1. Introduction:

Several studies comprise the importance of vegetables and fruits on human diet (Abas *et al*., 2006). Generally, different groups of vegetables are extremely rich in carbohydrates, proteins, minerals and vitamins (Ismail *et al.*, 2010), and some specific groups have high medicinal potential as prevention or cure of ailments such as diabetes, high blood pressure, cardiovascular disease, arthritis, fever, coughs, skin infections, stomach pains, and diarrhoea among others. Also, vegetables can lower the incidence of cancer and may also be extremely efficient controlling age related diseases (Abas *et al*., 2006).

A lot of vegetables and fruits have been used for thousands of years in what is called folk or traditional medicine, with a consequent relation and passage for scientific studies. Traditional medicine, known by the use of natural products to treat health disorders, was the first one to reveal the importance of much of aliments we know nowadays and to treat several types of diseases. Among others, chinese traditional medicine, is a particular and well documented folk medicine applied several thousands years ago, which found that natural compounds, are now recognized as anticancer drugs (Cai *et al*., 2004). Xu *et al*., (2000) reported that a chinese medical monograph called *Yellow Emperor's Canon of Medicine* had applied the term 'tumor' 437-221 B.C. Despite the great numbers of drugs developed and used today, therapies with anticancer medicinal plants are used all along the chinese country. As expected, in the XXI century a lot of information and knowledge about diseases and the importance of using specific aliments to treat several health problems are now available. Moreover, one of the most developing areas of Food Science Research is the antioxidants and their role on prevent/inhibit oxidative stress. According to Huang *et al*. (2005), on the past decade the number of publications in this field has nearly quadruplicated, because not only the science researchers but also nutritional and medical experts and general public demonstrated a growing interest by this subject. In this sense, it is important to study benefits of plants, fruits and vegetables available to take part on the human diet in order to satisfy their interest.

One type of food with increasing interest is represented by plants and it is indeed one specific plant, namely *Portulaca oleracea*, that this study concerns. Only little information about the antioxidant properties of this plant is available and so, some of

the methods used in this work were done in this plant for the first time. Due to this reason, this work becomes even more interesting since it opens the door to new research, new knowledge and possibly to new applications of this plant on the human health.

Antioxidant activity, phenolic compounds and flavonoids are of extreme importance regarding their role in human health. Thus, studying their presence, quantity and inhibition activity in such an important vegetable as *Portulaca oleraceae* enables a better understanding of this plant and also allows knowing how this may influence and help treating diseases or just meliorating human health. In this sense, this study goes through antioxidant activity in different parts of purslane aiming first of all to verify if antioxidant activity is high; if there is any difference in antioxidant activity depending on the part of the plant and also if it varies depending on the location where environmental conditions may vary. In a second part, the study aims to know if the compounds obtained in water extracts have a protective role for DNA when joining extracts of purslane with DNA exposed to Fenton solution.

2. Literature Review

2.1. *Portulaca oleracea*

A wide distributed vegetable, one of the most used medicinal plants listed by the World Health Organization, *Portulaca oleracea,* also known as purslane is the one this study examines (figure 1). Naturally occurring (Hongxing *et al.,* 2007), this plant of the Portulacaceae family (taxonomic hierarchy is present on table I) has succulent stems and leaves, and salty-acidic taste similar to spinach (Lim and Quah, 2007).

Table I – Taxonomic hierarchy of *Portulaca oleracea* (adapted from Danin *et al*.,1978)

Kingdom	Plantae – Plants
Sub-Kingdom	Tracheobionta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-Class	Caryophyllidae
Order	Caryophyllales
Family	Portulacaceae
Gender	*Portulaca* L.
Species	*Portulaca oleracea*

Leaves can reach between 1 to 5 cm long and 0.5 to 2 cm across, while stems are cylindrical, up to 30 cm long and 3 mm in diameter (Oliveira *et al*., 2009). Flowers are characterized by a small size and yellow colour, and the large production of seeds, is released 14 to 16 days after flowering and transported by wind, birds, other animals or human agricultural practices. Seeds germinate around March, near the spring period, usually after irrigation or rain, when soil temperature reaches about 15 ºC. The adult state is reached in 4 to 6 weeks, after which flowering occurs and seeds production initiates shortly thereafter. In favourable conditions, this plant completes its life cycle in approximately

Fig.1- *Portulaca oleracea* leaves, stems and flowers in the field. (from: http://free-photos.biz/photographs /food/vegetables /273496_portulaca_oleracea_stems.php)

60 days (Rashed *et al*., 2003).

