The enzymatic activity was calculated trough the slope determination from the absorbances read at 240nm versus time. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyses the reduction of 1µmol H_2O_2 per minute (see Eq. 4).

Table 2.5 - Volumes and concentrations in the reaction mix for the catalase activity.

Solutions	Assay	Blank	Final concentration
100mM Sodium phosphate buffer pH 7.8 with 1% (w/v) PVP, 0.1 mM EDTA and 0.2% (v/v) Triton X-100	800µl	850µl	80mM
40 mM H_2O_2	150µl	150µl	6mM
Propolis extract	50µl diluted extract 1:5	-	-

In a quartzo *cuvette* sodium phosphate buffer, H_2O_2 and propolis extracts were added in the quantities indicated in Table 2.5. The reaction was initiated after the addition of H_2O_2 and the absorbance at 240nm was measured in intervals of 10s for a period of 200s, using a spectrophotometer UV-160A Shimazu. The blank assay was prepared by substituting the propolis extract with sodium phosphate buffer and each assay was done in triplicates.

2.2.7.2. Superoxide dismutase activity determination

Superoxide dismutase (SOD) activity determination was based on the method of Beauchamp and Fridovich [70] which measures the percentage inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) caused by the presence of SOD. The enzymatic activity was calculated trough the slope determination from the absorbances read at 560nm versus time. With this method, photoreduced riboflavin produces $O_2^{\cdot-}$ radicals that reduce NBT to an unsoluble form, recognized by the appearance of a pink coloration in the solution.

One unit of enzymatic activity (U) was defined as the amount of enzyme that inhibits 50% of the reaction (Fig. 2.4).

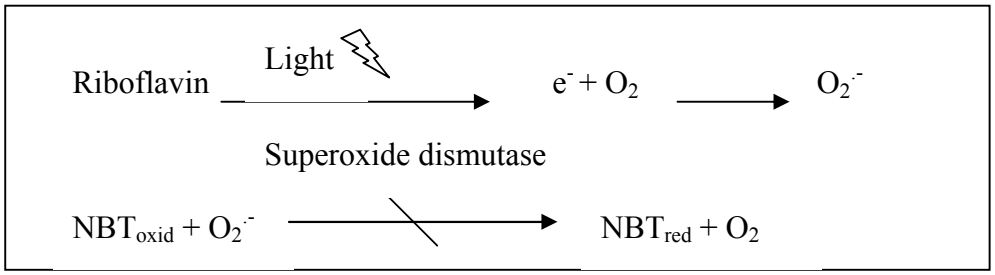

Figure 2.4 – Schematic representation of the photochemical reduction of nitroblue tetrazolium (NBT) caused by the presence of SOD used in it's activity determination.

In a test tube sodium phosphate buffer, EDTA, NBT, methionine, propolis extracts and riboflavin were added in the quantities indicated in Table 2.6. The reaction was initiated after the addition of riboflavin and the test tubes were placed in a water bath at 25ºC, with agitation and under the direct incidence of a fluorescent white light (30watts).

The absorbance at 560nm was measured in intervals of 1 minute for a period of 10 minutes, using a spectrophotometer UV-160A Shimazu. The blank assay was prepared by substituting the propolis extract with sodium phosphate buffer and each assay was done in triplicates.

Table 2.6- Volumes and concentrations in the reaction mix for the SOD activity.

Solutions	Assay	Blank	Final concentration
100mM Sodium phosphate buffer pH 7.8 with 1% (w/v) PVP, 0.1 mM EDTA and 0.2% (v/v) Triton X-100	2500µl	2600µl	-
0.1M EDTA	200µl	200µl	6.7mM
1.5mM NBT	100µl	100µl	0.5mM
1mM L-methionine	50µl	50µl	216.7µM
Propolis extracts	100µl	-	-
0.12mM Riboflavin	50µl	50µl	2µM

2.2.7.3. Guaiacol peroxidase activity determination

Peroxidase activity was determined by the guaiacol oxidation method [71]. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyses the reduction of 1µmol guaiacol per minute (Fig. 2.5).

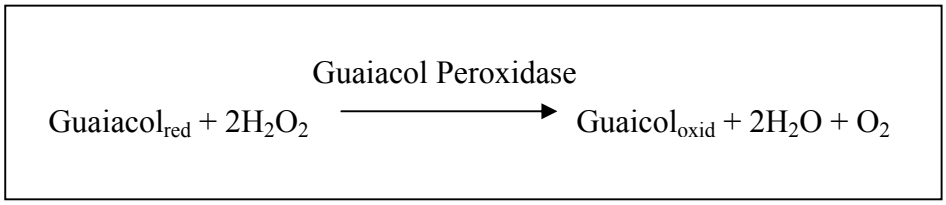

Figure 2.5 – Schematic representation of Guaiacol reduction used in Guaiacol peroxidase activity determination.

Table 2.7 - Volumes and concentrations in the reaction mix for the guaiacol peroxidase activity.

Solutions	Assay	Blank	Final concentration
100mM Sodium phosphate buffer pH 7.8 with 1% (w/v) PVP, 0.1 mM EDTA and 0.2% (v/v) Triton X-100	1210µl	1310	-
0.1M Guaiacol	40µl	40µl	2.6mM
Propolis extracts	100µl	-	-
40 mM H_2O_2	150µl	150µl	4mM

In a quartzo *cuvette* sodium phosphate buffer, guaiacol peroxidase and propolis extracts were added in the quantities indicated in Table 2.7. The reaction was initiated after the addition of H_2O_2 and the absorbance at 436nm was measured in intervals of 10s for a period of 200s, using a spectrophotometer UV-160A Shimazu. The blank assay was prepared by substituting the propolis extract with sodium phosphate buffer and each assay was done in triplicates.

2.2.7.4. Protein quantification assay

Total protein detection and quantification was determined using the Bradford method [72]. In this method, Coomassie Blue G-250 in an acidic environment binds to proteins. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, so that is the optimal wavelength to measure the blue color from the Coomassie dye-protein complex. The protein quantity was calculated using a standard curve with the absorbances read at 595nm versus standard solutions concentration.

For the standard curve standard solutions containing 0.2, 0.3, 0.4, 0.5, 0.6 e 0.8 mg/ml BSA were prepared. In a plastic *cuvette* 20µl of a BSA solution and 980µl Bio-Rad Protein Assay reagent diluted 5 times were added and incubated at room temperature for 5 minutes. Then the absorbance at 595nm was measured using a spectrophotometer UV-160A Shimazu.

For the propolis extracts the same procedure as above was used except propolis extracts were diluted 2 times. The blank assay was prepared by substituting the propolis extract with sodium phosphate buffer and each assay was done in triplicates.

2.2.8. HPLC evaluation of phenolic and polyphenolic acids from propolis

In this study we identified some phenolic and polyphenolic acids from extracts of propolis using the methodology described by Croci [73]. All phenolic and polyphenolic acids used are listed in table 2.8 and have a high degree of purity (99%). All the used standards of phenolic and polyphenolic acids solutions were prepared in the appropriate solvent and filtered (syringe filter 0,45µm PTFE membrane, VWR International) prior to the analysis by HPLC.

Analyses were carried out on an HPLC thermo-surveyor system equipped with automatic sample injection and diode array detector (DAD) (the spectral detection interval was 190-360 nm). The chromatographic data were processed using EZChrom Elite software, equipped with a spectral identification module of the compounds separated on the column.

Table 2.8 – Used standards of phenolic and polyphenolic acids.

1. Benzoic acid	10. Vanillic acid	19. Rosmarinic acid
2. p-Hydroxybenzoic acid	11. D(-)-Quinic acid	20. 3,4-Dihydroxybenzoic acid
3. (+)-Catechic hydrate	12. Naringin	21. Ursolic acid
4. Chlorogenic acid	13. (±)-Naringenin	22. Taxifolin
5. Caffeic acid	14. Galic acid	23. Diomin
6. p-Coumaric acid	15. Quercetin	24. Luteolin
7. trans-Cinnamic acid	16. Sinapic acid	25. Apigenin
8. Syringic acid	17. Galangin	26. Carnosol
9. Ferulic acid	18. Pinocembrin	27. Carnosic acid

A Purosphere® STAR RP18e column (250mm length, 4mm diameter, 5µm particle; LiChroCART®) was used. Column temperature was 25ºC and injection volume was 20µl. The flow rate was 0,9 ml/min and UV detection was performed at 280nm.

The mobile phase was a mixture of acetonitrile (Lab-Scan) and acetic acid 10% (ProLabo). A linear gradient was used for elution as described bellow in Table 2.9.

Table 2.9 – Elution gradient used in HPLC.

Time (min)	Acetic acid (10%)	Acetonitrile
0	92	8
30	9	91
35	92	8
37	92	8

2.2.9. Statistical analysis

The data were subjected to analysis of variance (ANOVA) and mean values were compared using SPSS for Windows (statistical program). Duncan Post-hoc tests were performed when significant differences occurred at 5 % level.

3. Results and Discussion

3.1. Determination of the antimicrobial potential of propolis extracts through agar diffusion method

The antimicrobial activity was determined by the agar diffusion method as previously described. Initially the antimicrobial activity was tested using 3µl of diluted propolis extracts (1:50) against *S. aureus* CFSA2*, L. monocytogenes* EGD and *S. enterica* serovar *thyphimurium*. With this method it is possible to evaluate the susceptibility of these bacteria on the basis of the presence or absence of growth around the disks where the propolis extract was placed. Hence, after the appropriate incubation period the growth inhibition zones were measured and the mean value and standard deviation were calculated and are represented in Fig. 3.1 A and B. The results were also subjected to statistical analysis that is listed in Table 7.1 in the annexes section.

The results obtained for *S. aureus* CFSA2 showed little growth inhibitory activity with growth inhibition zones very similar to the ones obtained with the negative control, ranging from 6.000 to 7.333mm. Although there were no statistical differences observed between the obtained values (Annexes, Table 7.1) it seems that there could be a tendency to increase the inhibitory activity when comparing different collection times, with samples collected at winter time having a lower activity than the ones collected at springtime (Fig. 3.1 A and B), but further testing with more concentrated extracts would be necessary to be certain.

Regarding the results for *L. monocytogenes* EGD, a small growth inhibitory activity was also observed although with slightly higher values than the obtained for *S. aureus* CFSA2. There were statistical differences observed between the obtained values (Annexes, Table 7.1) and these ranged from 7.000 to 9.333mm. Some of the extracts that had showed a higher growth inhibition against *Listeria monocytogenes* EGD were also tested against two other strains of *Listeria monocytogenes* C882 and T8, with results similar to the negative control (results not shown).

With *S. enterica* serovar *thyphimurium,* propolis extracts also showed a small growth inhibitory activity only a little better than the results obtained for *S. aureus* CFSA2. There were statistical differences observed between the obtained values (Annexes, Table 7.1) and these ranged from 6.000 to 8.333mm.

The small variations observed in antibacterial activity among the tested bacteria may be due differences in the extracts composition suggesting that it varies with the collection time and site, which therefore can influence its inhibitory activities or to differences in the type of tested bacteria. In fact there are several reports that indicate that Gram-positive and negative bacteria have different susceptibility towards propolis extracts, where usually Gram-negative bacteria

have little or no susceptibility to propolis extracts [36, 37, 40]. Despite this, the small differences in antibacterial activity between the two types of bacteria suggest that both types of bacteria are susceptible to propolis extracts of Portuguese origin. However, variations between samples were so small that further testing would be necessary to be certain.

A

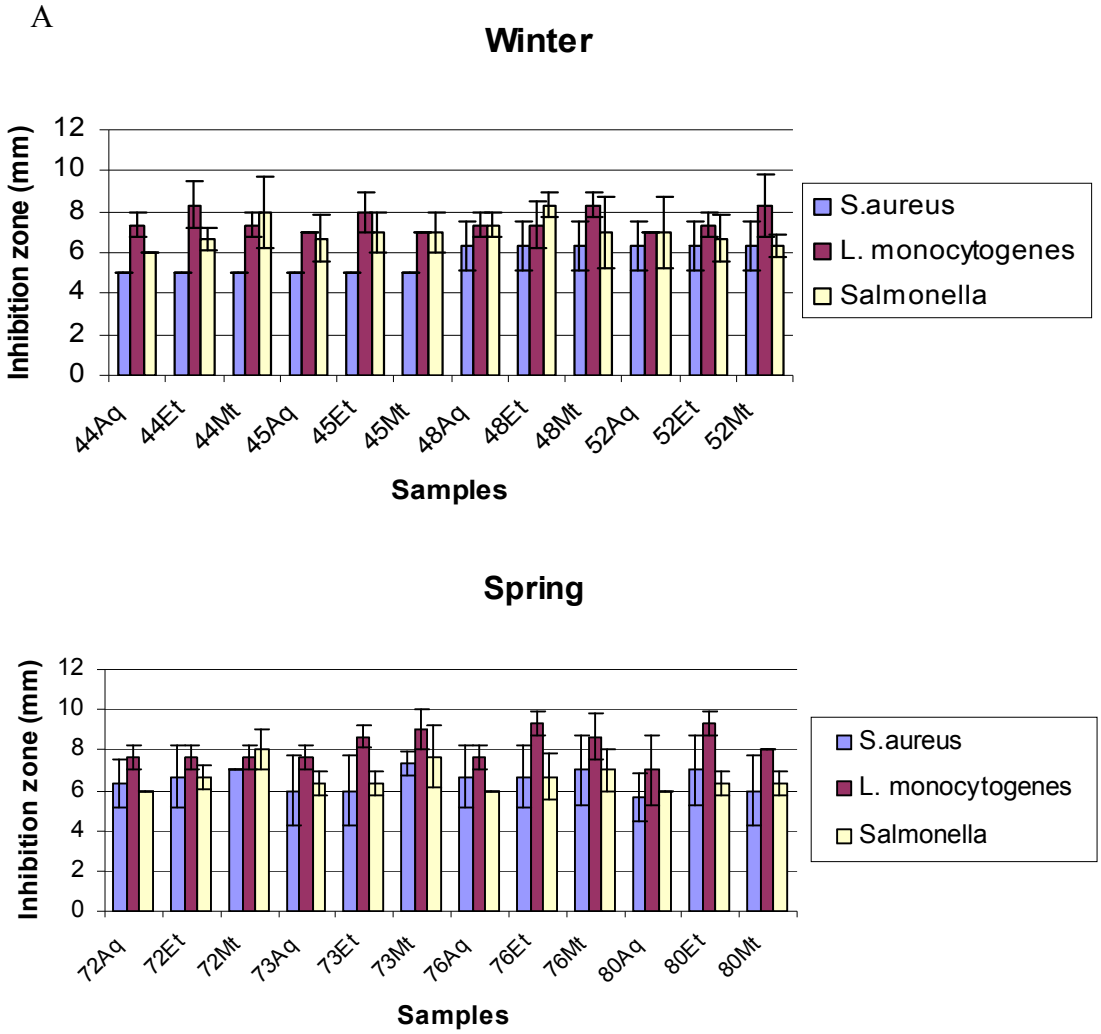

Figure 3.1 - Graphs showing antimicrobial activity of propolis extracts in A) wintertime and B) springtime for *S.aureus, L. monocytogenes* and *Salmonella*, results for the positive control not shown (18.375, 25.000 and 22.700mm, respectively); 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

So, as the results obtained with the 1:50 diluted extracts of propolis didn't show a very large growth inhibition activity for any of the tested bacteria, different volumes of a smaller dilution of the extracts were tested next. Therefore, the antimicrobial activity was tested using 5, 10, 15 and 20µl of diluted propolis extracts (1:10) against *S. aureus* CFSA and *S. enterica* serovar *thyphimurium*. After the appropriate incubation period the growth inhibition zones were

measured and in Fig. 3.2 are presented some photographs of the plates. All measurements were done in triplicates and the mean value and standard deviation were calculated. The results were also subjected to statistical analyses that are listed in Tables 7.2 and 7.3 in the annexes section.

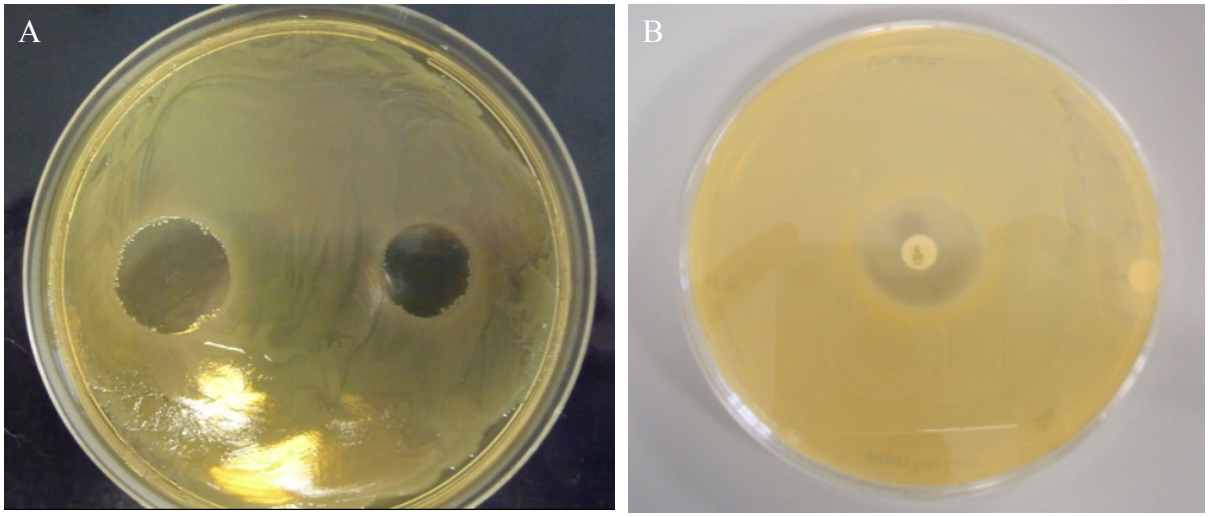

Figure 3.2 – Photographs of some of the plates used in the antibacterial activity determination using the agar diffusion method where it's visible the growth inhibition zones, A- *S. enterica* serovar *thyphimurium* with two different volumes of propolis extract and B- *S. aureus* CFSA with an antibiotic disk (30ng chloramphenicol).

After testing different volumes of less diluted propolis extracts (1:10) against *S. aureus* CFSA and *S. enterica* serovar *thyphimurium* a large increase in the antibacterial activity was observed which can be seen by the larger growth inhibition zones obtained (Fig. 3.3 and 3.4). This increase in activity was directly proportional to the increase of the tested volumes showing that propolis extracts exhibit a dose dependent antibacterial activity.