Despite eating the plant fresh or cooked, in raw salads or cut in small pieces (Oliveira *et al*., 2009), infusions such as tea or soups may be done to ingest the dried herb (Cai *et al*., 2004) without risk of toxicity or genotocity, since the water extracts of *Portulaca oleracea* was certified to be safe for daily consumption as a vegetable (Yen *et al*., 2001).

Regarding nutritional level, it was reported that purslane is a rich source of omega-3 fatty acids (Simopoulos, 2004) and minerals such as potassium (Dweck, 2001), magnesium (Teixeira & Carvalho, 2009), calcium and iron (Dweck, 2001). Other components include ascorbic acid, α-tocopherols, β-carotene and glutathione (Simopoulos *et al*., 1992), calcium oxalate, malic and citric acids, dopamine and dopa, coumarins, flavonoids and alkaloids and urea, among others (Dweck, 2001), and it was indicated that *Portulaca oleracea* provides better nourishment than the major cultivated vegetables (Liu *et al*., 2000).

Mentioned as the common Purslane, this plant is recognized by its potential in folk medicine to treat human ailments, where leaves are used all around the world to alleviate pain and swelling, working also as an antiseptic (Chan *et al*., 2000). Moreover, it can be used to treat dysentery and externally to eczema, erysipelas, and bites of snake or insects (Chen *et al*., 2009, Xie 2002), and specifically in Arabian peninsula it was also used as an anti-scorbutic, antispasmodic, vermifuge, and in oral ulcers and urinary disorders (Chen *et al*, 2009, Ghazanfar,1994), and some African populations attributed some esoteric properties to it (Dweck. 2001). Finally, due to its high water composition, it is still considered diuretic (Youguo *et al.*, 2009) and all this medical applications lead this plant to be considered in Chinese folklore as ‘the vegetable for long live’ (Chen *et al.*, 2009).

In the last years this plant has received increasing interest and has been concern of a lot of studies in several research areas. Rashed *et al*., (2003) showed that this vegetable may accelerate wound healing and effectively treat ulcers such as those induced by indomethacine and phenylbutazone. Simopoulos, (2004) reported that because of the omega-3 fatty acids content *Portulaca oleracea* is highly important when preventing heart attacks and improving the immune system. Parry *et al.*, (1993) has been study its muscle relaxant effect, associating this effect to the high content of potassium ions. Malek *et al.*, (2004) demonstrated a relatively potent bronchodilatatory effect in this plant and Sakai *et al.*, (1996), showed that purslane methanolic extracts

exhibit moderate antimicrobial activity against *Bacillus subtiliis*. Moreover, Chen *et al.*, (2009), concluded that extracts of *P.oleracea* present a notable anti-hypoxic activity in mice and Wang *et al.*, (2010), reported that betacyanins isolated from this plant can improve the activity of antioxidant enzymes in mouse brain. Abas *et al.*, (2006) and Lim & Quah, (2007) demonstrated that within its components, a high antioxidant activity is present, however only little information is available and much more can be done in this area.

Due to its high content of nutrients, especially antioxidants and omega-3 fatty acids, and its wound healing and antimicrobial effects as well as its traditional use in topical treatments, *Portulaca oleracea* is a highly candidate as a useful cosmetic ingredient (Leung & Foster, 1996). Regarding the importance of vegetables and all the information about purslane, it was decided that this study would focus on antioxidant activity.

2.2. Antioxidant Activity

According to Halliwell & Gutteridge, (1995) an antioxidant is "any substance that, when present at low concentration compared to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate", which can act by several chemical events like enzyme inhibition, metal chelation, hydrogen donation and oxidation to a non propagating radical (Parr & Bolwell, 2000).