Analysing the results obtained for *S. enterica* serovar *thyphimurium*, the three types of extracts (aqueous, ethanolic and methanolic) exhibited a similar antibacterial activity, with the exception of the extracts from B.S. Arneijoafra collected at winter time (Fig. 3.3 C), where the aqueous extract exhibits a higher activity than the ethanolic and methanolic extracts. However, this is not observed in extracts collected in springtime at the same location (Fig. 3.3 G) where all extracts had very similar activity or in other extracts collected in winter time. Most aqueous propolis extracts showed similar or slightly smaller activity than the other two types of extracts (ethanolic and methanolic) (Fig. 3.3).

Results with 20µl of propolis extracts were very good with most of the extracts having equal or higher activity than the positive control (30ng chloramphenicol), that was 24.667mm (Annexes, Table 7.2). Growth inhibition zones were as large as 27.333mm for the methanolic extract from B.N. Pé da Serra collected at springtime.

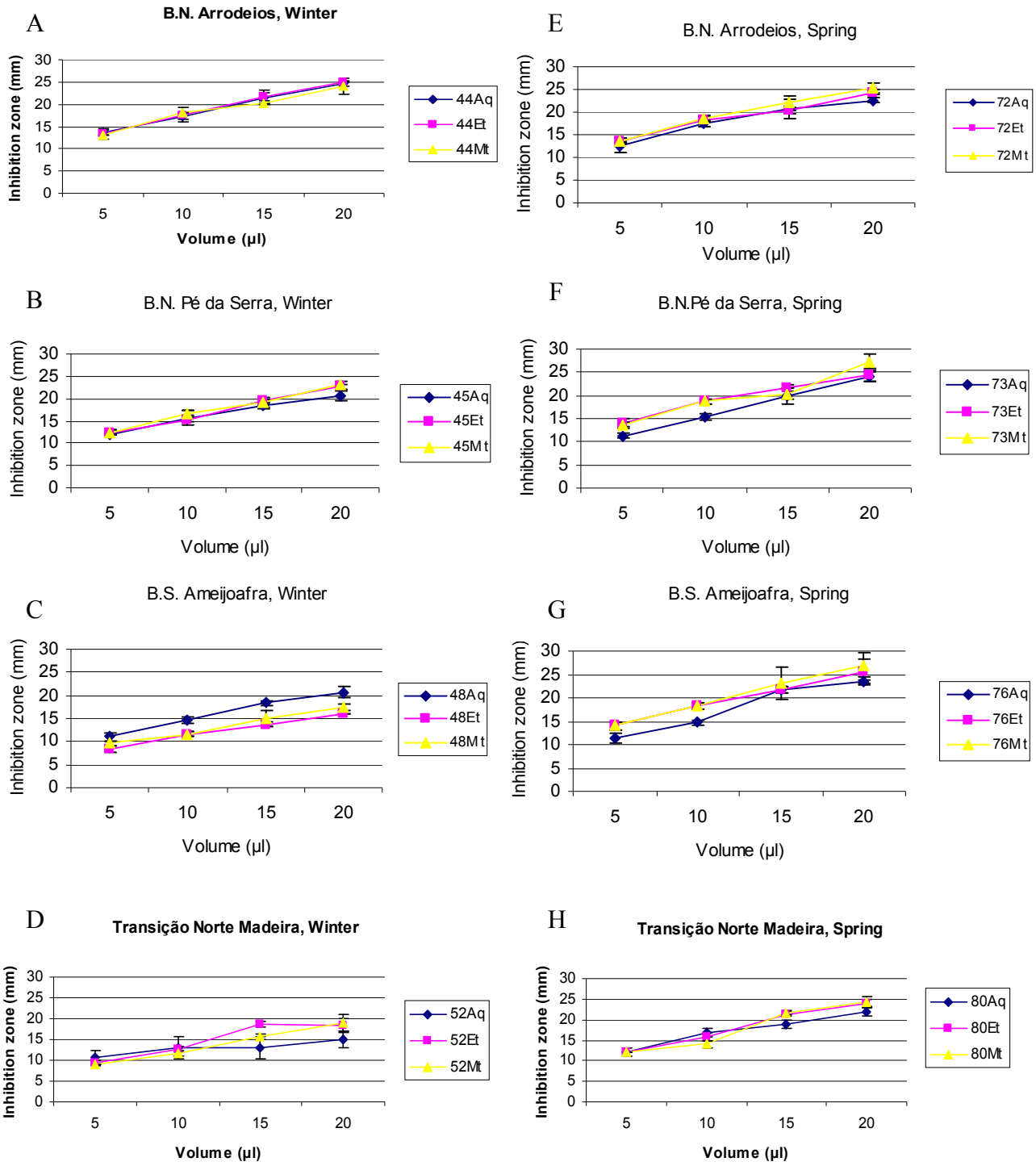

Figure 3.3 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and springtime (E, F, G, H) for *S. enterica* serovar *thyphimurium*; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

It was also noticeable a variation of the antibacterial activity in relation to the collection time, with samples collected at springtime displaying in almost all cases a higher activity than

the ones collected in wintertime. In terms of collection site, if we consider the highest values statistically equal obtained for each tested volume, then the extracts with highest activity as the ones with the highest values in all or most of the tested volumes. With *S. enterica* serovar *thyphimurium* the ones that showed highest activity were methanolic extracts from B.N. Pé da Serra and B.S. Arneijoafra at springtime (Annexes, Table 7.2).

In the case of *S. aureus* CFSA2, results were similar to the ones obtained with *S. enterica* serovar *thyphimurium* but differences in activity between different types of extracts were more visible with these bacteria. Methanolic and ethanolic extracts displayed very similar activity and the aqueous extract displayed a slightly smaller activity witch was clearly visible in samples from B.N. Arrodeios (winter time), B.N. Pé da Serra (winter and springtime), B.S. Arneijoafra and T.N. Madeira (springtime) (see Fig. 3.4 A, B, F, G and H).

Like with *S. enterica* serovar *thyphimurium*, it was also observed a variation in the antibacterial activity when comparing results from the two collection times, with samples collected in springtime showing higher activity than samples collected in wintertime. In terms of collection site, if we apply the same reasoning as before the extracts with highest activity were the methanolic extract from B.N. Pé da Serra and ethanolic extract from T. N. Madeira both collected at springtime (Annexes, Table 7.3).

S. aureus CFSA2 was more susceptible to propolis extracts than *S. enterica* serovar *thyphimurium*, since the measured growth inhibition zones were higher. In fact results with 15 and 20µl of propolis extracts for some of the samples were higher than the positive control (30ng chloramphenicol), that was 21.667mm (Annexes, Table 7.3). The best result using 15µl was 25.333mm using the methanolic extract from B.N. Pé da Serra and the ethanolic extract from T. N. Madeira both collected in spring time. With 20µl the best result was 28.333mm using the ethanolic extract from T.N. Madeira also collected in spring time (Annexes, Table 7.3). This difference in susceptibility towards propolis extracts was not unexpected given that the two bacteria belong to different categories, as has been mentioned before. Despite the fact that *S. enterica* serovar *thyphimurium* is a Gram-negative bacteria, results were very close to the ones obtained for *S. aureus* CFSA2 demonstrating that Gram-negative bacteria can also be susceptible to propolis extracts.

After the good results from the tests performed with *S. enterica* serovar *thyphimurium* we continued testing the antibacterial activity of propolis extracts against Gram-negative bacteria, namely two strains of *H. pylori* J99 and 26695. The same volumes of diluted propolis extracts (1:10) were tested using the agar diffusion method, as described before. After the incubation period the growth inhibition zone were measured and in Fig. 3.5 is presented a photograph of one of the plates. All measurements were done in triplicates and the mean value and standard

deviation were calculated. The results were also subjected to statistical analyses that are listed in Tables 7.4 and 7.5 in the annexes section.

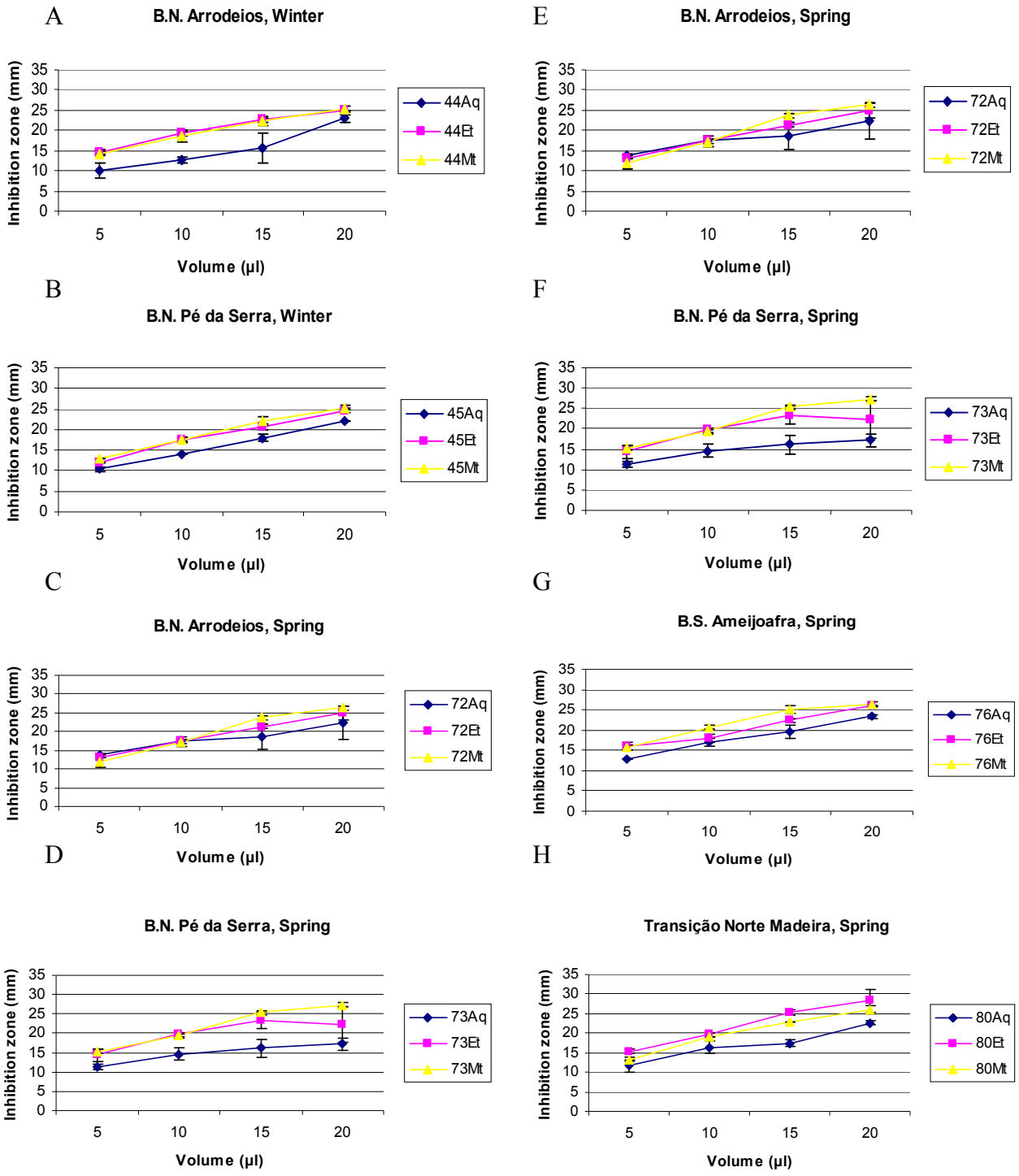

Figure 3.4 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and spring time (E, F, G, H) for *S.aureus* CFSA2 ; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

Analysing the results obtained with the two strains *H. pylori* we can verify that they are similar to the ones obtained before with *S. enterica* serovar *thyphimurium*. Most aqueous propolis extracts showed similar or slightly smaller activity than the other two types of extracts (ethanolic and methanolic) (Fig. 3.6 and 3.7), with the exception of the extracts from B.N. Pé da Serra and T. N. Madeira collected at winter time (Fig. 3.6 B and D) with the strain J99. In the case of the extracts collected in B.N. Pé da Serra both aqueous and ethanolic extracts present a smaller activity contrasting with the higher activity of the methanolic extract and the extracts collected in T. N. Madeira the aqueous extract exhibits a much lower activity than the other two extracts.

Figure 3.5 – Photograph of one of the plates used in the antibacterial activity determination using the agar diffusion method where it's visible the growth inhibition zones for *H. pylori* with two different volumes of propolis.

The propolis samples collected at springtime showed higher antibacterial activity, in comparison with samples harvested at wintertime (Fig. 3.6 and 3.7). This difference in antibacterial activity was observed in almost all samples and was especially noticeable in *H.pylori* J99.

Both of the tested strains were susceptible to the propolis extracts; however *H.pylori* 26695 was slightly more sensible to propolis extracts than J99. Results with 20µl of propolis extracts were very good although not, as high as, the positive control (30ng chloramphenicol), that was 42.250mm with *H.pylori* J99 and 37.667mm with *H.pylori* 26695 (Annexes, Table 7.4 and 7.5). The best results obtained with *H.pylori* J99 was 33.667mm for the ethanolic extract from T.N. Madeira collected at springtime and with *H.pylori* 26695 it was 35.667mm for the

ethanolic extract from B.N. Arrodeios collected at wintertime, although there were several other samples with statistically equal results (Annexes, Table 7.4 and 7.5).

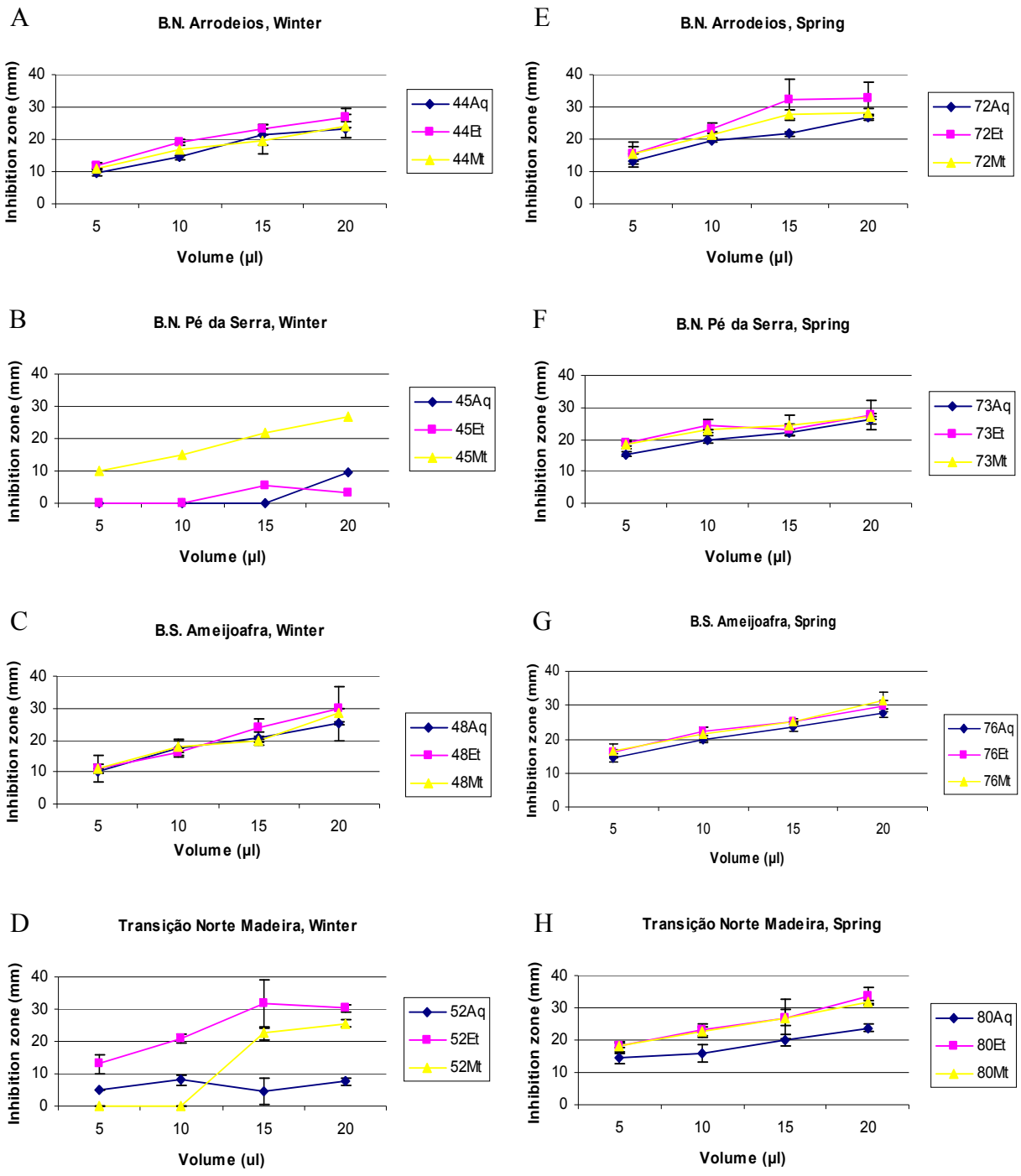

Figure 3.6 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and spring time (E, F, G, H) for *H. pylori* J99; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

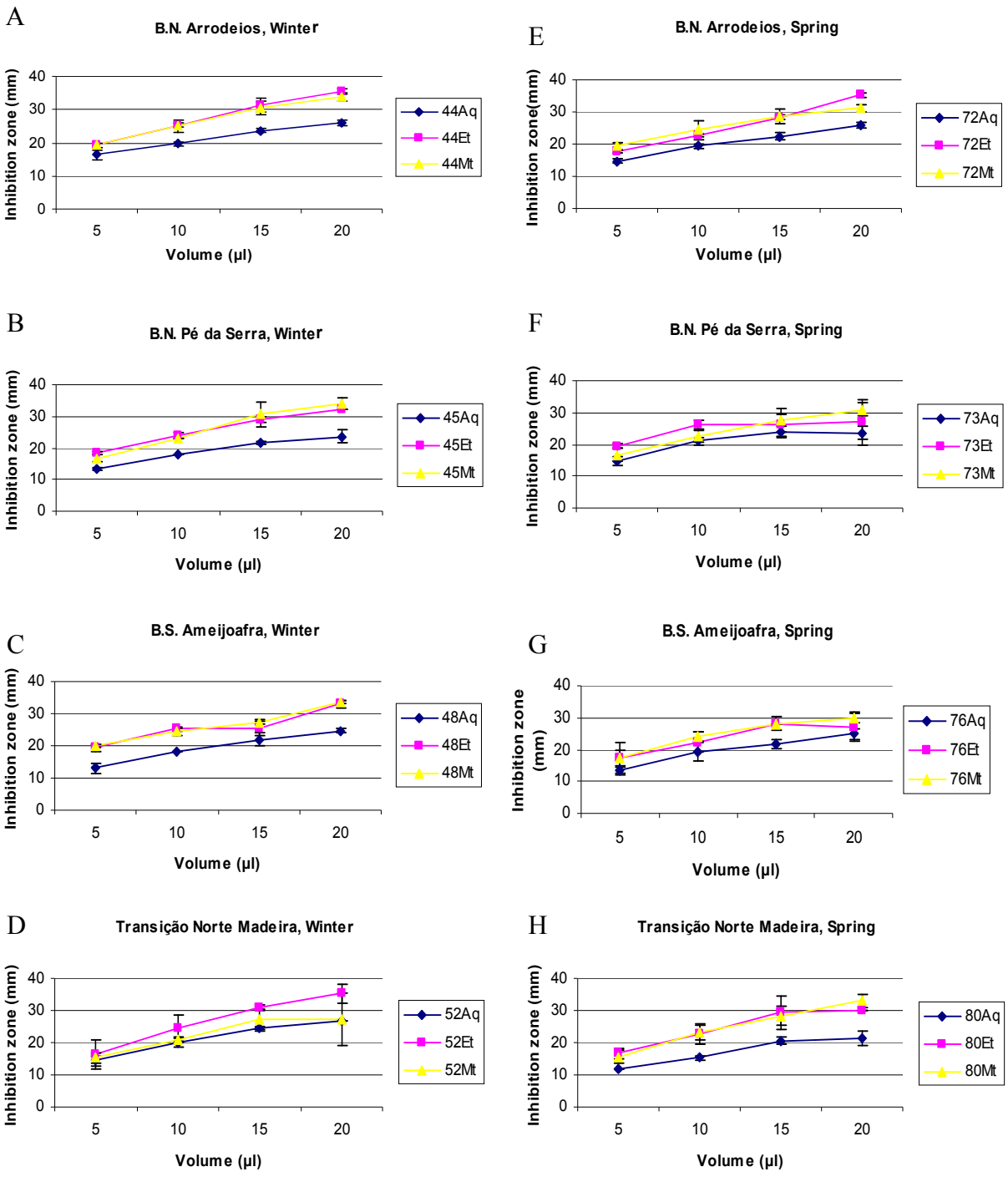

Figure 3.7 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and spring time (E, F, G, H) for *H. pylori* 26695; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

In terms of collection site, with *H.pylori* J99 there were a number of extracts with statistically equal results unlike with *H.pylori* 26695 where there were only one extract that had high results for almost all the tested volumes (Annexes, Table 7.4 and 7.5). So, with *H.pylori* J99

the extracts with highest activity were ethanolic extract from T. N. Madeira at winter time, the ethanolic and methanolic extracts from B.N. Arrodeios, the ethanolic extracts from B.N. Pé da Serra and B.S. Arneijoafra at springtime and the ethanolic and methanolic extracts from T. N. Madeira at springtime. With *H.pylori* 26695, the extract with the highest activity was the ethanolic extract from B.N. Arrodeios at wintertime.

We further studied the antibacterial activity of propolis extracts against *S. pneumoniae* D39 and *H. influenza* TD-4, the first being a Gram-positive bacteria and the second a Gram-negative bacteria. We used the same methodology as before and also tested the same volumes of diluted propolis extracts (1:10) using the agar diffusion method. After the appropriate incubation period the growth inhibition zones were measured and in Fig. 3.8 are presented photographs of some of the plates. All measurements were done in triplicates and the mean value and standard deviation were calculated. The results were also subjected to statistical analyses that are listed in Tables 7.6 and 7.7 in the annexes section.

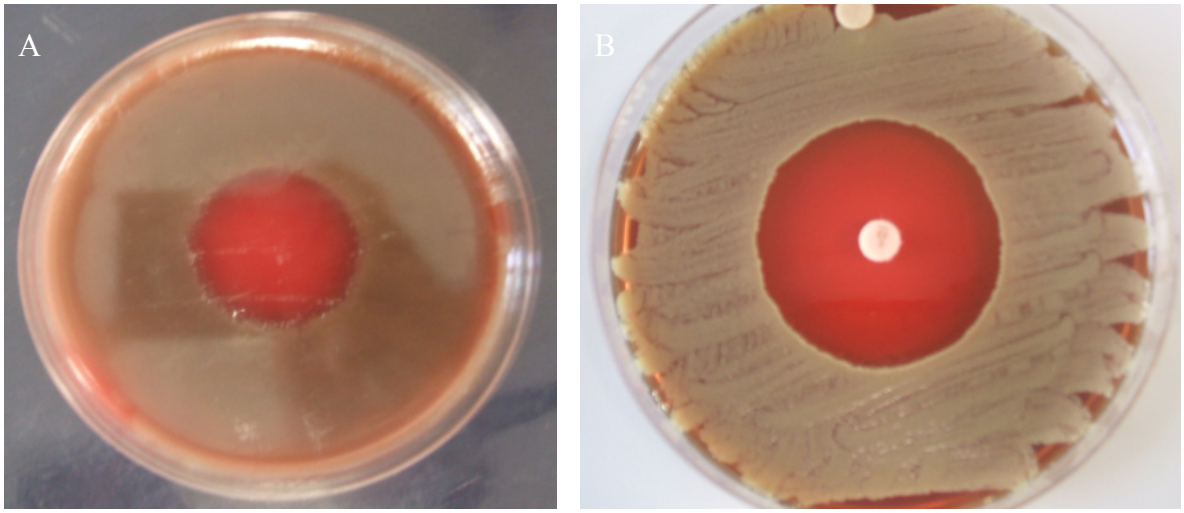

Figure 3.8 – Photographs of some of the plates used in the antibacterial activity determination using the agar diffusion method where it's visible the growth inhibition zones, A- *S. pneumoniae* D39 with propolis extract and B- *H. influenza* TD-4 with an antibiotic disk (30ng chloramphenicol).

With *S. pneumoniae* D39 the difference of activity of the different types of propolis extracts was particularly noticeable since it could be observed in all the tested samples (Fig. 3.9). All samples showed similar activity for the ethanolic and methanolic extracts and smaller activity for the aqueous extracts. Contrary to what had been observed for the other bacteria with *S. pneumoniae* D39 propolis extracts seem to have a decrease in their antibacterial activity when we compare results from samples collected at wintertime to results from samples collected at springtime.

In terms of collection site, the extract with highest activity was the ethanolic extract from B.N. Pé da Serra at wintertime (Annexes, Table 7.6).

As observed before with *S. aureus* CFSA2 and *S. enterica* serovar *thyphimurium*, results with 15 and 20µl of propolis extracts were close to the ones obtained with the positive control. In this study we used two positive controls chloramphenicol (30ng) and penicillin G (10ng) that had growth inhibition zones of 22.833 and 33.167mm respectively. The best results with 15µl of propolis extract was 27.000mm with the ethanolic extract from the sample collected at B.N. Arrodeios collected at wintertime; with 20µl the best result was 32.333mm with the ethanolic extract from the sample collected in B.N. Pé da Serra at winter time (Annexes, Table 7.6).

Results for *H. influenza* TD-4 were very similar to the ones obtained for *S. pneumoniae* D39, presenting the same differences in activity when comparing different types of extracts and different collection times (Fig. 3.10). This might mean that the compounds that are responsible for the antibacterial activity with these two bacteria not only vary with collection time but are also different to the ones that responsible for the antibacterial activity in the other tested bacteria.

We used the same positive controls that we used previously for *S. pneumoniae* D39 and the growth inhibition zones obtained were 37.667mm for chloramphenicol (30ng) and 43.667mm for penicillin G (10ng) (Annexes, Table 7.7). Although the bacteria were susceptible to the propolis extracts none of the tested volumes of propolis extracts exhibited an activity as high as the positive control, unlike what had been observed for the other tested bacteria. The best result was obtained when using 20µl of ethanolic propolis extract from B.S. Arneijoafra collected at winter time with a growth inhibition zone of 31.000mm.

In terms of collection site, the extract with highest activity was the ethanolic extract from B.S Arneijoafra at winter time (Annexes, Table 7.7).

While analysing our results we tried to compare our results to results obtained from different authors, however, it was very difficult because not only of the variety of methods used (agar dilution, agar diffusion, broth dilution) but also the variety of strains of bacteria and difference in virulence of the tested organisms. Although most studies on the antimicrobial activity of propolis have been carried out using Petri dish methods (well or disk diffusion and agar dilution) these can't be correlated either since they are directly influenced by the solubility of constituents in agar [74].

The results showed, for almost all samples, that both ethanolic and methanolic extracts have a very similar activity, as expected since both solvents have similar polarity and therefore can extract the some of the same compounds. Surprisingly, the aqueous extracts showed in most samples a similar activity or a slightly smaller activity to the ethanolic and methanolic extracts. It was unexpected since that most of the compounds that are reportedly responsible for the

antibacterial activity of propolis extracts are mostly phenols and flavonoids that are not so easily extracted when water is used as an extraction solvent and so usually aqueous extracts have a lower phenols concentration and consequently a lower activity.

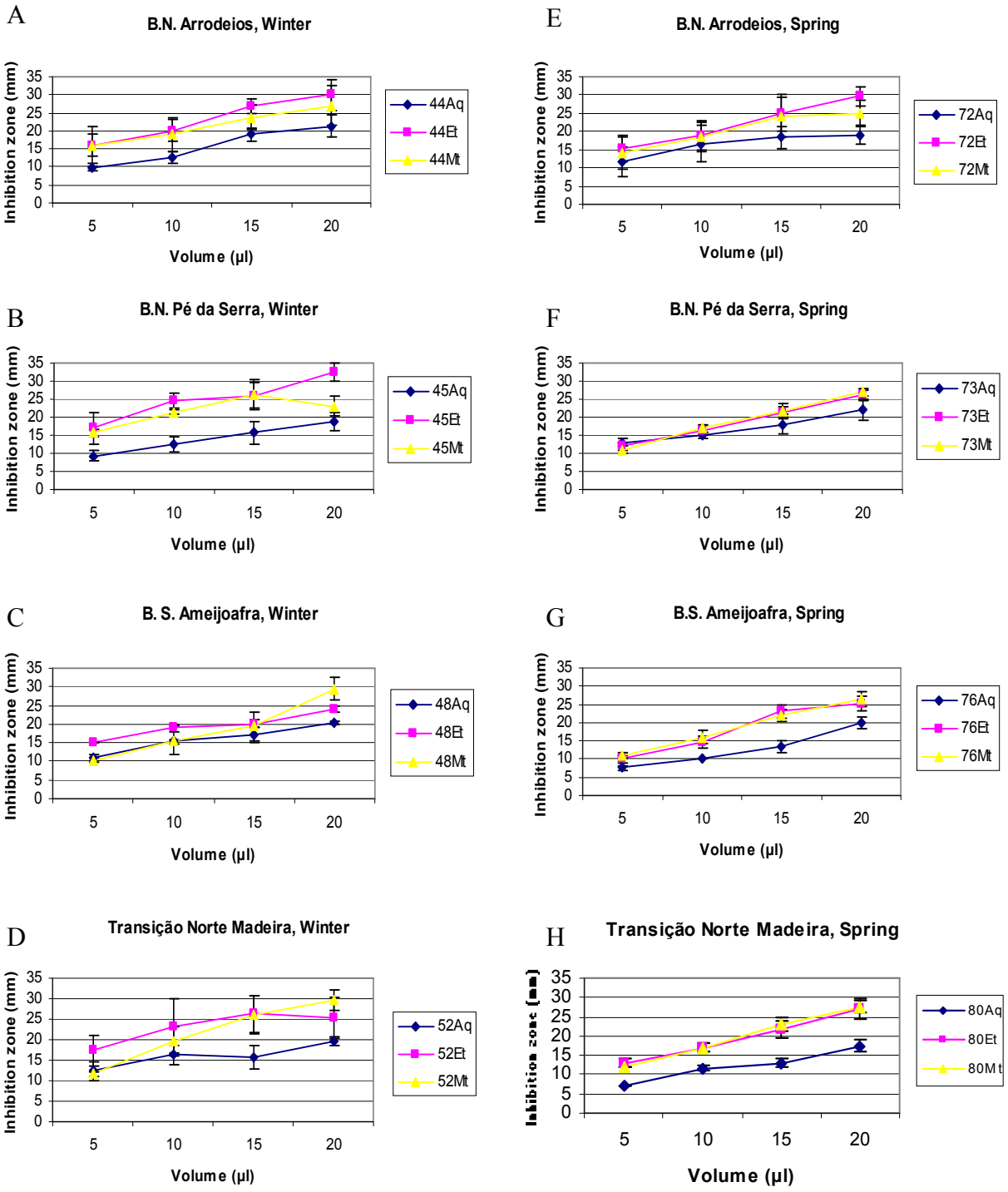

Figure 3.9 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and springtime (E, F, G, H) for *S. pneumoniae* D39; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

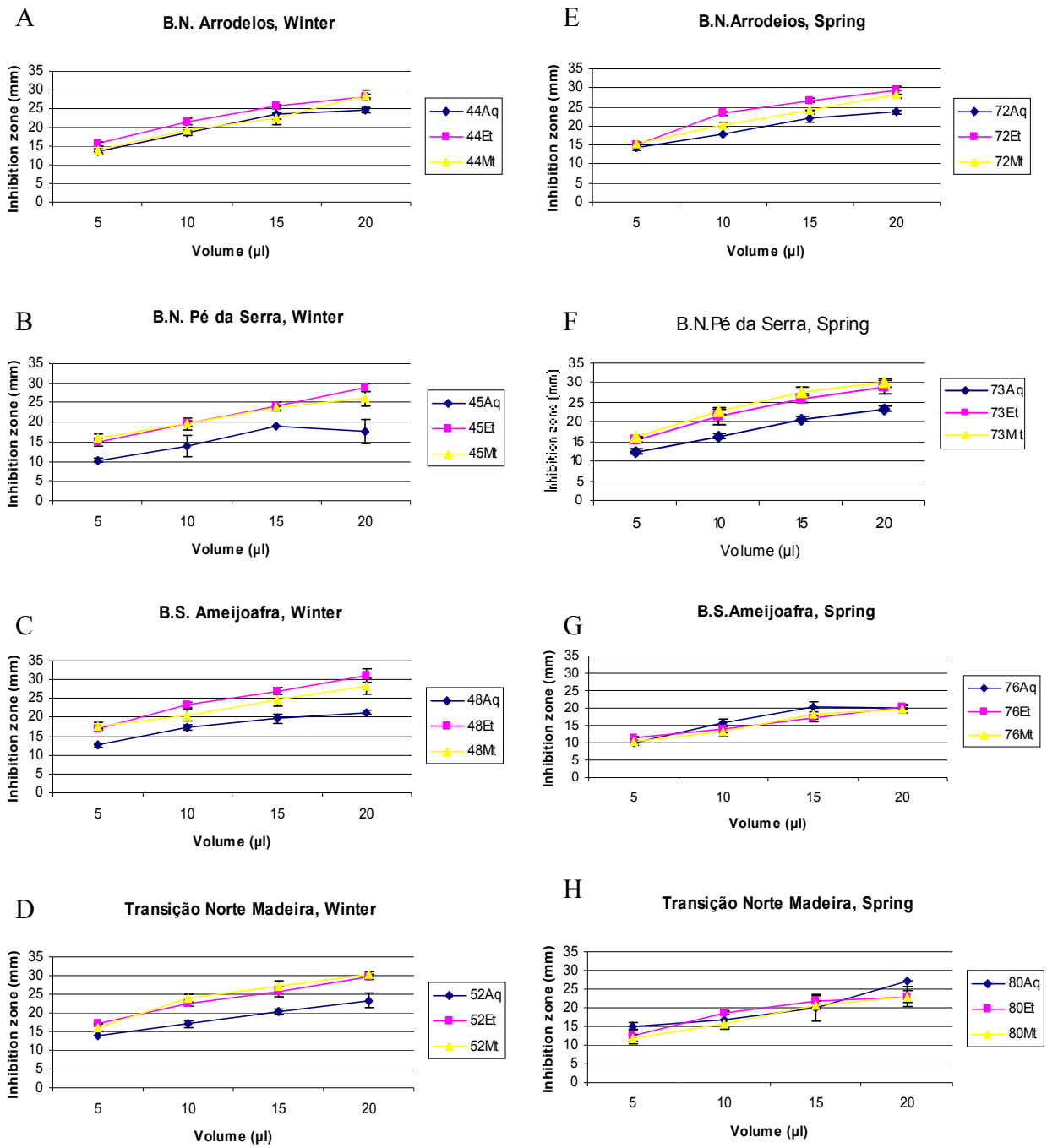

Figure 3.10 - Graphs showing antimicrobial activity of propolis extracts in winter (A, B, C, D) and springtime (E, F, G, H) for *H. influenza* TD-4; 44, 72 - B. N. Arrodeios, 45, 73 - B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts.

While the determination of the antibacterial activity of the propolis extracts was being done, the determination of its phenol concentration was simultaneously being done (S. Nunes, unpublished results). From those results we can see that although in most cases all three types of

propolis extracts had similar activity their phenol concentration were very different, with ethanolic and methanolic having in some cases a concentration ten times higher (see the annexes section, Table 7.8). This means that despite having a lower concentration on phenolic compounds, aqueous extracts can display an antibacterial activity similar to the one obtained with the other two extracts. Therefore, aqueous extracts may have fewer phenols but the ones that it has are capable of a good antibacterial activity or they could have other compounds different from phenolic compounds that are responsible for its antibacterial activity. So, it may not be a question of the quantity of phenols present in the extracts but a question of the quality.

3.2. Determination of citotoxicity of propolis extracts through the colorimetric MTT assay

Based on the results from the antibacterial activity, three propolis extracts with the highest results were selected and the effect of them on the *in vitro* growth of Caco-2 cell lines was studied using the colorimetric MTT assay, in order to determine if there was a citotoxic effect of the propolis extracts at the concentrations that showed to have antibacterial activity.

On the basis of the selection of the propolis extracts for testing were as mentioned before the ones with the highest results in the antibacterial study and simultaneously with larger amounts of extracts still available since their were limited amounts of propolis extracts and limited amounts of propolis from which we could produce more extract. So, based on that we chose to test the aqueous and ethanolic extract from B.N. Arrodeios (highest value obtained 26.667mm and 35.333mm respectively, Annexes, Table 7.4 and 7.5) and the aqueous extract from T.N. Madeira (highest value obtained 27.000mm, Annexes, Table 7.7) all collected at springtime.