The reactive oxygen species (ROS), produced continuously in the cells (Devaraj *et al*., 2002) in response to environmental stresses (Meot-Duros & Magné, 2009) play an important role in the two major causes of death in Western societies: heart disease and cancer (Parr & Bolwell, 2000) and also in other major problems of industrialized countries like diabetes, neurological and age-related diseases (Oliveira *et al.*, 2009), atherosclerosis, rheumatoid arthritis and cataracts (Parr & Bolwell, 2000). They can cause as well DNA damages, destabilization in membranes and oxidation in low-density lipoprotein (LDL), contribute to cellular aging and mutagenesis (Heim *et al.*, 2002) and enhance the inflammation through activation and phophorylation of MAP-kinases and some transcription factors (Rahman, 2008).

In general antioxidants can decrease the concentration of these radicals by two different ways: hydrogen atom transfer or simple electron transfer (Huang *et al.*, 2005). Both are represented on a simplified scheme reaction on figure 2, where R• is a free radical, LH is the substrate and AH is the antioxidant, assuming that oxygen is in excess. Reducing the concentration of free radicals, antioxidants reduce the likelihood that these radicals will cause the previously spoken pathologies (Parr & Bolwell, 2000). Several epidemiologic and intervention studies have even proved the beneficial health effects of dietary fruits and vegetables, at least in part due to these antioxidant content (Carvalho *et al.*, 2010, Nijveldt *et al.*, 2001).

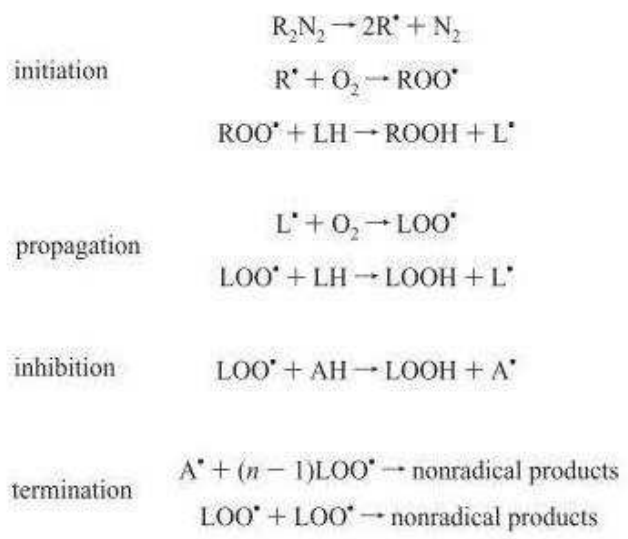

Fig.2 – Representative scheme of the initiation, propagation, inhibition and termination of a free radical reactions chains, adapted from Huang et al. (2005).

Some antioxidants can be synthesized by the cells, such as the enzymatic antioxidants that include superoxide dismutase, catalase, and glutathione peroxidase, Zn-superoxide dismutase and high levels of Cu in the liver, as well as the peptides with thiol groups, as glutathione (Junqueira *et al.*, 2004). Cu is not totally accepted as antioxidant because it was unable to act alone and Cu(I) can initiate more free radicals chain reactions by the decomposition of peroxides (LOOH) in a Fenton-type reaction, however is unquestionable that it participates on the LDL oxidation process.

Despite that in the absence of another antioxidants Cu(II) does not induce the autoxidation of lipids, in the presence of them it develops an important role on the process. Tocopherol, present in the LDL, was first converted to free radical by donating an electron to Cu(II) [generating Cu(I)], and then the tocopherol radical slowly induces the autoxidation of LDL. So, it is viewed like an 'indirect' antioxidant, able to act as a catalyst in the presence of excessive antioxidants and the antioxidants may act as pro-oxidants in their presence (Huang *et al.*, 2005).

Other antioxidants, common known as dietary antioxidants, are obtained through nutrition such as ascorbic acid (Shin *et al.*, 2008), selenium, carotenoids and vitamins C

and E (Junqueira *et al.*, 2004), with the last one being considered the major lipid-soluble antioxidant (Schinella *et al.*, 1999).