The propolis extracts dilutions were done using culture medium without phenol red and the volumes used were 1, 5, 10, 15 and 20µl for 200µl of medium and the incubation periods were 1, 4 and 24 hours. The volumes used were the same tested in the antibacterial study but in the case of a high citotoxicity a smaller volume, 1µl, was also tested. The incubation periods were chosen taking into account the time of interaction between bacteria and cells and also the cell's doubling time.

Besides testing the effect of propolis extracts on cell viability four different controls were also used. As a positive control it was used culture medium and another three controls were also used: culture medium with n-propanol and water and culture medium with n-propanol and 70% ethanol to eliminate any activity in the extracts from the extraction solvents used and culture medium with 5% hydrogen peroxide as a negative control.

The optical densities measured after the incubation period were then used to calculate the relative percentage of cell growth considering the values obtained with culture medium as 100% of cell growth. And so the rest of the values appear as a relative percentage of the values obtained with just culture medium [75]. These results were also subjected to statistical analyses that are listed in Tables 7.9 to 7.11 in the annexes section.

Analysing the cell viability results when using the aqueous extract from B.N. Arrodeios collected at springtime it is visible that there isn't a significant increase or decrease in cell viability except when using hydrogen peroxide where an increase in cell viability is observed after incubation for 4h, which will be discussed later on (Fig. 3.11A). Considering the results obtained with the ethanolic extract from the same sample a small decrease in viability is observed which is especially noticeable after incubation for 4h (Fig. 3.11B). These differences were somewhat expected since although using a propolis sample from the same collection time and site, the extraction solvent was different and so is the composition of the extract. By testing the cell viability with controls for the extraction solvents, where the same amounts of water and 70% ethanol were used as in the sample with 20µl of propolis extract, we can eliminate any citotoxic effect from the extraction solvents. If there were any effects from either n-propanol, water or 70% ethanol the results from these controls would be very similar to the results obtained with the propolis extracts. In fact, the results show that in most cases the activity of these controls is very similar to the activity in the control with just culture medium (Annexes, Tables 7.9, 7.10 and 7.11). So, the positive effect observed in cell viability when using the ethanolic extract from B.N. Arrodeios collected at springtime is probably due to propolis constituents.

The results for cell viability when using the aqueous extract from T.N. Madeira collected at springtime doesn't show any statistical differences between the results mainly because of the considerable standard deviation between values. This might be explained by the formation of bubbles in the wells due to the pipetting of several solutions prior to the measurement of the optical density that might cause a variation in optical densities. It wasn't possible to compare these results to the results with the ethanolic extract because of the limited amounts of extract available that didn't allow performing the assay.

Regarding the results obtained when using 5% hydrogen peroxide a big decrease in cell viability was expected, since it has been described that concentrations above 0.05mM cause oxidative injuries in cells, but instead no effect on viability was observed or in some cases an increase in viability was observed [76]. This increase in cell viability was also observed with the aqueous extract from B.N. Arrodeios collected at springtime after 4h of incubation or aqueous extract from Transição Norte Madeira collected at springtime after 4h and 24h of incubation (Fig. 3.11 A and C).

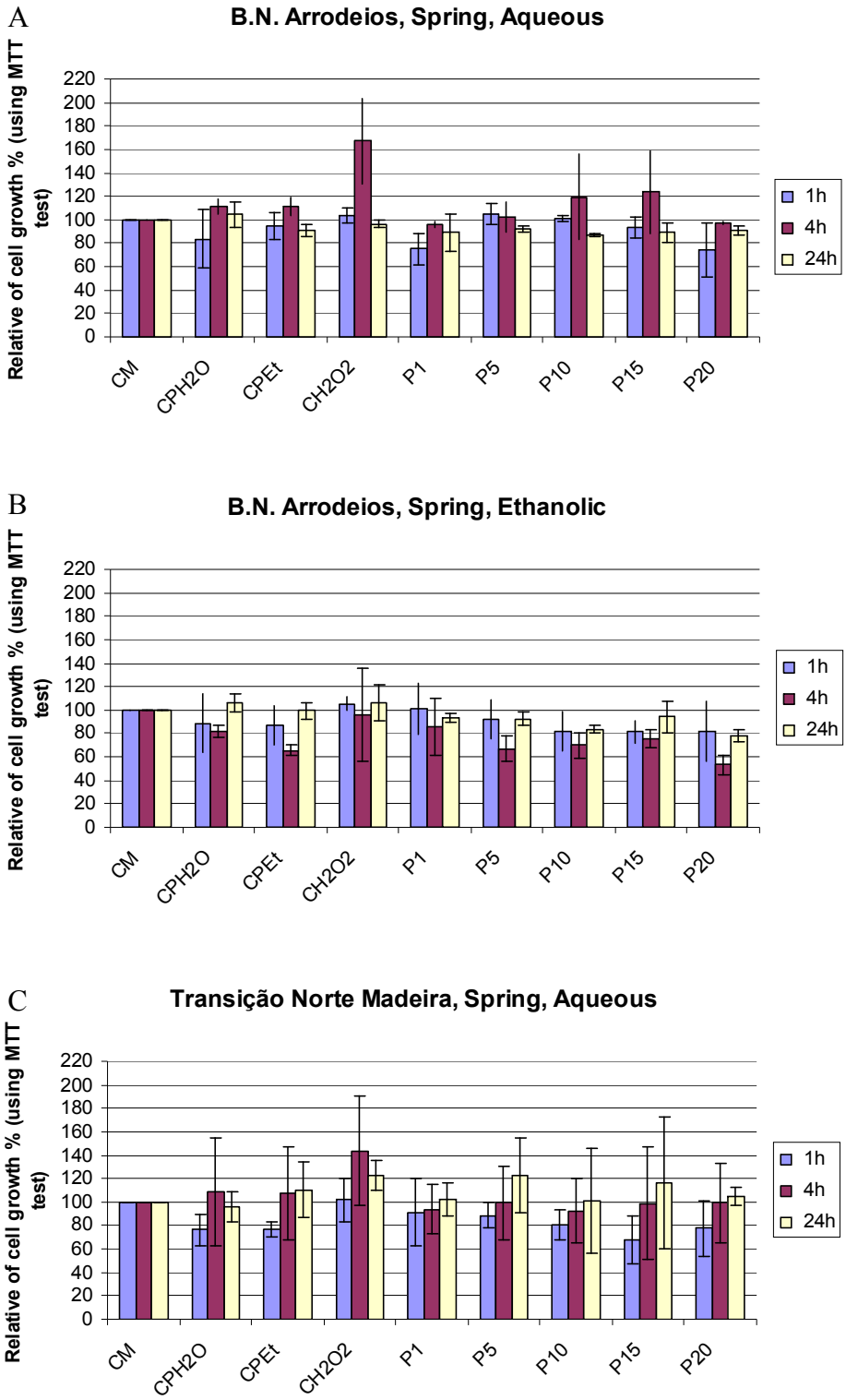

Figure 3.11- Relative percentage of cell growth using the MTT assay, A – aqueous extract from B.N. Arrodeios, B- ethanolic extract from B.N. Arrodeios, C- aqueous extract from Transição Norte Madeira, all collected at springtime; CM – control with culture medium, CPH2O – control with culture medium, *n*-propanol and water, CPEt - control with culture medium, n-propanol and etanol 70%, CH2O2 - control with culture medium and H_2O_2 5%, P1 to P20 – Culture medium with 1 to 20µl of propolis extracts.

In the later cases a proliferative effect of hydrogen peroxide was observe in the cells. This has been described before when using much smaller concentrations (1nM-1μM) than the ones used in this assay (approximately 7M)[77]. This suggests that the hydrogen peroxide stock solution used for the preparation of the 5% solution might have not been in proper conditions and was in fact degraded, leading to the use of a much smaller concentration of hydrogen peroxide in the assay rather then 5%.

3.3. Enzymatic antioxidant activity study

The enzymatic antioxidant activity of propolis was determined using three different methods one for each of the enzymatic activity. All the extracts were prepared freshly as described before.

For the determination of guaiacol peroxidase and catalase activity, the reactions were followed by measuring the absorbances, which were plotted versus time and the slope of each of the obtained lines were determined. This slope corresponded to the variation in absorbance per second, which was then converted to variation in absorbance per minute. Considering that one unit of enzymatic activity (U) was defined as the amount of enzyme that catalyses the reduction of 1μmol guaiacol per minute for guaiacol peroxidase activity and the amount of enzyme that catalyses reduction of 1μmol H_2O_2 per minute for catalase activity, the enzymatic activity of the propolis extracts was calculated. For this calculation Beer-Lambert's law (Eq. 9), the volume of extract used in the reaction, the volume of the reaction, the total volume of the extract and the molar extinction coefficient of guaiacol (ε tetraguaiacol = 25.5$mM^{-1}cm^{-1}$) and hydrogen peroxide (ε H_2O_2 = 39.4 $mM^{-1}cm^{-1}$), respectively were taken into consideration [78].

Since one unit of enzymatic activity for superoxide dismutase was defined as the amount of enzyme that inhibits 50% of the reaction the calculations for the determination of SOD activity were a little different. We plotted the measured absorbances versus time and determined the slope of each of the obtained lines that corresponded to the variation in absorbance per minute. Considering the variation in absorbance for the blank as 100% of reaction, the percentage of reaction inhibition was calculated. Then taking into account the dilution factor SOD activity was determined.

Equation 9. $Abs = \varepsilon \times c \times l$, ε = molar extinction coefficient,

c = concentration,

l = length of the light through the sample

These values for enzymatic activity were then converted to specific enzymatic activity (U/mg) by dividing the values obtained by the respective protein concentration of the extract in milligrams. The determination of the protein concentration will be addressed later on this section. All measurements and calculations were done in triplicate and the mean value and standard deviation were calculated. These results were also subjected to statistical analyses that are listed in Table 7.12 in the annexes section.

Considering results from propolis guaiacol peroxidase activity, no statistical differences were observed between samples. The samples with the highest activity were from B.S. Arneijoafra collected at springtime (3.835U/mg) and from B.N. Pé da Serra also collected at springtime (2.637U/mg)(Fig. 3.12 A).

Regarding propolis catalase activity, statistical differences were observed between samples and the sample from B.N. Arrodeios collected at springtime had the highest activity. Results showed differences among the samples in terms of collection site but not in terms of collection time, except for the sample from B.N. Arrodeios. It is important to note that the catalase activity nearly doubles from winter to springtime for this particular sample going from 31.692U/mg in wintertime to 59.313U/mg in springtime (Fig. 3.12 B).

Concerning SOD activity, statistical differences were observed between samples and its activity was dependent on the collection time. SOD activity decreased drastically from winter to springtime in samples from B. N. Arrodeios and B. S. Arneijoafra. The opposite was observed in samples from B. N. Pé da Serra and Transição Norte Madeira. These differences in activities relatively to the collection time may be due to differences in the propolis composition that reflect phytogeographical differences (Fig. 3.12 C).

The samples with the highest activity were from B.N. Pé da Serra collected at spring (30.663 U/mg), B.N. Arrodeios collected at winter (26.831 U/mg) and B.S. Arneijoafra collected winter (24.827 U/mg).

SOD activity generates hydrogen peroxide and oxygen molecules. These hydrogen peroxide molecules can be converted into water molecules and oxygen by the action of catalase and guaiacol peroxidase. So, the samples with higher SOD activities should have higher CAT activities also. Such was not observed, which may suggest that the hydrogen peroxide elimination could be done by other antioxidant enzymes such as, glutathione peroxidase or guaiacol peroxidase.

The protein content of the propolis extracts was also determined, and for that the Bradford method and calibration curve using a series of standard solutions containing a defined concentration were used. Then, using the measured absorbances of the extracts the protein concentration was determined. All measurements were done in triplicate and the mean value and

standard deviation were calculated. These results were also subjected to statistical analyses that are listed in Table 7.12 in the annexes section.

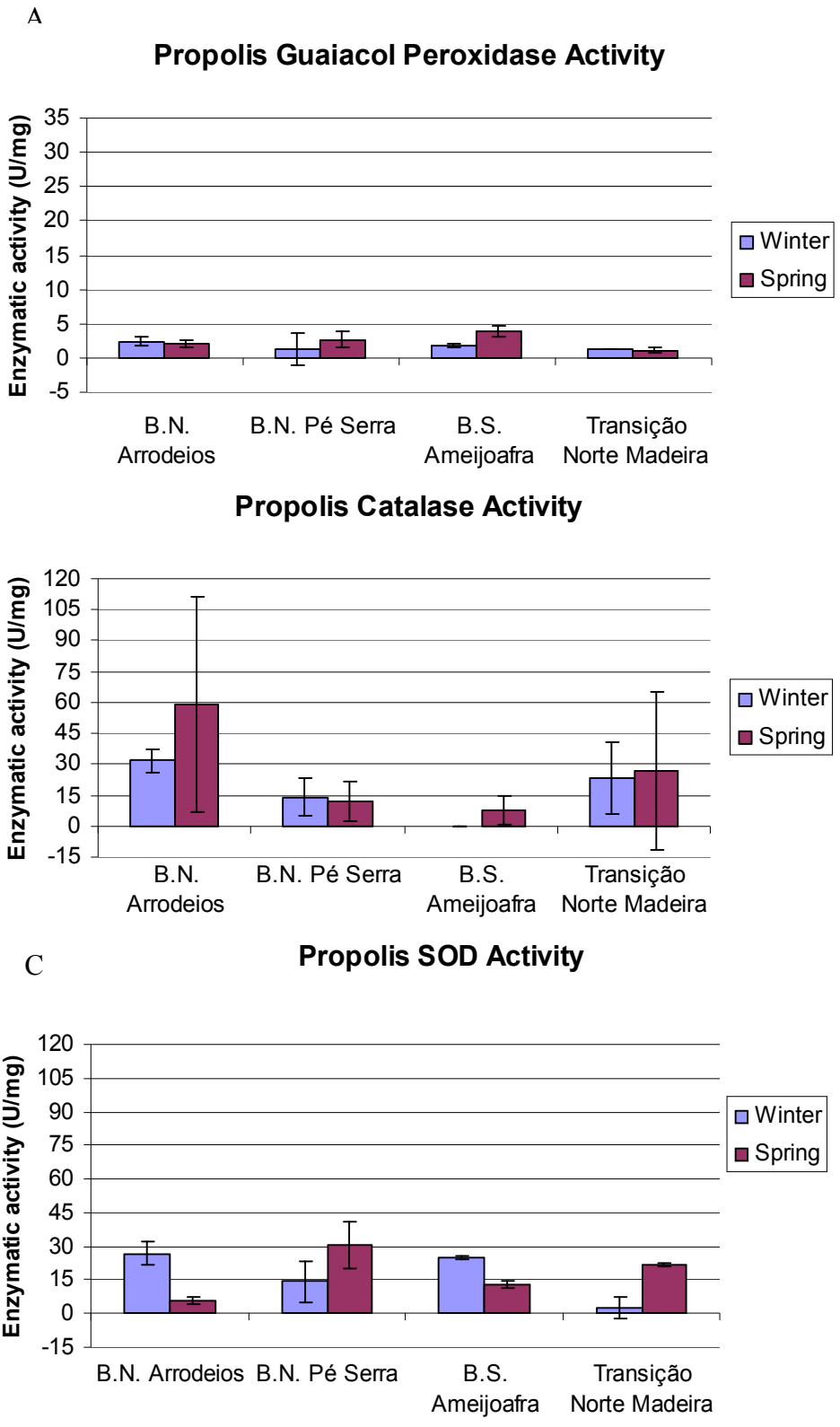

Figure 3.12 – Enzymatic propolis extracts activity for A - guaiacol peroxidase, B - catalase and C - superoxide dismutase (SOD), results are presented as specific enzymatic activity (U/mg).

Regarding propolis extracts protein concentration, no significant differences were observed between samples collected at different times except for samples from B. S. Arneijoafra. In this case, a decrease occurred from the sample collected at winter (1.444mg/ml) to the sample collected at spring (0.682mg/ml)(Fig. 3.13). However despite this difference in protein content we didn't observe large differences in the enzymatic activity, except in the case of SOD activity where a decrease between the activities of the two extracts is also observed (Fig. 3.12 C, for B.S. Arneijoafra). So, it seems there is no correlation between propolis protein content and the measured enzymatic activity. This could be caused probably by the existence of other enzymes in the extracts different to the ones tested or the presence of other molecules capable of a similar antioxidant activity, such as vitamins C, E and A, carotenoids or phenols [20]. Although most of these compounds wouldn't be easily extracted with the extraction method used since they are liposoluble.

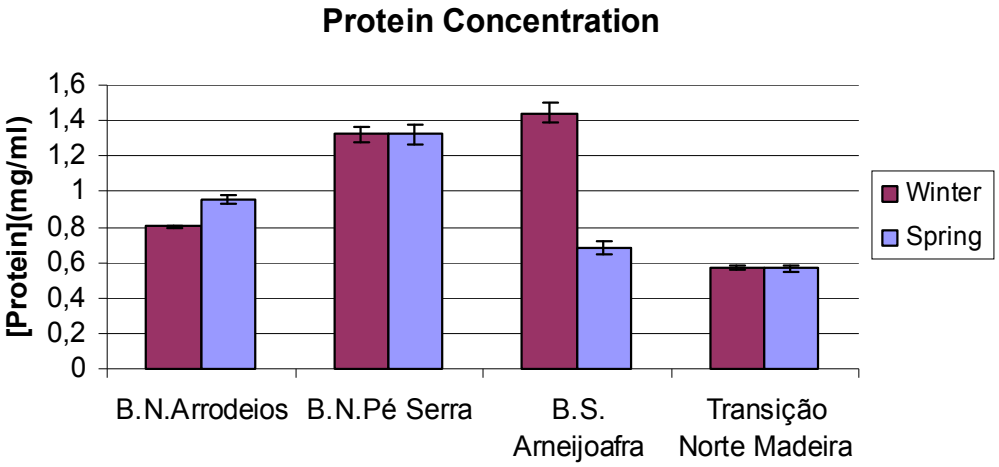

Figure 3.13 - Protein concentration of propolis enzymatic extracts.

In terms of geographical location, the samples with the highest protein concentration were from B.S. Arneijoafra collected at winter (1.444mg/ml) and B.N. Pé da Serra collected at winter (1.322mg/ml) and spring (1.323mg/ml).

Studies on the effect of propolis on the activities of SOD and catalase in human erythrocytes, *in vitro*, showed an increase in SOD and CAT activity and other studies also demonstrated a positive effect of propolis in CAT and SOD activities, acting as a detoxifier when used combined with a pharmaceutical drug such as cypermethrin or paclitaxel in rats [79-81]. However, these studies determine the effect that propolis extracts alone or combined with another substance have on antioxidant markers such as SOD, CAT and GPX and not the activity

of these enzymes in propolis extracts. Therefore, this is the first study on the enzymatic activity of propolis extracts.

3.4. HPLC evaluation of phenolic and polyphenolic acids from propolis

Based on the results obtained from the antibacterial activity, where differences in the activity of the three types of propolis extracts had been observed, an analysis of the extracts was done using HPLC. All the extracts and standard samples were analysed by HPLC, as described before.

The identification of the compounds was done by comparing the retention times of the standard samples with the ones from the samples of propolis and simultaneously by co-elution of the standard samples with the samples of propolis. The standard samples used were selected based on the compounds that had been previously identified on samples of European propolis [3, 5, 82]. An example of one the obtained chromatograms from an aqueous extracts is presented in Fig. 3.14 where the identified compounds are indicated and listed in Table 3.1.

Almost all of the identified compounds were identified in the aqueous extracts and some of these, namely caffeic acid, ferulic acid and galagin had already been described previously as compounds that have antimicrobial activity [2, 3, 5]. So, these compounds might be responsible, or at least partially responsible, for the antibacterial activity of the aqueous extracts.

As had been suggested by the antibacterial activity results, ethanolic and methanolic extracts which had similar activity also showed similarities in their chromatograms, as can be observed in Fig. 3.15 A e B. This reflects the similarity in the composition of the two extracts which correlates with the antibacterial activity results.

On the other hand, aqueous extracts which had fairly good results in the antibacterial activity, despite the difference in concentration between the other two types of extracts, gave origin to very different chromatograms (Fig. 3.14). This shows that, in fact, aqueous extracts have a different composition than ethanolic and methanolic extracts, as can be noticed when comparing the chromatograms of the different types of extracts (Fig. 3.14 and 3.15). The difference in composition is especially noticeable in Fig. 3.16 when chromatograms from two types of extracts are superimposed.

So, it seems that the antibacterial activity of the extracts is in fact related to its composition, as had been reported previously.

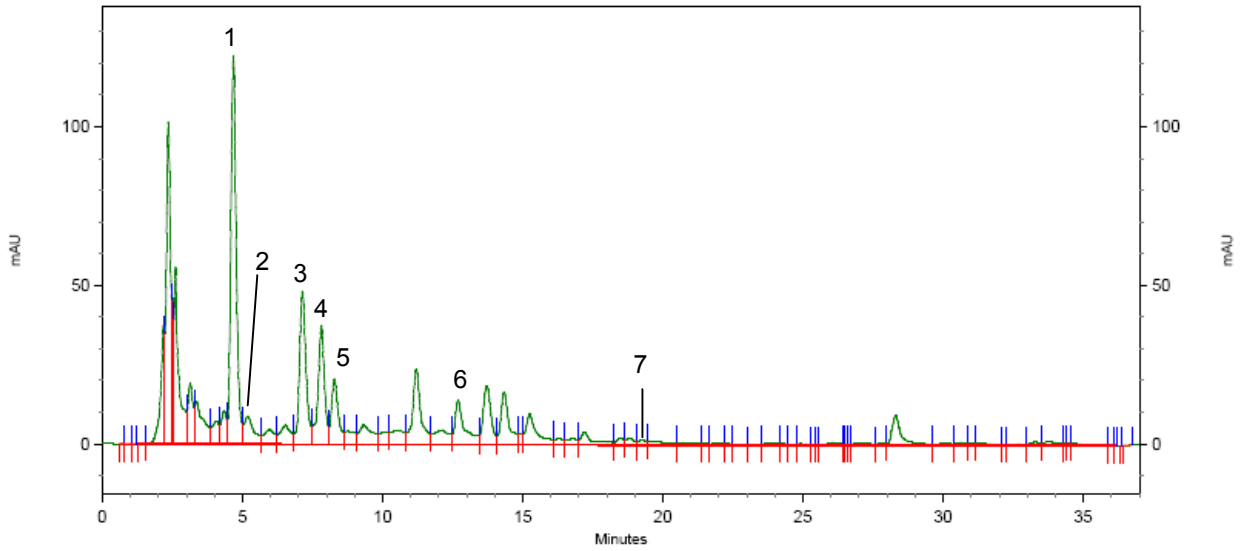

Figure 3.14 - HPLC chromatogram for a sample of a propolis aqueous extract.

Table 3.1 - Retention times of the standard samples.

Standard sample	Retention time (min)
1 – Caffeic acid	4.673
2 – Syringic acid	5.193
3 – Taxifolin	7.140
4 – Ferulic acid	7.813
5 – Diosmin	8.280
6 – Apigenin	12.120
7 – Galangin	19.907

The chromatograms were also compared to try to establish a correlation between the collection sites and times. In terms of location, propolis samples from B.N. Arrodeios, B.N. Pé da Serra and B.S. Arneijoafra are very similar among themselves and different from T.N. Madeira. This can be observed in Fig. 3.17 when chromatograms of extracts from B.N. Arrodeios and T. N. Madeira are superimposed.

The fact that composition variations are more noticeable in the samples from T.N. Madeira could be a reflection of differences in vegetation since this collection site was located in the northern side of the Salir region.

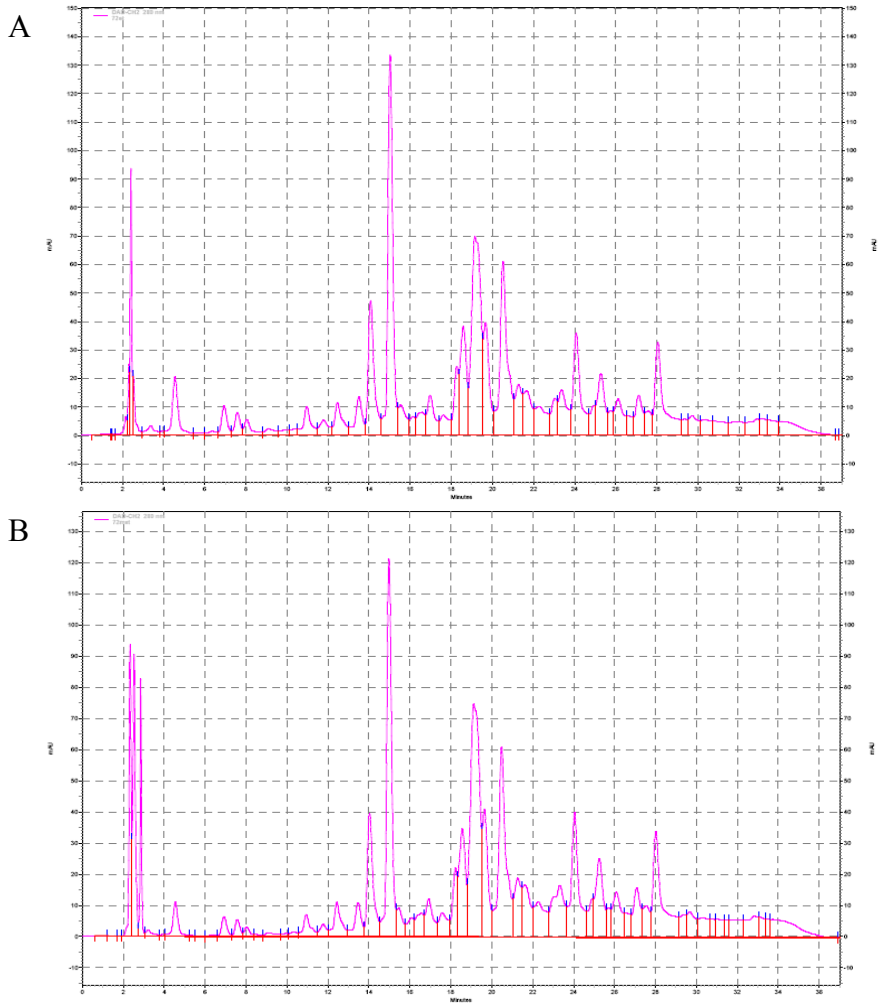

Figure 3.15 - HPLC chromatogram for a sample of an ethanolic (A) and a methanolic (B) extract.

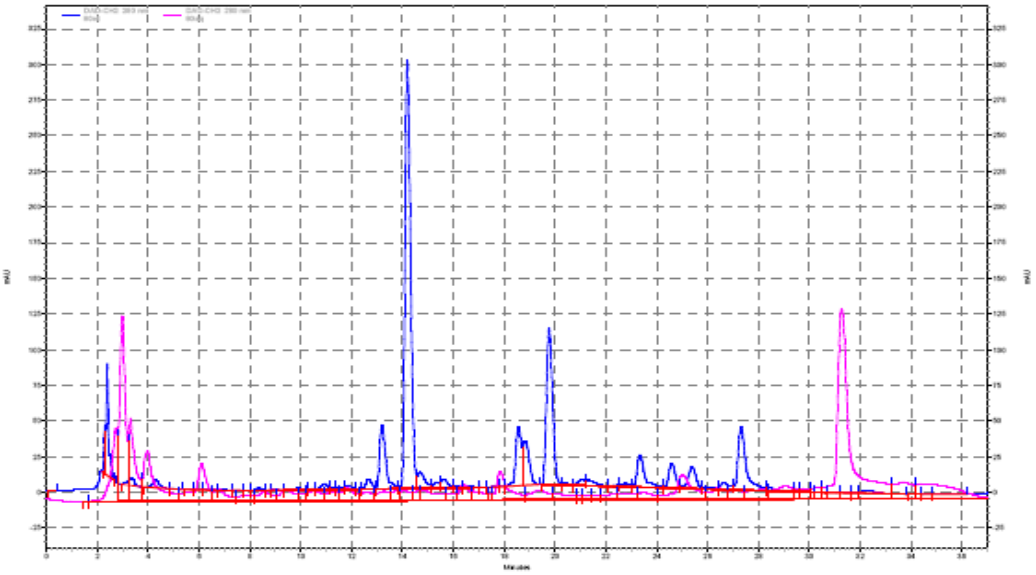

Figure 3.15 – Superimposed HPLC chromatograms of an aqueous extract (pink) with and ethanolic extract (blue).

In terms of collection time, small differences were observed for all the propolis samples, an example is given in Fig. 3.18. From the analyses of the superimposed chromatograms of extracts collected in different times it is noticeable that most retention times are the same indicating the presence of the same compounds and most peak areas vary indicating a change in concentration. So, the differences observed between collection times are mainly in concentration and not in composition. These results correlate with the antibacterial activity results that also demonstrated a variation in activity in relation to collection time.

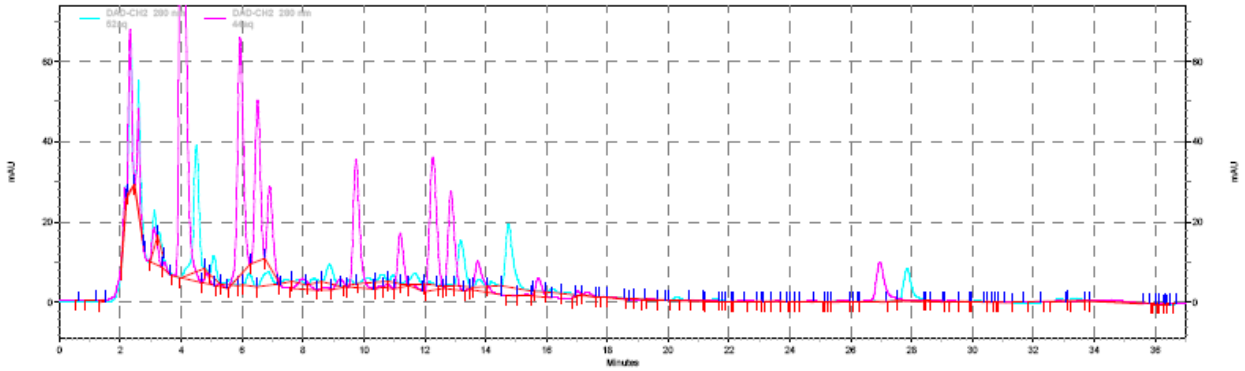

Figure 3.17 – Superimposed HPLC chromatograms of an aqueous extract from B.N. Arrodeios (pink) and T. N. Madeira (green).

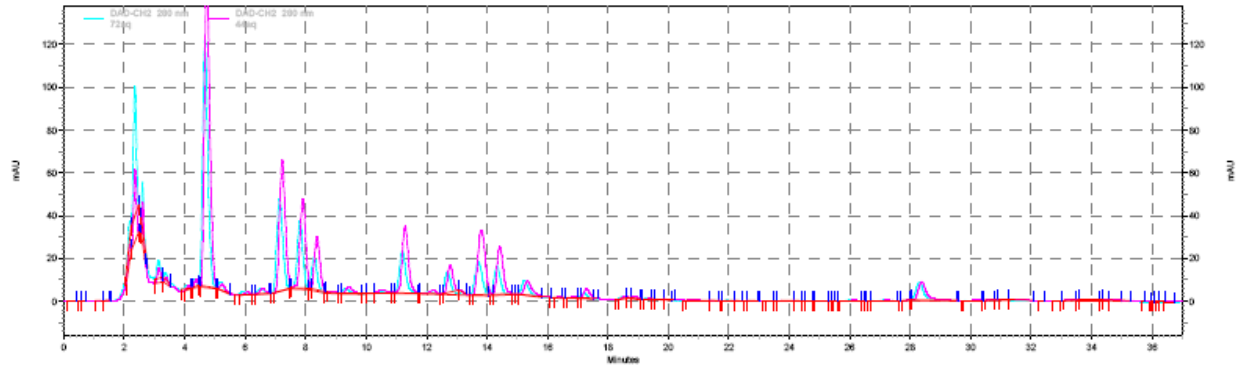

Figure 3.18 – Superimposed HPLC chromatograms of an aqueous extract from B.N. Arrodeios collected in winter (pink) and springtime (green).

4. Conclusions

On this study some of the biological activities of propolis extracts were evaluated. In addition, their chemical composition was also studied.

All of the strains of bacteria tested showed susceptibility to the diluted propolis extracts (1:10) and in the majority of cases in a dose-dependent way. Their sensitivity to propolis varied and considering the highest growth inhibition zone measured for each one, *Salmonella enterica* subspecie *enterica* serovar *thyphimurium* ATCC 14028 (27.333mm) was the least sensitive followed by *Staphylococcus aureus* CFSA2 (28.333mm), *Haemophilus influenza* TD-4 (31.000mm), *Streptococcus pneumonia* D39 (32.333mm), *H.pylori* J99 (33.667mm) and the most sensitive was *H.pylori* 26695 (35.667mm). Therefore, our results showed that propolis with Portuguese origin is active against both Gram-positive and Gram-negative bacteria, contrary to what most literature reports.

It is also important to note that the observed results were for different propolis extracts obtained with different solvents and that the use of a different extraction procedure and/or extraction solvent may have led to extracts exhibiting different antibacterial activity, as reported previously. In fact, our results showed for almost all samples that both ethanolic and methanolic extracts have a very similar activity, as expected. Aqueous extracts also showed, in most samples, a similar activity or slightly smaller activity to the ethanolic and methanolic extracts. This suggests that different solvents can extract compounds in propolis with antibacterial activity and that both types of solvents can be used since three types of propolis extracts showed antibacterial activity.

For a possible use in pharmacology it would be useful to know not only which compounds in propolis or propolis extracts have the highest activity but also the time of year in which they are present in higher concentration in propolis in order to facilitate extraction and purification. Our results showed a variation in antibacterial activity when comparing propolis samples collected in winter and spring time. With most of the tested bacteria there was an increase in activity in the samples collected in spring time but this was not true for *Haemophilus influenza* TD-4 and *Streptococcus pneumonia* D39. In this two there was a decrease in activity. These differences might reflect differences in propolis composition since it is known to vary with both location and collection time and also differences in the susceptibility of the tested bacteria.

In addition, the citotoxicity of propolis extracts in Caco-2 cells was studied. The overall result is positive since it was necessary to determine if propolis extracts when used in the same quantities as the ones that showed antibacterial activity would affect cell viability and what was

observed was that aqueous propolis extracts have no effect and ethanolic propolis extracts have a small decrease in cell viability. However, further studies are necessary in order to quantify the citotoxicity of propolis extracts, namely the determination of its IC_{50}.

Regarding the enzymatic antioxidant activity, superoxide dismutase, catalase and guaiacol peroxidase activities of samples of propolis harvested at two different times were tested. Our results showed that SOD activity was dependent on the collection time. SOD activity decreased drastically from winter to spring in samples from B. N. Arrodeios and B. S. Arneijoafra. An opposite feature was observed in samples from B. N. Pé da Serra and Transição Norte. SOD generates hydrogen peroxide and oxygen molecules. These hydrogen peroxide molecules can be converted into water molecules and oxygen by the action of catalase and guaiacol peroxidase. So, the samples with higher SOD activities should have higher CAT activities also. Such was not observed, which may suggest that the hydrogen peroxide elimination could be done by other antioxidant enzymes.

In respect with protein contents, major differences were not observed for samples harvested at winter and spring time, except for samples from B. S. Arneijoafra. In this case, a decrease occurred from the sample collected at winter to sample collected at spring.

The HPLC analysis confirmed what the antibacterial results had suggested. Analysing the chromatograms, ethanolic and methanolic extracts have similar chromatograms hence a similar composition, and aqueous extracts have a different composition in relation to the other two extracts. Small differences between chromatograms of samples from different collection times were also observed, and in terms of collection site all the samples were very similar except for the ones from T.N. Madeira that were different from the other three locations. Differences observed between collection times were mainly in concentration and not in composition, and correlated with the antibacterial activity results which also demonstrated a variation in activity in relation to collection time.

It is also important to note that this was the first study of enzymatic antioxidant activity of propolis and the first study of the antibacterial activity and citotoxicity of Portuguese propolis.

5. Future work

This study we evaluated some of the biological activities of propolis extracts. The antibacterial activity was analysed using the agar diffusion method which has some limitations in respect to the comparison of results. So, for comparison of results of antibacterial activity of Portuguese propolis with results from other countries it would be helpful to also test the antibacterial activity using another method and determining the MIC values. The good results obtained with *H. pylori* make interesting the continuation of the characterization of the antibacterial activity of propolis extracts with these bacteria. This includes assays that simulate the gastric environment to determine if the extracts maintain their activity in such conditions.

Citotoxicological studies showed that propolis extracts had a small negative effect on the cellular viability of Caco-2 cells. However, further studies are necessary in order to quantify the citotoxicity of propolis extracts, namely the determination of its IC_{50}. This should also include other types of cells and preferably larger numbers of propolis extracts.