Due to their interactions with free radicals, antioxidants have potential therapeutic applications on slow down or reverse symptoms of neurodegenerative disorders, including Alzheimer's and Parkinson diseases, amyotrophic lateral sclerosis, multiple sclerosis, as well as encephalopathies (Brambilla *et al.*, 2008). It can also be used as prophylactic and therapeutic agents for cardiac complications and therapy in protecting against cardiovascular disease inflicted by accentuated oxidative stress from the diabetic state (Hill, 2008). Other authors defended their use in supplements to obese people, considering that obesity may lead to a condition of increased oxidative stress, and so obese individuals may benefit from food supplements rich in antioxidants, preventing the risk of cardiovascular diseases and hypertension and increasing levels of HDL cholesterol and triacylglycerols (Higdon & Frei, 2011).

It was also demonstrated that antioxidants such as vitamin E and resveratrol significantly suppress some oxidative stress biomarkers, such as nitrotyrosine, in both humans and animals with increased oxidative stress (Hill, 2008). High intakes of vitamin E, conjugated with flavonoids and phenols are referred by Visioli & Galli, (1998) as being related to a lower incidence of CHD, and Brambilla *et al.*, (2008) associate these compounds to the maintenance of an effective immune response.

Moreover, according to Servili *et al.*, (2009), antioxidants are also important in preventing damages of the colonic mucosa in inflammatory diseases, such as ulcerative colitis, because it can be related to the accumulation in the epithelium surface of ROS produced in excess.

A recent study from H. Li *et al.*, (2008) concluded that the antioxidant capacity is strongly related to the total phenolic content, and particularly to flavonoids (Carvalho *et al.*, 2010, Wang *et al.*, 1999).

2.2.1. Phenolic Compounds

In plants, phenolic compounds are a group of pytochemicals with potentially beneficial effects preventing some diseases due to its antioxidant and anti-inflammatory properties (Rahman, 2008).

In chemical terms its structure ranges from simple molecules to macromolecular polymers, with the presence of a hydroxyl-substitute benzene ring as a common feature

(Parr & Bolwell, 2000). Although apparently the *trans* form predominate they can exist in *cis* or *trans* conformation, but the general properties of the compound are not influenced by their form. However, the interactions in the human body may be different in *cis* or *trans* conformation (Parr & Bolwell, 2000).

In plants, phenolics take part on growth and reproduction processes (Meot-Duros & Magné, 2009), act as antioxidants, UV screens and play an important role in the relationship with the animals, attracting the pollinating animals and defending against predators (Parr & Bolwell, 2000). The action of coloured phenolics such as anthocyanins and betalains turns the flower to be more attractive to pollinating animals (Stintzing et al. 2004), but even non-pigmented compounds may attract these animals by other senses (Parr & Bolwell, 2000).

In the opposite way, some phenols present a role in non-specific defence mechanisms. One reported example of the defence role of phenolics in plants is presented by Parr & Bolwell, (2000), where some studied phenolics, like tannins, made the plant unpalatable to the herbivores by his astringent taste and also presented an ability to inhibit digestive enzymes. These tannins consist mainly in catechin components such as epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (Cook & Samman, 1996). In addition, other types of phenols have the ability of interacting with some host receptors and change their normal function causing toxicity (Parr & Bolwell, 2000).

Moreover, phenolic acids and other flavonoids, especially anthocyanins, sinapate esters and other hydroxylated or methoxylated cinnamic acid derivatives, have been pointed out to play a crucial role in photoprotection (Carvalho *et al.*, 2010.). In humans, phenolics participate actively in biological molecular processes as the inhibition of xanthine oxidase and other enzymes involved on damaging of ROS, or the inhibition of tyrosine kinase and so modulate the signal transduction and enhance anticancer actions (Parr & Bolwell, 2000).

It is still mentioned that phenolics present anti-thrombotic, cardioprotective and vasodilatory activities (Trabelsi *et al.*, 2010, Siddhuraju, 2007), as well as antibacterial, antiviral, anti-carcinogenic, anti-inflammatory properties (Aberoumand & Deokule, 2008) and may interact with specific receptors, however, their mainly function is as antioxidants. Phenolics are also able to scavenge free radicals by different ways, but generally they donate a hydrogen atom to the radical, originating a stable

macromolecule and in this way, breaking the chain of formation of new radicals (Parr & Bolwell, 2000).

Another way of action is the indirect inhibition of radical formation. In this case, some enzymes such as cyclo-oxygenases or lipoxygenases are potencially pro-oxidant since they can generate radicals, but several phenolic compounds can inhibit these enzymes, which allow reducing the formation of free radicals (Parr & Bolwell, 2000).