We only focused on part of the enzymatic antioxidant activity, so it would also be interesting to extent the determination of antioxidant activity *in vivo*, as well as, the anti-inflammatory activity using adequate cell lines

The HPLC analysis of the extracts only enabled us the identify some of the compounds in propolis extracts. The identification of the rest of the compounds should be continued, as well as, the quantification of the ones that all ready have been identified. This would allow us to better understand the differences between propolis extracts.

6. Bibliography

1. Burdock, G.A. - Review of the Biological Properties and Toxicity of Bee Propolis (Propolis), Food and Chemical Toxicology 36, 347-363 (1998)

2. Castaldo, S., Capasso, F. – Propolis, an old remedy used in modern medicine, Fitoterapia 73 Suppl. 1, S1–S6 (2002)

3. Bankova, V.S., De Castro, S.L., Marcucci, M.C. - Propolis: recent advances in chemistry and plant origin, Apidologie 31, 3–15 (2000)

4. www.propolis-sana.com

5. Marcucci, M.C. - Propolis: chemical composition, biological properties and therapeutic activity, Apidologie 26, 83-99 (1995)

6. Bankova, V -Recent trends and important developments in propolis research, eCAM 2 (1), 29–32 (2005)

7. Banskota, A.H., Tezuka, Y., Kadota, S. - Recent Progress in Pharmacological Research of Propolis, Phytotherapy Research 15, 561–571 (2001)

8. Ahn, M.R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T. - Antioxidant activity and constituents of propolis collected in various areas of China, Food Chemistry 101, 1383–1392 (2007)

9. Pekmez, H., Kus, I., Colakoglu, N., Ogeturk, M., Ozyurt, H., Turkoglu, A.O., Sarsilmaz, M. - The protective effects of caffeic acid phenethyl ester (CAPE) against liver damage induced by cigarette smoke inhalation in rats, Cell Biochemistry and Function 25, 395–400 (2007)

10. Bankova, V. - Chemical diversity of propolis and the problem of standardization, Journal of Ethnopharmacology 100, 114–117 (2005)

11. Savickas, A., Majiene, D., Ramanauskiene, K., Pavilonis, A., Muselik, J., Masteikova, R., Chalupova, Z. - Chemical composition and antimicrobial activity of Lithuanian and Czech propolis, BIOLOGIJA 4, 59-63 (2005)

12. Trusheva, B., Popova, M., Bankova, V., Simova, S., Marcucci, M.C., Miorin, P.L., Pasin, F.R., Tsvetkova, I. - Bioactive Constituents of Brazilian Red Propolis, eCAM 3(2), 249–254 (2006)

13. Yaghoubi, S.M.J., Ghorbani, G.R., Soleimanian Zad, S., Satari, R. - Antimicrobial activity of Iranian propolis and its chemical composition, DARU 15(1), 45-48 (2007)

14. Mohammadzadeh, S., Shariatpanahi, M., Hamedi, M., Ahmadkhaniha, R., Samadi, N., Ostad, S.N. - Chemical composition, oral toxicity and antimicrobial activity of Iranian propolis, Food Chemistry 103, 1097–1103 (2007)

15. Vardar-Ünlü, G., Silici, S., Ünlü, M. - Composition and in vitro antimicrobial activity of *Populus* buds and poplar-type propolis, World J. Microbiol. Biotechnol. 24, 1011–1017 (2008)

16. Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S. - Antibacterial, antifungal and antiviral activity of propolis of different geographic origin, Journal of Ethnopharmacology 64, 235–240 (1999)

17. Halliwell, B. - Tell me about free radicals, doctor: a review, Journal of the Royal Society of Medicine 82, 747- 752 (1989)

18. Carpes, S.T. – Estudo das características físico-químicas e biológicas do pólen de Apis melífera *L. da região do Sul do Brasil*, Tese doutoramento, Universidade Federal do Paraná (2008)

19. Matés, J. M., Péres-Gómez, C., Castro, I.N. - Antioxidant Enzymes and Human Diseases, Clinical Biochemistry 32 (8), 595–603 (1999)

20. Bianchi, M.L.P., Antunes, L.M.G. - Free radicals and the main dietary antioxidants, Rev. Nutr. 12 (2), 123-130 (1999)

21. Matés, J.M., Segura, J.A., Alonso, F.J., Márquez, J. - Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis, Arch Toxicol 82, 273-299 (2008)

22. Mika, A., Lüthje, S. - Properties of Guaiacol Peroxidase Activities Isolated from Corn Root Plasma Membranes, Plant Physiology 132, 1489–1498 (2003)

23. Ghamsari, L., Keyhani, E., Golkhoo, S. - Kinetic Properties of Guaiacol Peroxidase Activity in *Crocus sativus L.* Corn during Rooting, Iranian Biomedical Journal 11 (3), 137-146 (2007)

24. Tsibris, J.C.M., Thomason, J.L., Kunigk, A., Khan-Dawood, F.S., Kirschner, C.V., Spellacy, W.N. - Guaiacol peroxidase levels in human cervical mucus: A possible predictor of ovulation, Contraception 25 (1), 59-67 (1982)

25. Cadenas, E., Packer, L. - Handbook of Antioxidants, Chap.1: Food-derived antioxidants, How to evaluate their importance in food and *in vivo*, Marcel Dekker Inc., 2nd edition (2002)

26. Yamasaki, H., Sakihama, Y., lkehara, N. - Flavonoid-Peroxidase Reaction as a Detoxification Mechanism of Plant Cells against H_2O_2, Plant Physiology 11 (5), 1405-1 412 (1997)

27. Sun, F., Hayami, S., Haruna, S., Ogiri, Y., Tanaka, K., Yamada, Y., Ikeda, K., Yamada, H., Sugimoto, H., Kawai, N., Kojo, S. - *In Vivo* Antioxidative Activity of Propolis Evaluated by the Interaction with Vitamins C and E and the Level of Lipid Hydroperoxides in Rats, J. Agric. Food Chem. 48 (5), 1462-1465 (2000)

28. Sheng, J., Zhou, J., Wang, L., Xu, J., Hu, Q. - Antioxidant activity of ethanol and petroleum ether extracts from Brazilian propolis, European Food Research Technology 225, 249–253 (2007)

29. Jun, X. - Comparison of Antioxidant Activity of Ethanolic Extracts of Propolis Obtained by Different Extraction Methods, The Canadian Journal of Chemical Engineering 84, 447-451 (2006)

30. Kumazawa, S., Hamasaka, T., Nakayama, T. - Antioxidant activity of propolis of various geographic origins, Food Chemistry 84, 329–339 (2004)

31. Moreira, L., Dias, L.G., Pereira, J.A., Estevinho, L. - Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal, Food and Chemical Toxicology 46, 3482-3485 (2008)

32. Orsi, R.O., Sforcin, J.M., Rall, V.L.M., Funari, S.R.C., Barbosa, L., Fernandes JR, A. - Susceptibility Profile of Salmonella against the Antibacterial Activity of Propolis Produced in Two Regions of Brazil, J. Venom. Anim. Toxins incl. Trop. Dis.11 (2), 109-116 (2005)

33. Orsi, R.O., Sforcin, J.M., Funari, S.R.C., Junior, A.F., Bankova, V. – Synergistic Effect of Propolis and Antibiotics on the *Salmonella* Typhi, Brazilian Journal of Microbiology 37, 108-112 (2006)

34. Endler, A.L., Oliveira, S.C., Amorim, C.A., Carvalho, M.P., Pileggi, M. - Test of the Efficiency of Propolis in Combating Pathogenic Bacteria of the Respiratory System, Ci. Biol. Saúde 9 (2), 17-20 (2003)

35. Todar's Online Textbook of Bacteriology, www.textbookofbacteriology.net

36. Boyanova, L., Gergova, G., Nikolov, R., Derejian, S., Lazarova, E., Katsarov, N., Mitov, I., Krastev, Z. - Activity of Bulgarian propolis against 94 Helicobacter pylori strains *in vitro* by agar-well diffusion, agar dilution and disc diffusion methods, Journal of Medical Microbiology 54, 481-483 (2005)

37. Gonsales, Z., Orsi, R. O., Fernandes JR, A., Rodrigues, P., Funari, S.R.C. - Antibacterial Activity of Propolis Collected in Different Regions of Brazil, J. Venom. Anim. Toxins incl. Trop. Dis. 12 (2), 276-284 (2006)

38. Nostro, A., Cellini, L., Di Bartolomeo, S., Cannatelli, M.A., Di Campli, E., Procopio, F., Grande, R., Marzio, L., Alonzo, V. - Effects of Combining Extracts (from Propolis or *Zingiber officinale*) with Clarithromycin on *Helicobacter pylori*, Phytotheraphy Research 20, 187–190 (2006)

39. Orsi, R. O., Sforcin, J. M., Funari, S. R. C., Fernandes-JR., A., Rodrigues, P., Bankova, V. – Effects of Propolis from Brazil and Bulgaria on *Salmonella* serovars, J. Venom. Anim. Toxins incl. Trop. Dis. 13 (4), 748-757 (2007)

40. Silici, S., Ünlü, M., Vardar-Ünlü, G. - Antibacterial activity and phytochemical evidence for the plant origin of Turkish propolis from different regions, World J. Microbiol. Biotechnol. 23, 1797-1803 (2007)

41. Salomão, K., Dantas, A.P., Borba, C.M., Campos, L.C., Machado, D.G., Aquino Neto, F.R., de Castro, S.L. - Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis, Letters in Applied Microbiology 38, 87-92 (2004)

42. Santos, F.A., Bastos, E.M.A., Uzeda, M., Carvalho, M.A.R., Farias, L.M., Moreira, E.S.A., Braga, F.C. - Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria, Journal of Ethnopharmacology 80, 1-7 (2002)

43. Banskota, A. H., Tezuka, Y., Adnyana, I. K., Ishii, E., Midorikawa, K., Matsushige, K., Kadota, S. - Hepatoprotective and anti-Helicobacter pylori activities of constituents from Brazilian propolis, Phytomedicine 8 (1), 16–23 (2001)

44. Nostro, A., Cellini, L., Di Bartolomeo, S., Di Campli, E., Grande, R., Cannatelli, M. A., Marzio, L., Alonzo, V. - Antibacterial Effect of Plant Extracts against *Helicobacter pylori*, Phytotherapy Research 19, 198-202 (2005)

45. Koo, H., Rosalen, P. L., Cury, J.A., Ambrosano, G.M.B.,. Murata, R.M, Yatsuda, R., Ikegaki, M., Alencar, S.M., Park, Y.K. - Effect of a New Variety of *Apis mellifera* Propolis on Mutans Streptococci, Current Microbiology 41, 192–196 (2000)

46. Castro, M.L., Cury, J.A., Rosalen, P.L., Alencar, S.M., Ikegaki, M., Duarte, S., Koo, H. – Propolis from Southeastern and Northeastern of Brazil: The Influence of Seasonality in Antibacterial Activity and Phenolic Composition, Quim. Nova 30 (7), 1512-1516 (2007)

47. Marcucci, M.C., Ferreres, F., García-Viguera, C., Bankova, V.S., De Castro, S.L., Dantas, A.P., Valente, P.H.M., Paulino, N. - Phenolic compounds from Brazilian propolis with pharmacological activities, Journal of Ethnopharmacology 74, 105–112 (2001)

48. Salomão, K., de Souza, E.M., Henriques-Pons, A., Barbosa, H.S., de Castro, S.L. - Brazilian Green Propolis: Effects *In Vitro* and *In Vivo* on *Trypanosoma cruzi*, eCAM, 1-12 (2009)

49. Sforcin, J. M., Fernandes-JR, A., LOPES, C. A. M., FUNARI, S. R. C., BANKOVA, V. - Seasonal Effect of Brazillian Propolis on *Candida albicans* and *Candida tropicalis*, J. Venom. Anim. Toxins 7 (1), 1-3 (2001)

50. Koc, A.N., Silici, S., Mutlu-Sariguzel, F., Sagdic, O. - Antifungal Activity of Propolis in Four Different Fruit Juices, Food Technol. Biotechnol. 45 (1), 57-61 (2007)

51. Alencar, S.M., Oldoni, T.L.C., Castro, M.L., Cabral, I.S.R., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L., Ikegaki, M. - Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis, Journal of Ethnopharmacology 113, 278–283 (2007)

52. Banskota, A.H., Nagaoka, T., Sumioka, L.Y., Tezuka, Y., Awale, S., Midorikawa, K., Matsushige, K., Kadota, S. - Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines, Journal of Ethnopharmacology 80, 67-73 (2002)

53. Bazo, A.P., Rodrigues, M.A.M., Sforcin, J.M., Camargo, J.L.V., Ribeiro, L.R., Salvadori, D.M.F. - Protective Action of Propolis on the Rat Colon Carcinogenesis, Teratogenesis, Carcinogenesis, and Mutagenesis 22, 183–194 (2002)

54. Amoros, M., Sauvager, F., Girre, L., Cormier, M. - In vitro antiviral activity of propolis, Apidologie 23, 231-240 (1992)

55. Gekker, G., Hua, S., Spivak, M., Lokensgard, J.R., Peterson, P.K. - Anti-HIV-1 activity of propolis in CD4+ lymphocyte and microglial cell cultures, Journal of Ethnopharmacology 102, 158–163(2005)

56. Bhadauria, M., Nirala, S.K., Shukla, S. - Propolis protects CYP 2E1 enzymatic activity and oxidative stress induced by carbon tetrachloride, Mol Cell Biochem. 302, 215–224 (2007)

57. Mirzoeva, O. K., Calder, P. C. - The effect of propolis and its components on eicosanoid production during the inflammatory response, Prostaglandins, Leukotrienes and Essential Fatty Acids 55(6), 441-449 (1996)

58. Sforcin, J.M., Orsi, R.O., Bankova, V. - Effect of propolis, some isolated compounds and its source plant on antibody production, Journal of Ethnopharmacology 98, 301–305(2005)

59. Sforcin, J.M. - Propolis and the immune system: a review, Journal of Ethnopharmacology 113, 1–14 (2007)

60. Ilhan, A., Koltuksuz, U., Ozen, S., Uz, E., Ciralik, H., Akyol, O. - The effects of caffeic acid phenethyl ester (CAPE) on spinal cord ischemia/reperfusion injury in rabbits, European Journal of Cardio-thoracic Surgery 16, 458-463 (1999)

61. www.natus.com.br

62. www.colgate.com.my

63. www.prodapys.com.br

64. Maps from Instituto Geográfico do Exército, www.igeoe.pt

65. Midorikawa, K., Banskota, A.H., Yasuhiro, T., Nagaoka, T., Matsushige, K.,Message, D., Huertas, A.A.G., Kadota, S.- Liquid Chromatography-Mass Spectrometry Analysis of Propolis, Phytochemical Anal. 12, 366-373 (2001)

66. Popova, M., Bankota, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B., Sabatini, A.G., Marcazzan, G.L., Bogdanov, S. – Validated Methods for the Quantification of Biologically Active Constituents of Poplar-type Propolis, Phytochemical Analytical 15, 235-240 (2004)

67. Faleiro, M.L., Miguel, M.G., Ladeiro, F., Venâncio, F., Tavares, R., Brito, J.C., Figueiredo, A.C., Barroso, J.G., Pedro, L.G. - Antimicrobial activity of essential oils isolated from

Portuguese endemic species of *Thymus*, Letters in Applied Microbiology 36, 35-40 (2003)

68. Hazzit, M., Baaliouamer, A., Veríssimo, A.R., Faleiro, M.L., Miguel, M.G. - Chemical composition and biological activities of Algerian *Thymus* oils, Food Chemistry 116, 714-721 (2009)

69. Beers, R.F., Sizer, I.A. – Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, Journal of Biological Chemistry 195, 133-140 (1952)

70. Beauchamp, C., Fridovich, I. – Superoxide Dismutase: improved assays and an assay applicable to acrylamide gels, Analytical Biochemistry 44, 276-287 (1971)

71. Bajji, M., Kinet, J.M., Lutts, S. - Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures, Plant Science 137, 131-142 (1998)

72. Bradford, M.M. – A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72, 248-254 (1976)

73. Croci, A.N., Cioroiu, B., Lazar, D., Corciova, A., Ivanescu, B., Lazar, M.I. - HPLC evaluation of phenolic and polyphenolic acids from propolis, Farmacia, 57 (1), 52-57 (2009)

74. Seidel, V., Peyfoon, E., Watson, D.G., Fearnley, J. - Comparative Study of the Antibacterial Activity of Propolis from Different Geographical and Climatic Zones, Phytotheraphy Research, 22, 1256–1263 (2008)

75. Russo, A., Cardile, V., Sanchez, F., Troncoso, N., Vanella, A., Garbarino, J.A. - Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines, Life Sciences 76, 545–558 (2004)

76. Watson, A. J., Askew, J. N., Sandle, G. I. - Characterisation of oxidative injury to an intestinal cell line (HT-29) by hydrogen peroxide, Gut, 35, 1575-1581 (1994)

77. Ohguro, N., Fukuda, M., Sasabe, T., Tano, Y. - Concentration dependent effects of hydrogen peroxide on lens epithelial cells, British Journal of Ophthalmology, 83, 1064-1068 (1999)

78. Eisenthal, R. Danson, M.J. – Enzyme assays, Practical approach:257, Chap. 2: Photometric assays, Oxford University Press, 2nd edition (2002)

79. Girgin, G., Baydar, T., Tuylu, A.O., Sorkun, K., Salih, B., Sahin, G. - Effect of various propolis on erythrocyte superoxide dismutase and catalase activities: A preliminary study, Toxicology Letters 164S, S138 (2006)

80. Eraslan, G., Kanbur, M., Sicili, S., Altinordulu, S., Karabacak, M. – Effects of Cypermetherin on Some Biochemical Changes in Rats: The Protective Role of Propolis, Experimental Anim. 57 (5), 453-460 (2008)

81. Padmavathi, R., Senthilnathan, P., Chodon, D., Sakthisekaran, D. - Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7,12-dimethyl

benz(a)anthracene-induced breast cancer in female Sprague Dawley rats, Life Sciences 78, 2820-2825 (2006)

82. Bankova, V., Popova, M., Bogdanov, S., Sabatini, A.G. - Chemical Composition of European Propolis: Expected and Unexpected Results, Z. Naturforschung 57c, 530-533 (2002)

7. Annexes

7.1. Statistical analysis of the antibacterial activity results

Table 7.1 – Growth inhibition zone diameters (mm).