Phenolic compounds, such as phenolic monomers, polyphenols, and tannins can form insoluble complexes considered to interfere with iron absorption. Phenolic molecules with aromatic rings bearing three hydroxyls (galloyl group), two hydroxyls (catechol group) or on adjacent carbons have iron binding properties *in vitro*. However, the inhibition of iron absorption by phenolic compounds *in vivo* has been positively correlated with the presence of galloyl groups but not with catechol groups (Heim *et al.*, 2002).

Due to the properties and mechanisms of action previously referred, phenolic compounds present several implications on human health and this evidence was proved by different studies. Servilli *et al.*, (2009) suggest that anti-carcinogenic properties of olive oil are due to the high concentration of these compounds, and Tuck & Hayball, (2002) also reported that high concentration of hydroxytyrosol, the major phenolic compound in olive oil is related with reduced risk of coronary health disease. This reduced risk is associated with the action of hydroxyltyrosol and other phenols in inhibit the oxidation of LDL and consequently the formation of atherosclerotic plaques.

These compounds may vary due to differences in plant growth conditions and even to intrinsic factors of the plant, such as genus species and cultivars (Aberoumand & Deokule, 2008). In the study of Wang & Zheng, (2001), the highest day/night temperature resulted in greatest phenolic content along with strongest antioxidant activities against hydroxyl and peroxide radicals. Howard *et al.*, (2002) also suggest that in vegetables like spinach, higher temperatures and light intensity enhance the phenolic content and the total antioxidant activity. Their antioxidant activity increases according to the degree of hydroxylation, as well (Aberoumand & Deokule, 2008).

Polyphenols present both hydrophilic and hydrophobic natures, standing the hydrophilic in aqueous environments and the hydrophobic into lipid environment (Parr & Bolwell, 2000). Among all the types of polyphenols, Wang & Zheng, (2001) choose the flavonoids as the most important single group, and the same can be concluded according to the general opinion of scientific society which presents that the antioxidant

capacity is directly correlated with phenolic compounds, including the anthocyanins, but especially with flavonoids (Cordenunsi *et al.*, 2005).

2.2.2. Flavonoids

According to Heim *et al.*, (2002), there are over 4000 types of flavonoids in vascular plants, but recently Moon *et al.*, (2006) pointed out that more than 8000 compounds with a flavonoid structure has been identified. The majority arises from combinations of hydroxyl and methoxyl substituent groups on the basic formula of flavonoids. Based on their structure they are classified into classes, such as flavonols, flavones, flavanones, catechins (or flavanols), anthocyanidins, isoflavones, among others (Cook & Samman, 1996) and these classes can be found specially in different dietary sources. For instance, the major source of flavanols is the tea, but flavanone is more common in citrus, flavones in fruit and vegetables skins and red wine, flavonols in vegetables as broccoli and lettuce, isoflavones in soybean, anthocyanins in colored fruits like cherrys and berrys (Heim *et al*., 2002), ferulic acid in corn and rice, chlorogenic acid in fruits like apples, pears and apricots, coumaric acid in tomatos and caffeic acid in white grapes/wine, olives and coffe of course (Rice-Evans *et al.*, 1995).

Flavonoids fulfill many functions on plants like the production of yellow, red or blue pigmentation but also more important functions including the protective roles of different flavonoids subclasses in plant tissues (Carvalho *et al.*, 2010).

Presenting a hydrophobic nature, flavonoids are generally concentrated in or around epidermal tissues (Parr & Bolwell, 2000), especially after light stress induction (Carvalho *et al.*, 2010) or localized among photosynthesizing cells (Schubert *et al.*. 1999). Although widely distributed, flavonoids may vary within vegetables or fruits species in type and quantity due to variations in plant growth conditions and maturity (Cook & Samman, 1996), such as alterations in temperature, humidity or drought and also in light intensity during growth period. Wang & Zheng, (2001) demonstrated that high growing temperatures (25 and 30 °C) significantly enhanced the content of flavonoids and the ROO• absorbance capacity of strawberry fruit juice. UV light also interferes, inducing the enhancement of flavonoid accumulation (Parr & Bolwell, 2000). Liu *et al.*, (2007) still demonstrated that in lettuce flavonoids are sensitive to environmental conditions, since higher temperatures and light intensity enhance the

content of these compounds and the same was suggested to happen with phenols in general.

Such as phenolic compounds, flavonoids also present several mechanisms of antioxidant actions, including chelate metal catalysts (Heim *et al.*, 2002) and divalent cations (Cook & Samman, 1996), activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases and have the ability to transfer electrons to free radicals (Heim *et al.*, 2002), essentially due to their hydroxyl groups (Parr & Bolwell, 2000).

In biological systems these actions can turn into several biological effects like antibacterial, antiviral, vasodilatory actions, anti-allergic, anti-inflammatory, and anti-carcinogenic activities (Yao *et al.*, 2004), lipid peroxidation inhibition (LPO), decrease of capillary fragility, and inhibition of enzyme systems including cyclo-oxygenase and lipoxygenase (Cook & Samman, 1996), some P450 isoforms (Heim *et al.*, 2002), telomerase, xanthine oxidase, matrix metalloproteinase, angiotensin-converting enzyme and sulfotransferase, as well as interactions in other signal transduction pathways, drug transport systems, compete with glucose for transmembrane transport and affect the platelet function (Halliwell *et al.*, 2005). Schubert *et al.*, (1999) proposed that flavonoids may be effective anti-inflammatory agents against parodentitis and local pain, without the gastric irritating effects of aspirin and other non-steroidal anti-inflammatory drugs.

In a review work, Heim *et al.*, (2002) discuss about the studies of Facino *et al*,. (1999), which demonstrated that diets rich in flavonoids reduce de myocardial post-ischemic on rats. Other studies predicted a protective role in human beings, since high flavonoid uptake predicted lower incidence of myocardial infarction and stroke in older persons, lower mortality from coronary heart disease and reduced the risk of coronary heart disease by 38% in postmenopausal women (Heim *et al.*, 2002; Boyer & Liu, 2004). Yao *et al.*, (2004) still mentioned that flavonoids may help prevent steroid hormone-dependent cancers, however the certain mechanism of action is still unknown and clinical trials are still in progress.

Understanding the absorption and metabolism mechanisms is still limited to a small number of dietary flavonoids. Until now, it was reported that flavonoids are poorly absorbed on oral administration and after this the absorption rate increases, especially on gastrointestinal tube (Cook & Samman, 1996). It was even proposed that the peak of flavonoids concentrations typically occur approximately 2 hours after

ingesting food (Heim *et al.*, 2002). This make sense since due to the molecular size of compounds, the absorption occurs across the intestinal epithelium (Halliwell *et al.*, 2005) but requires preliminary degradation to smaller and lower molecular weight compounds, done with the contribution of caecal bacteria and low gastric pH (Heim *et al.*, 2002).

Flavonoids and other phenolic compounds may interact with each other in potentiating interactions (additive effects or synergies), inhibition interactions or other interactions with concomitant compounds that are not themselves bioactive but may enhance bioavailability or absorption of flavonoids (Lila, 2004).

Despite all benefits, adverse reactions from flavonoids have been proposed following administration of chronic pharmacological doses that exceed the appropriate dietary intake of 23 to 170 mg/day. Secondary effects have been documented like toxic effects, acute renal failure, hemolytic anemia, thrombocytopenia, hepatitis, fever, and skin reactions from doses of 1 to 1.5 g/day of flavonoid drugs such as cianidanol (Cook & Samman, 1996), however this values are much higher than the value consumed by population.

2.2.3. Carotenes

Carotenes, a sub-division of carotenoids, are natural pigments with lypophilic nature, responsible for red, orange and yellow colors of fruits and vegetables that have gained more and more attention on natural diet (Ganesan *et al.*, 2008). Recent studies proved reduced risk of coronary heart disease and other degenerative diseases for rich diets in carotenoid-rich fruits and vegetables (Prasad *et al.*, 2011), mainly due to its antioxidant activity by scavenging free radicals (Tlili *et al.*, 2010) or retard the extent of oxidative deterioration (Ganesan *et al.*, 2008). It was reported that carotenes are more efficient scavengers of free radicals than xanthophylls, the other sub-group of carotenoids (Miller *et al.*, 1996).