Collection Time	Sample	*S. aureus*		*L. monocytogenes*		*S. enterica*	
		Mean[1]	SD	Mean	SD	Mean	SD
Winter	44Aq	6.000^{b}	0.000	7.333^{def}	0.577	6.000^{c}	0.000
	44Et	6.000^{b}	0.000	8.333^{bcde}	1.155	6.667^{bc}	0.577
	44Mt	6.000^{b}	0.000	7.333^{def}	0.577	8.000^{bc}	1.732
	45Aq	6.000^{b}	0.000	7.000^{ef}	0.000	6.667^{bc}	1.155
	45Et	6.000^{b}	0.000	8.000^{bcdef}	1.000	7.000^{bc}	1.000
	45Mt	6.000^{b}	0.000	7.000^{ef}	0.000	7.000^{bc}	1.000
	48Aq	6.667^{b}	0.577	7.333^{def}	0.577	7.333^{bc}	0.577
	48Et	6.667^{b}	0.577	7.333^{def}	1.155	8.333^{bc}	0.577
	48Mt	6.667^{b}	0.577	8.333^{bcde}	0.577	7.000^{bc}	1.732
	52Aq	6.667^{b}	0.577	7.000^{ef}	0.000	7.000^{bc}	1.732
	52Et	6.667^{b}	0.577	7.333^{def}	0.577	6.667^{bc}	1.155
	52Mt	6.667^{b}	0.577	8.333^{bcde}	1.527	6.333^{bc}	0.577
Spring	72Aq	6.667^{b}	0.577	7.667^{cdef}	0.577	6.000^{c}	0.000
	72Et	7.000^{b}	1.000	7.667^{cdef}	0.577	6.667^{bc}	0.577
	72Mt	7.000^{b}	0.000	7.667^{cdef}	0.577	8.000^{bc}	1.000
	73Aq	6.667^{b}	1.155	7.667^{cdef}	0.577	6.333^{bc}	0.577
	73Et	6.667^{b}	1.155	8.667^{bcd}	0.577	6.333^{bc}	0.577
	73Mt	7.333^{b}	0.577	9.000^{bc}	1.000	7.667^{bc}	1.527
	76Aq	7.000^{b}	1.000	7.667^{cdef}	0.577	6.000^{c}	0.000
	76Et	7.000^{b}	1.000	9.333^{b}	0.577	6.667^{bc}	1.155
	76Mt	7.333^{b}	1.155	8.667^{bcd}	1.155	7.000^{bc}	1.000
	80Aq	6.333^{b}	0.577	7.000^{ef}	1.732	6.000^{c}	0.000
	80Et	7.333^{b}	1.155	9.333^{b}	0.577	6.333^{bc}	0.577
	80Mt	6.667^{b}	1.155	8.000^{bcdef}	0.000	6.333^{bc}	0.577
	C+	18.375^{a}	1.408	25.000^{a}	0.000	22.700^{a}	1.888
	C-	6.167^{b}	0.389	6.583^{f}	0.793	6.000^{c}	0.000

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C+ - positive control (30µg chloranphenicol), C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.2 - Inhibition zone diameters (mm) for *Salmonella enterica* serovar *thyphimurium*.

Collection time	Volume (µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	13.667^{b}	0.577	17.333^{bcd}	0.577	21.333^{bcde}	1.155	24.667^{cdef}	0.577
	44Et	13.333^{bc}	1.155	17.667^{bcd}	1.527	21.667^{bcd}	1.527	25.000^{bcde}	0.000
	44Mt	13.000^{bcd}	0.000	18.000^{bc}	0.000	20.333^{cdefg}	0.577	24.000^{cdefg}	1.732
	45Aq	12.000^{def}	0.000	15.667^{fgh}	1.527	18.333^{h}	0.577	20.667^{hi}	1.155
	45Et	12.333^{cde}	0.577	15.333^{gh}	0.577	19.667^{efgh}	0.577	22.667^{fgh}	0.577
	45Mt	12.333^{cde}	0.577	16.667^{def}	0.577	19.000^{fgh}	1.000	23.000^{efg}	1.000
	48Aq	11.000^{fg}	1.000	14.667^{hi}	0.577	18.333^{h}	0.577	20.667^{hi}	1.155
	48Et	8.333^{i}	0.577	11.667^{l}	0.577	13.667^{jk}	0.577	16.000^{kl}	0.000
	48Mt	9.667^{h}	0.577	11.667^{l}	0.577	15.000^{ij}	1.732	17.500^{jk}	0.707
	52Aq	10.667^{g}	1.527	13.000^{jk}	2.646	13.000^{k}	2.828	15.000^{l}	2.000
	52Et	9.333^{hi}	0.577	12.667^{kl}	0.577	18.667^{gh}	0.577	18.333^{j}	1.527
	52Mt	9.000^{hi}	0.000	11.667^{l}	0.577	15.667^{i}	0.577	19.000^{ij}	2.000
Spring	72Aq	12.333^{cde}	1.155	17.333^{bcd}	0.577	20.667^{cdef}	2.082	22.667^{fgh}	0.577
	72Et	13.667^{b}	0.577	18.333^{bc}	0.577	20.333^{cdefg}	0.577	24.333^{cdef}	0.577
	72Mt	13.667^{b}	0.577	18.667^{b}	0.577	22.000^{bc}	1.732	25.333^{c}	1.155
	73Aq	11.333^{efg}	0.577	15.333^{gh}	0.577	20.000^{defgh}	1.732	24.000^{cdefg}	1.000
	73Et	14.000^{b}	1.000	18.667^{b}	0.577	21.667^{bcd}	0.577	24.333^{cdef}	1.155
	73Mt	13.667^{b}	0.577	18.667^{b}	0.577	20.333^{cdefg}	1.155	27.333^{a}	1.527
	76Aq	11.333^{efg}	1.155	14.667^{hi}	0.577	21.667^{bcd}	0.577	23.333^{defg}	0.577
	76Et	14.000^{b}	1.000	18.333^{bc}	0.577	21.667^{bcd}	0.577	25.667^{abc}	2.517
	76Mt	14.000^{b}	1.000	18.333^{bc}	0.577	23.000^{b}	3.464	27.000^{ab}	2.646
	80Aq	12.000^{def}	0.000	17.000^{cde}	1.000	19.000^{fgh}	1.000	22.000^{gh}	1.000
	80Et	12.000^{def}	1.000	16.000^{efg}	1.000	21.333^{bcde}	0.577	24.000^{cdefg}	1.000
	80Mt	12.000^{def}	0.000	14.000^{ij}	1.000	21.667^{bcd}	0.577	24.333^{cdef}	1.155
	C+	24.667^{a}	0.577	24.667^{a}	0.577	24.667^{a}	0.577	24.667^{cdef}	0.577
	C-	6.000^{j}	0.000	6.000^{m}	0.000	6.000^{l}	0.000	6.000^{m}	0.000

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C+ - positive control (30µg chloranphenicol), C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.3 - Growth inhibition zone diameters (mm) for *S. aureus*CFSA2.

Collection time	Volume (µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	10.000^{i}	1.732	12.667^{l}	0.577	15.667^{h}	3.786	23.000^{defg}	1.000
	44Et	14.667^{bcd}	0.577	19.333^{bcd}	0.577	22.667^{bc}	0.577	25.000^{bcde}	0.000
	44Mt	14.333^{bcd}	0.577	18.667^{cdef}	1.527	22.333^{bc}	1.155	25.333^{bcd}	0.577
	45Aq	10.333^{hi}	0.577	14.000^{kl}	0.000	18.000^{fgh}	1.000	22.000^{ghi}	0.000
	45Et	12.000^{efgh}	0.000	17.333^{efg}	0.577	20.667^{cde}	0.577	24.667^{cdef}	0.577
	45Mt	13.000^{defg}	0.000	17.667^{defgh}	0.577	22.000^{bc}	1.000	25.333^{bcd}	0.577
	48Aq	10.667^{hi}	0.577	13.333^{kl}	0.577	17.333^{fgh}	1.155	20.000^{i}	0.000
	48Et	10.000^{i}	1.000	13.667^{kl}	0.577	17.667^{fgh}	0.577	20.333^{hi}	1.155
	48Mt	11.000^{hi}	1.414	16.000^{hij}	1.000	18.333^{efg}	0.577	21.000^{ghi}	0.000
	52Aq	11.000^{hi}	0.000	14.000^{kl}	0.000	19.000^{efg}	4.243	20.000^{i}	2.828
	52Et	10.000^{i}	1.000	14.333^{jkl}	0.577	18.667^{efg}	0.577	23.000^{defg}	0.000
	52Mt	10.667^{hi}	1.527	14.000^{kl}	2.000	16.667^{gh}	1.527	20.000^{i}	0.000
Spring	72Aq	13.667^{cde}	0.577	17.333^{efg}	1.154	18.667^{efg}	3.214	22.333^{fghi}	4.619
	72Et	13.000^{defg}	0.000	17.333^{efg}	0.577	21.333^{bcd}	0.577	25.000^{bcde}	1.732
	72Mt	12.000^{efgh}	1.732	17.000^{fgh}	1.000	23.667^{ab}	0.577	26.333^{abc}	0.577
	73Aq	11.333^{ghi}	0.816	14.667^{ijk}	1.633	16.167^{gh}	2.317	17.167^{j}	1.472
	73Et	14.333^{bcd}	1.527	19.667^{bc}	0.577	23.500^{ab}	2.121	22.333^{fghi}	4.619
	73Mt	15.333^{bc}	0.577	19.333^{bcd}	0.577	25.333^{a}	0.577	27.333^{ab}	0.577
	76Aq	13.000^{defg}	0.000	17.000^{fgh}	1.000	19.667^{def}	1.527	23.333^{defg}	0.577
	76Et	16.000^{bcd}	1.000	18.000^{cdefg}	1.000	22.333^{bc}	0.577	26.000^{abc}	0.000
	76Mt	15.667^{b}	0.577	20.667^{ab}	0.577	25.000^{a}	1.000	26.333^{abc}	0.577
	80Aq	11.667^{fghi}	1.527	16.333^{ghi}	1.527	17.500^{fgh}	0.707	22.667^{efgh}	0.577
	80Et	15.333^{bc}	0.577	19.667^{bc}	0.577	25.333^{a}	0.577	28.333^{a}	2.887
	80Mt	13.333^{def}	0.577	19.000^{bcde}	0.000	23.000^{abc}	0.000	26.000^{abc}	1.000
	C+	21.667^{a}	1.633	21.667^{a}	1.633	21.667^{bcd}	1.633	21.667^{ghi}	1.633
	C-	6.500^{j}	0.548	6.500^{m}	0.548	6.500^{i}	0.548	6.500^{k}	0.548

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C+ - positive control (30µg chloranphenicol), C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.4 - Inhibition zone diameters (mm) for *H.pylori* J99.

Collection time	Volume (µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	9.333^{bc}	0.577	14.333^{bc}	0.577	21.333^{bcde}	3.055	23.000^{bcd}	2.646
	44Et	11.667^{bc}	1.155	19.000^{b}	1.000	23.000^{bcd}	1.732	26.667^{abc}	2.887
	44Mt	11.000^{bc}	0.000	17.000^{bc}	2.000	19.333^{bcde}	3.786	24.000^{bc}	3.605
	45Aq	0.000^{c}	0.000	0.000^{d}	0.000	0.000^{f}	0.000	9.500^{cde}	0.707
	45Et	0.000^{c}	0.000	0.000^{d}	0.000	5.667^{def}	4.933	3.000^{e}	5.196
	45Mt	10.000^{bc}	1.732	15.000^{bc}	0.000	22.000^{bcde}	3.464	27.000^{ab}	2.646
	48Aq	10.333^{bc}	0.577	17.667^{b}	2.517	20.500^{bcde}	2.121	25.333^{abc}	0.577
	48Et	11.000^{bc}	1.414	16.000^{bc}	1.414	24.000^{bc}	2.828	30.000^{ab}	0.000
	48Mt	11.000^{bc}	4.000	18.000^{b}	2.000	19.667^{bcde}	0.577	28.333^{ab}	8.505
	52Aq	5.000^{bc}	0.000	8.000^{bc}	1.732	4.667^{ef}	4.041	7.667^{de}	1.155
	52Et	13.000^{bc}	2.828	21.000^{b}	1.414	31.667^{ab}	7.638	30.333^{ab}	1.155
	52Mt	0.000^{c}	0.000	0.000^{d}	0.000	22.667^{bcd}	2.082	25.667^{abc}	1.155
Spring	72Aq	13.333^{bc}	2.082	19.667^{b}	0.577	21.667^{bcde}	0.577	26.667^{abc}	0.577
	72Et	15.667^{bc}	3.512	23.333^{b}	1.527	32.333^{ab}	6.429	32.667^{ab}	4.933
	72Mt	15.333^{bc}	2.309	21.333^{b}	1.155	27.667^{ab}	1.527	28.000^{ab}	1.732
	73Aq	15.333^{bc}	0.577	20.000^{b}	1.000	22.000^{bcde}	1.000	26.000^{abc}	1.000
	73Et	19.000^{b}	1.000	24.333^{b}	2.082	23.000^{bcd}	2.000	27.667^{ab}	4.619
	73Mt	18.333^{bc}	1.155	23.000^{b}	0.000	24.333^{bc}	3.055	27.000^{ab}	1.000
	76Aq	14.333^{bc}	1.155	19.667^{b}	0.577	23.333^{bcd}	1.155	27.667^{ab}	1.155
	76Et	16.000^{bc}	1.000	22.333^{b}	1.155	25.333^{ab}	0.577	29.667^{ab}	1.527
	76Mt	16.333^{bc}	2.082	21.333^{b}	2.082	25.000^{b}	0.000	31.333^{ab}	2.517
	80Aq	14.333^{bc}	1.527	16.000^{bc}	2.646	20.000^{bcde}	1.732	23.667^{bc}	1.155
	80Et	18.000^{bc}	1.732	23.000^{b}	2.000	27.000^{ab}	2.646	33.667^{ab}	2.517
	80Mt	18.333^{bc}	0.577	22.667^{b}	1.527	26.667^{ab}	6.028	31.667^{ab}	0.577
	C+	42.250^{a}	16.499	42.250^{a}	16.499	42.250^{a}	16.499	42.250^{a}	16.499
	C-	6.875^{c}	1.642	6.875^{c}	1.642	6.875^{cdef}	1.642	6.875^{e}	1.642

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C+ - positive control (30µg chloranphenicol), C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.5 - Inhibition zone diameters (mm) for *H.pylori* 26695.

Collection time	Volume (µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	16.333^{bcdef}	1.527	19.667^{fgh}	0.577	23.667^{ghij}	0.577	26.000^{fg}	1.000
	44Et	19.333^{b}	0.577	25.333^{bc}	0.577	31.333^{b}	2.082	35.667^{ab}	0.577
	44Mt	19.333^{b}	0.577	25.000^{bc}	2.000	30.667^{bc}	2.082	33.667^{bcd}	1.155
	45Aq	13.333^{fgh}	0.577	18.000^{hi}	0.000	21.667^{ij}	0.577	23.667^{gh}	2.082
	45Et	18.333^{bc}	0.577	24.000^{bcd}	1.000	29.000^{bcd}	1.000	32.000^{bcd}	0.000
	45Mt	16.667^{bcde}	1.155	23.000^{bcde}	0.000	30.667^{bc}	3.786	34.000^{abc}	1.732
	48Aq	13.000^{gh}	1.732	18.000^{hi}	0.000	22.000^{ij}	2.000	24.667^{gh}	0.577
	48Et	19.333^{b}	1.155	25.333^{bc}	0.577	25.667^{defgh}	2.309	33.000^{bcd}	1.000
	48Mt	19.997^{b}	0.577	24.333^{bc}	1.155	27.333^{cdef}	0.577	33.667^{bcd}	0.577
	52Aq	14.333^{defgh}	1.55	20.000^{efgh}	1.412	24.5^{efghi}	0.707	27.000^{efg}	0.000
	52Et	16.333^{bcdef}	4.726	24.667^{bc}	4.163	31.000^{bc}	1.000	35.333^{ab}	3.055
	52Mt	15.333^{cdefg}	1.527	21.000^{defgh}	1.000	27.333^{cdef}	3.055	27.333^{efg}	8.083
Spring	72Aq	14.667^{defgh}	0.577	19.333^{gh}	0.577	22.333^{hij}	1.155	26.000^{fg}	1.000
	72Et	17.667^{bcd}	0.577	22.667^{cdef}	0.577	28.333^{bcd}	0.577	35.333^{ab}	0.577
	72Mt	19.333^{b}	1.155	24.333^{bc}	2.887	28.667^{bcd}	2.309	31.333^{cd}	1.155
	73Aq	14.667^{defgh}	1.527	21.000^{defgh}	1.000	24.000^{fghij}	1.732	23.667^{gh}	2.082
	73Et	19.500^{b}	0.707	26.000^{b}	1.414	26.000^{defg}	3.464	27.000^{efg}	7.071
	73Mt	16.667^{bcde}	2.082	22.667^{cdef}	2.082	27.667^{bcde}	3.786	31.000^{cd}	2.000
	76Aq	13.500^{efgh}	1.643	19.167^{h}	2.858	21.833^{ij}	1.472	25.000^{gh}	1.673
	76Et	17.333^{bcd}	4.933	22.333^{cdefg}	3.214	28.000^{bcde}	0.000	27.000^{efg}	4.582
	76Mt	17.200^{bcd}	2.683	24.000^{bcd}	1.414	28.167^{bcd}	2.041	29.833^{def}	1.602
	80Aq	12.000^{h}	0.000	15.333^{i}	0.577	20.667^{j}	1.155	21.333^{h}	2.309
	80Et	16.667^{bcde}	1.527	22.667^{cdef}	3.055	29.333^{bcd}	5.033	30.000^{de}	0.000
	80Mt	15.667^{cdefg}	2.082	23.333^{bcd}	2.309	28.333^{bcd}	2.887	33.000^{bcd}	2.000
	C+	37.667^{a}	2.517	37.667^{a}	2.517	37.667^{a}	2.517	37.667^{a}	2.517
	C-	6.000^{i}	0.000	6.000^{j}	0.000	6.000^{k}	0.000	6.000i	0.000

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C+ - positive control (30µg chloranphenicol), C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.6 - Inhibition zone diameters (mm) for *S. pneumonea*.