2.2.4. Anthocyanins

Anthocyanins are natural pigments, considered the great responsible for flowers and fruit coloration (Stintzing & Carle, 2004). They were also used many centuries ago because many traditional herbs used on folk medicines by North American Indians,

Europeans, and Chinese populations present high concentrations of these compound, usually on dried leaves, fruits, storage roots, or seeds (Konczak & Zhang, 2004).

These compounds play an important role in biological activities including anti-inflammatory, antimicrobial and anti-carcinogenic activities, improvement of vision (Mazza, 2007) and cardioprotection by maintaining vascular permeability, however, the principal benefit are the antioxidant protection and maintenance of DNA integrity, and consequent role in neuronal age-related and risk of coronary heart diseases (Bagchi *et al.*, 2004; Mazza, 2007).

Moreover, is known that anthocyanins are highly present in berries, and Bagchi *et al.*, (2004) referred that berry extracts have demonstrated to exhibit benefits for health related with urinary tract, anti-diabetic, anti-angiogenic and anti-aging properties. Some of these effects are also found on red wine (Mazza, 2007), and shared between anthocyanins and the other major components of red wine: tannins.

2.2.5. Tannins

Tannins are natural polyphenols usually classified into two groups: condensed tannins also known as proanthocyanidins, and hydrolyzable tannins (Cai *et al.*, 2004) and it was proposed that they have higher antioxidant activity than other phenolic compounds (Awika *et al.*, 2003). Moreover, anti-carcinogenic, cardiovascular, gastroprotective, anti-ulcerogenic and cholesterol-lowering properties are attributed to these compounds, as well as urinary tract health promotion (Dykes & Rooney, 2007), anti-mutagenic activity and chemoprevention (Okuda, 2005). Okuda, (2005) also referred that one of the most important activities of tannins related to health is to inhibit the lipid peroxidation on liver mitochondrias. Still according to the same author, some hydrolysable tannins may inhibited some virus, such as Herpes simplex.

3. Material and methods

Antioxidant abilities of plant extracts depend on large way on the extract composition, on the extraction conditions and on the assay system. Measuring antioxidant abilities may be influenced by several factors, which cannot be accurately and quantitatively described with one single method (Oliveira *et al.*, 2009). In order to avoid this and to complement as much as possible the results, in the present work, the antioxidant properties of *Portulaca oleracea* were measured using different assays: the total antioxidant activity (TAA) assay, the reducing power (RP) assay, the ferric-reducing antioxidant power (FRAP) assay and the scavenging effect of the samples on DPPH and ABTS free radicals.

3.1. Reagents

1,1-Diphenyl-2-picrylhdrazyl (DPPH), Folin- reagent, 2-2'-Azino-bis(3ethylbenzozthiazoline-6-sulfonic acid) diammonium salt (ABTS), ammonium molybdate, sodium phosphate dibasic dehydrate, monosodium phosphate hydrous, gallic acid, vainilin quercetin and Trolox were purchased from Sigma-Aldrich Co. Ltd (Poole, UK). Iron (III) chloride, methanol, trichloroacetid acid, sodium acetate, sulfuric acid, glacial acetic acid, 2,4,6-tripyridyl-2-triazine (TPTZ), hydrochloridic acid and ascorbic acid were purchased from VWR (Pennsylvania, USA), Aluminium chloride and absolute ethanol were purchased from Merck (Nottingham, UK). All reagents were of analytical grade.

3.2. Plant materials

Samples of *Portulaca Oleracea* were harvested at the end of September and beginning of October, 2010 at two different locations: Tavira, Algarve (N 37º 20' 0.7" W 7º 49' 3.58") and Vendas Novas, Alentejo (N 38º 40' 38.52'' W 8º 27' 18.76''), from now on referred as location 1 and location 2, respectively, which were treated separately (figure 3).