Collection time	Volume(µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	10.000^{fghi}	0.000	13.500^{ghij}	0.707	19.000^{defghij}	1.732	21.333^{efghij}	3.214
	44Et	16.000^{cde}	5.000	20.000^{bcdef}	3.000	27.000^{b}	2.000	30.000^{abc}	4.359
	44Mt	16.000^{cde}	3.000	19.000^{bcdefg}	4.582	23.667^{bcdef}	3.512	30.000^{abc}	2.828
	45Aq	9.333^{ghi}	1.527	12.333^{hij}	2.082	17.500^{fghij}	0.707	18.667^{ij}	2.517
	45Et	17.000^{cd}	4.359	24.667^{b}	2.082	26.000^{bc}	3.605	32.333^{ab}	2.517
	45Mt	15.667^{cdef}	1.155	21.500^{bcde}	0.707	26.333^{bc}	4.163	23.000^{defghij}	2.646
	48Aq	11.000^{defghi}	1.000	15.333^{efghij}	0.577	17.000^{ghij}	2.000	20.500^{fghij}	0.707
	48Et	15.000^{cdefg}	0.000	19.000^{bcdefg}	1.414	20.000^{cdefgh}	1.000	24.000^{cdefghi}	1.000
	48Mt	10.333^{efghi}	0.577	15.667^{efghij}	4.041	19.333^{defghi}	4.041	29.333^{abc}	3.055
	52Aq	12.333^{cdefgh}	2.309	16.333^{efghi}	2.309	15.667^{hij}	2.887	19.667^{ghij}	1.155
	52Et	17.333^{c}	3.786	23.333^{bc}	6.506	26.333^{bc}	4.509	25.333^{cdefg}	4.933
	52Mt	11.667^{cdefghi}	0.577	19.667^{bcdef}	3.512	26.000^{bc}	4.582	29.667^{abc}	2.517
Spring	72Aq	11.667^{cdefghi}	4.163	16.667^{defghi}	4.933	18.333^{efghij}	2.887	19.000^{hij}	2.646
	72Et	15.333^{cdefg}	3.512	19.000^{bcdefg}	4.000	25.000^{bcd}	5.000	29.667^{abc}	2.517
	72Mt	14.000^{cdefg}	4.359	18.667^{bcdefgh}	4.041	24.000^{bcde}	5.568	25.000^{cdefgh}	3.605
	73Aq	13.000^{cdefgh}	1.000	15.000^{efghij}	1.000	18.000^{efghij}	2.646	22.000^{defghij}	2.646
	73Et	12.000^{cdefghi}	1.000	16.333^{efghi}	1.155	21.333^{bcdefgh}	1.527	26.333^{bcdef}	1.155
	73Mt	11.000^{defghi}	1.000	17.000^{cdefghi}	1.000	21.667^{bcdefgh}	2.082	27.000^{bcde}	1.000
	76Aq	7.667^{hi}	0.577	10.000^{jk}	0.000	13.333^{ij}	1.527	20.000^{ghij}	1.732
	76Et	10.000^{efghi}	1.000	14.667^{fghij}	1.527	23.000^{bcdefg}	2.000	25.333^{cdefg}	2.082
	76Mt	11.000^{defghi}	1.000	16.000^{efghij}	1.732	22.000^{bcdefgh}	1.732	26.333^{bcdef}	2.082
	80Aq	7.000^{hi}	0.000	11.667^{ijk}	0.577	13.000^{j}	1.000	17.333^{j}	1.527
	80Et	13.000^{cdefgh}	1.000	17.000^{cdefghi}	1.000	21.667^{bcdefgh}	2.082	27.000^{bcde}	2.646
	80Mt	12.000^{cdefghi}	0.000	17.000^{cdefghi}	1.000	23.000^{bcdefg}	1.732	27.667^{abcd}	1.527
	C1+	22.833^{b}	2.562	22.833^{bcd}	2.562	22.833^{bcdefg}	2.562	22.833^{defghij}	2.562
	C2+	33.167^{a}	6.242	33.167^{a}	6.242	33.167^{a}	6.242	33.167^{a}	6.242
	C-	6.167^{i}	0.408	6.167^{k}	0.408	6.167^{k}	0.408	6.167^{k}	0.408

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C1+ - positive control (30µg chloranphenicol),C2+ - positive control (10ng penicillin) C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.7 - Inhibition zone diameters (mm) for *Haemophilus influenza*.

Collection time	Volume(µl)	5		10		15		20	
	Sample	Mean[1]	SD	Mean	SD	Mean	SD	Mean	SD
Winter	44Aq	13.667^{cdefg}	0.577	18.667^{defghi}	0.577	23.667^{cdefghi}	1.527	24.667^{defghi}	0.577
	44Et	15.667^{cde}	0.577	21.333^{cdefg}	1.155	25.667^{cdef}	0.577	28.333^{cdefg}	0.577
	44Mt	14.000^{cdefg}	1.000	19.333^{cdefgh}	1.527	22.333^{cdefghi}	1.527	28.667^{cdef}	1.155
	45Aq	10.333^{fgh}	0.577	14.000^{ij}	2.646	19.000^{hij}	0.000	17.667^{j}	3.055
	45Et	15.000^{cdefg}	1.000	19.667^{cdefgh}	1.527	24.000^{cdefgh}	1.173	29.000^{cde}	1.000
	45Mt	16.000^{cde}	1.000	19.667^{cdefgh}	0.577	23.667^{cdefghi}	0.577	26.000^{cdefgh}	2.000
	48Aq	12.667^{cdefg}	0.577	17.333^{fghij}	0.577	19.667^{ghij}	1.155	21.333^{hij}	0.577
	48Et	17.000^{cd}	0.000	23.333^{cd}	0.577	27.000^{cd}	1.000	31.000^{c}	1.73
	48Mt	17.667^{c}	1.155	20.667^{cdefgh}	1.527	24.667^{cdefg}	1.527	28.333^{cdefg}	2.082
	52Aq	14.000^{cdefg}	0.000	17.000^{ghij}	1.000	20.333^{fghij}	0.577	23.333^{fghi}	2.082
	52Et	17.000^{cd}	0.000	22.333^{cdef}	0.577	25.667^{cdef}	1.527	29.667^{cd}	0.577
	52Mt	16.000^{cde}	1.000	24.000^{c}	1.000	27.000^{cd}	1.732	30.500^{c}	0.707
Spring	72Aq	14.333^{cdefg}	0.577	18.000^{efghij}	0.000	22.000^{defghij}	1.000	23.667^{efghi}	0.577
	72Et	15.000^{cdefg}	0.000	23.333^{cd}	0.577	26.667^{cd}	0.577	29.333^{cd}	1.155
	72Mt	15.333^{cdef}	0.577	20.333^{cdefgh}	0.577	24.000^{cdefgh}	0.000	28.333^{cdefg}	1.155
	73Aq	12.333^{defg}	0.577	16.333^{ghij}	0.577	20.667^{efghij}	0.577	23.333^{fghi}	0.577
	73Et	15.333^{cdef}	0.577	21.333^{cdefg}	2.082	26.000^{cde}	1.000	29.000^{cde}	1.732
	73Mt	16.000^{cde}	0.000	22.667^{cde}	0.577	27.667^{c}	1.155	30.000^{cd}	1.000
	76Aq	10.000^{gh}	0.000	15.667^{hij}	1.155	20.333^{fghij}	1.527	20.000^{ij}	0.000
	76Et	11.333^{efg}	0.577	14.000^{ij}	1.000	17.000^{j}	1.000	20.333^{ij}	0.577
	76Mt	10.333^{fgh}	1.155	13.333^{j}	1.527	18.333^{ij}	1.527	19.667^{ij}	1.155
	80Aq	15.000^{cdefg}	1.000	16.667^{ghij}	2.309	20.000^{ghij}	3.464	27.000^{cdefg}	0.000
	80Et	12.667^{cdefg}	1.527	18.667^{defghi}	0.577	21.667^{defghij}	1.527	23.000^{ghi}	1.732
	80Mt	11.667^{efg}	1.155	15.667^{hij}	1.155	20.667^{efghij}	1.155	23.000^{ghi}	2.646
	C1+	37.667^{b}	0.577	37.667^{b}	0.577	37.667^{b}	0.577	37.667^{b}	0.577
	C2+	43.667^{a}	7.767	43.667^{a}	7.767	43.667^{a}	7.767	43.667^{a}	7.767
	C-	6.000^{h}	0.000	6.000^{k}	0.000	6.000^{k}	0.000	6.000^{k}	0.000

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; C1+ - positive control (30µg chloranphenicol),C2+ - positive control (10ng penicillin) C- -negative control (*n*-propanol); Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

Table 7.8 – Results from the phenols concentration determination.

Collection Time	Sample	Phenols concentration (mg/ml)	
		Mean[1]	SD
Winter	44Aq	1.062	0.177
	44Et	9.978	1.598
	44Mt	8.480	1.242
	45Aq	0.696	0.059
	45Et	8.850	0.439
	45Mt	6.915	1.354
	48Aq	1.374	0.162
	48Et	10.871	0.903
	48Mt	9.846	1.309
	52Aq	0.407	0.181
	52Et	5.418	0.409
	52Mt	5.015	0.436
Spring	72Aq	1.027	0.130
	72Et	9.571	1.192
	72Mt	3.518	1.446
	73Aq	1.190	0.263
	73Et	10.310	1.483
	73Mt	8.543	0.718
	76Aq	1.346	0.284
	76Et	9.175	1.057
	76Mt	7.714	1.301
	80Aq	0.602	0.170
	80Et	3.848	0.753
	80Mt	3.088	0.520

[1]Values expressed here where obtained with the undiluted propolis extracts as equivalents to pinocembrin and are the mean value of three replicates. 44, 72 -B. N. Arrodeios, 45, 73 -B. N. Pé da Serra, 48, 76 - B. S. Arneijoafra and 52, 80 - Transição Norte Madeira; Aq – Aqueous, Et – Ethanolic and Mt – Methanolic extracts

7.2. Statistical analysis of the citotoxicity of propolis extracts results

Table 7.9 - Relative percentage of cell growth using the MTT assay after incubation for 1 hour.

Sample	B.N. Arrodeios-Aq (1h)		B.N. Arrodeios-Et (1h)		T. N. Madeira-Aq (1h)	
	Percentage of mean value[1]	SD	Percentage of mean value	SD	Percentage of mean value	SD
CM	100^{abc}	0.000	100^{a}	0.000	100^{a}	0.000
CPH2O	83.684^{abc}	24.708	88.819^{a}	25.497	76.601^{a}	13.305
CPEt	94.174^{abc}	11.542	87.570^{a}	16.605	76.632^{a}	6.547
CH2O2	104.063^{a}	6.460	105.270^{a}	6.128	101.687^{a}	18.408
P1	74.832^{bc}	14.013	100.871^{a}	21.382	91.339^{a}	29.112
P5	104.562^{a}	8.640	92.429^{a}	16.587	88.602^{a}	10.893
P10	100.765^{ab}	2.670	81.885^{a}	16.332	80.384^{a}	12.417
P15	93.196^{abc}	9.255	81.738^{a}	9.687	67.975^{a}	20.087
P20	74.118^{c}	22.675	81.876^{a}	25.662	77.624^{a}	24.046

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; CM – control with culture medium, CPH2O – control with culture medium, n-propanol and water, CPEt - control with culture medium, *n*-propanol and etanol 70%, CH2O2 - control with culture medium and H_2O_2 5%, P1 to P20 – Culture medium with 1 to 20µl of propolis extracts, Aq – Aqueous extract, Et – Ethanolic extracts

Table 7.10 - Relative percentage of cell growth using the MTT assay after incubation for 4 hours.

Sample	B.N. Arrodeios-Aq (4h)		B.N. Arrodeios-Et (4h)		T. N. Madeira-Aq (4h)	
	Percentage of mean value[1]	SD	Percentage of mean value	SD	Percentage of mean value	SD
CM	100^{b}	0.000	100^{a}	0.000	100^{a}	0.000
CPH2O	111.462^{b}	6.437	81.821^{abc}	5.114	109.073^{a}	46.042
CPEt	111.117^{b}	8.069	65.826^{bc}	4.128	107.284^{a}	40.037
CH2O2	167.114^{a}	36.165	96.094^{ab}	40.010	143.513^{a}	46.826
P1	95.599^{b}	2.266	85.544^{abc}	24.148	93.860^{a}	20.741
P5	102.094^{b}	13.095	66.902^{bc}	10.843	99.193^{a}	31.618
P10	119.485^{b}	36.966	69.995^{abc}	11.151	92.669^{a}	27.209
P15	123.522^{b}	35.168	75.181^{abc}	7.475	99.020^{a}	48.194
P20	96.945^{b}	1.469	53.398^{c}	8.507	99.388^{a}	34.163

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; CM – control with culture medium, CPH2O – control with culture medium, n-propanol and water, CPEt - control with culture medium, *n*-propanol and etanol 70%, CH2O2 - control with culture medium and H_2O_2 5%, P1 to P20 – Culture medium with 1 to 20µl of propolis extracts, Aq – Aqueous extract, Et – Ethanolic extracts

Table 7.11 - Relative percentage of cell growth using the MTT assay after incubation for 24 hours.

Sample	B.N. Arrodeios-Aq (24h)		B.N. Arrodeios-Et (24h)		T. N. Madeira-Aq (24h)	
	Percentage of mean value[1]	SD	Percentage of mean value	SD	Percentage of mean value	SD
CM	100^{ab}	0.000	100^{a}	0.000	100^{a}	0.000
CPH2O	104.487^{a}	11.006	105.658^{a}	7.775	95.422^{a}	12.870
CPEt	90.855^{ab}	4.920	99.175^{a}	7.223	110.525^{a}	23.365
CH2O2	96.502^{ab}	2.878	106.345^{a}	15.162	122.627^{a}	12.783
P1	89.339^{b}	16.003	93.373^{ab}	3.613	102.220^{a}	14.224
P5	91.778^{ab}	2.624	92.604^{abc}	6.132	123.029^{a}	31.830
P10	86.751^{b}	1.042	83.530^{bc}	2.960	100.773^{a}	44.815
P15	88.917^{b}	8.163	94.639^{ab}	13.432	116.332^{a}	55.804
P20	90.985^{ab}	3.998	78.065^{c}	4.680	104.547^{a}	7.404

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; CM – control with culture medium, CPH2O – control with culture medium, n-propanol and water, CPEt - control with culture medium, *n*-propanol and etanol 70%, CH2O2 - control with culture medium and H_2O_2 5%, P1 to P20 – Culture medium with 1 to 20µl of propolis extracts, Aq – Aqueous extract, Et – Ethanolic extracts

7.3. Statistical analysis of the enzymatic antioxidant activity results

Table 7.12 - Protein concentration of propolis enzymatic extracts and its enzymatic activity.

Collection time	Sample	Protein concentration (mg/ml)		SOD Activity (U/mg prot)		CAT Activity (U/mg prot)		GPX Activity (U/mg prot)	
		Means	SD	Means	SD	Means	SD	Means	SD
Winter	B.N. Arrodeios	0.801^{d}	0.008	26.831^{ab}	5.269	31.692^{ab}	5.809	2.333^{a}	0.660
	B.N. Pé da Serra	1.322^{b}	0.039	14.350^{bcd}	9.128	13.822^{b}	9.214	1.319^{a}	2.285
	B.S. Ameijoafra	1.444^{a}	0.053	24.827^{abc}	0.786	0.000^{b}	0.000	1.811^{a}	0.366
	Transição Norte Madeira	0.567^{f}	0.011	2.842^{d}	4.923	23.256^{ab}	17.252	1.317^{a}	0.000
Spring	B.N. Arrodeios	0.950^{c}	0.024	5.723^{d}	1.935	59.313^{a}	52.142	1.967^{a}	0.556
	B.N. Pé da Serra	1.323^{b}	0.055	30.663^{a}	10.636	12.280^{b}	9.586	2.637^{a}	1.176
	B.S. Ameijoafra	0.682^{e}	0.040	13.000^{cd}	1.739	7.442^{b}	6.821	3.835^{a}	0.775
	Transição Norte Madeira	0.566^{f}	0.016	21.549^{abc}	0.824	26.920^{ab}	38.071	1.057^{a}	0.349

1Values expressed are the mean value of three replicates. Values within each row followed by different letters are statistically different; SD – Standard deviation.

7.4. Poster presentations

Poster presented at the 13th International Congress Phytopharm, 29-31 July 2009, Bonn, Germany

Portuguese propolis: The effect of collection time and localization on enzyme antioxidant activities

Oliveira, AV[1], Ferreira AL[1], Nunes, S[1], Dandlen, SA[1], Cavaco A[1], Antunes, MD[1], Faleiro, L[1]., Miguel, MG[1]

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Propolis or bee glue is a resinous substance collected by bees mixing their own waxes with resins from plant sources. Propolis extracts are used as a folk medicine from ancient times. Nowadays, it was found to have a wide range of biological activities, namely antibacterial, anti-inflammatory, antioxidative, hepatoprotective effects and anti-tumoral activities. These pharmacological and antioxidant actions are probably due the presence of antioxidant compounds e.g. phenolic constituents, especially flavonoids and phenolic acids.

In this work, enzymatic antioxidant activities, namely: superoxide-dismutase (SOD), catalase (CAT) and guaiacol-peroxidase (GPX) of samples of propolis harvested at two different times (winter and spring) from several locations of the Algarve region (B. N. Arrodeios, B. N. Pé da Serra, B. S. Ameijoafra and Transição Norte) were tested.

In respect with protein contents, major differences were not observed for samples harvested at winter and spring time, except for samples from B. S. Ameijoafra. In this case, a decrease occurred from the sample collected at Winter to sample collected at Spring.

Concerning antioxidant enzymatic results, SOD activity was dependent on the collection time. SOD activity decreased drastically from winter to spring in samples from B. N. Arrodeios and B. S. Ameijoafra. An opposite feature was observed in samples from B. N. Pé da Sera and transição Norte. SOD generates hydrogen peroxide and oxygen molecules. These hydrogen peroxide molecules can be converted into water molecules and oxygen by the action of catalase and guaiacol peroxidase. So, the samples with higher SOD activities should have higher CAT activities also. Such was not observed, which may suggest that the hydrogen peroxide elimination could be done by other antioxidant enzymes such as, glutathione peroxidase or guaiacol peroxidase.

Acknowledgment: This study was partially funded by Cruz Alta Agricultura, Lda. Loulé

Poster presented at the 57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 16-20 August 2009, Geneva, Switzerland

Portuguese propolis: The effect of collection time and localization on anti-Helicobacter activity

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Propolis or bee glue is a resinous substance collected by bees mixing their own waxes with resins from plant sources. It has been used as a folk medicine from ancient times. Nowadays, it was found to have a wide range of biological activities, namely antibacterial, anti-inflammatory, antioxidative, hepatoprotective effects and anti-tumoral activities.

In this work, extracts (aqueous, ethanolic and methanolic) of propolis harvested at two different times from several locations of the Algarve region were tested for their antibacterial activity against Helicobacter pylori.

The propolis samples collected at springtime showed significant higher anti-helicobacter activity, in comparison with samples harvested at winter time. The majority of the extract samples showed a dose dependent activity. Statistical differences for samples provided from different locations were obtained. These differences may be linked to a different chemical composition of propolis which in turn can be due to the plant source from which this natural product is done.

Acknowledgment: This study was partially funded by Cruz Alta Agricultura, Lda. Loulé