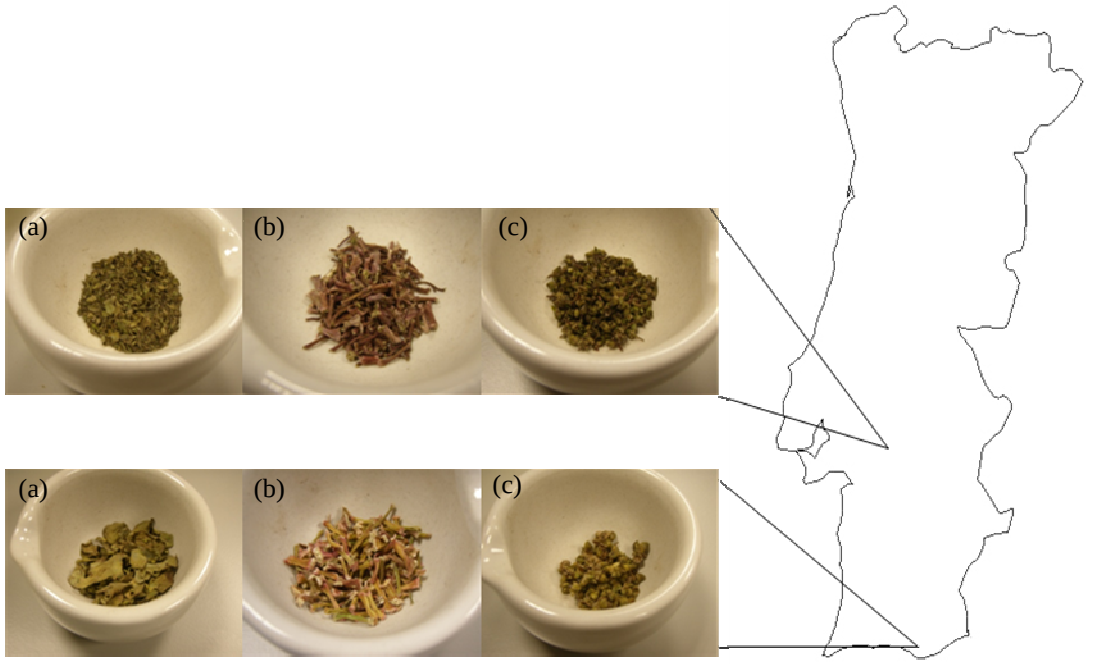

Fig. 3 – Different harvest locations of *Portulaca oleracea* leaves (a), stems (b) and flowers (c).

Leaves, stems and flowers of each sample were separated, dried in an incubator with air circulation for 24 hours at 60ºC and powdered, because according to Spigno *et al.*, (2007) reduction of particle size should increase the superficial area available for mass transfer and, then, increase extraction yield. In order to reproduce as best as possible how people use this plant in a non-scientific environment, for instance applied in teas, water extracts (WE) were performed (figure 4) by adding 1g of each sample to 25mL of distilled water at 95ºC during 10 min with magnetic stirring and filtered through a Whatman Nº4 filter paper. Then, infusions were transferred to Eppendorfs and stored at -4ºC. Later, it was also performed methanol extracts (ME) in order to analyze individual phenolic compounds and flavonoids by liquid chromatography.

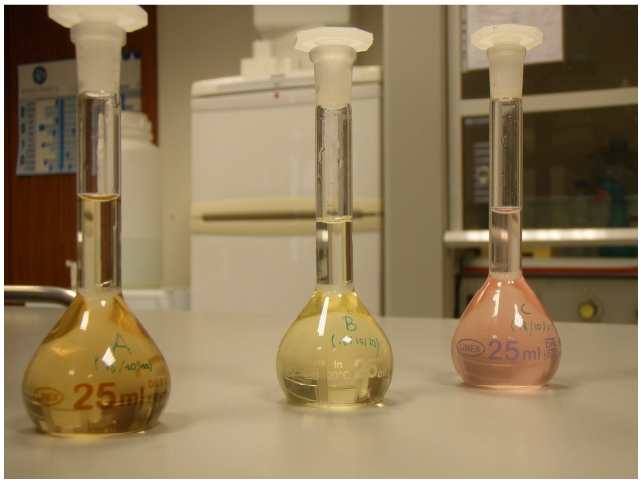

Fig. 4 – Water extracts of different parts of *Portulaca oleracea*

3.3. Total Content Assays

In a general way, these methods are based on electron-transfer reactions, involving two major components: antioxidants present in the extract samples and oxidants solutions done *in vitro*. The last one also acts as a probe since it changes its color when it receives an electron from the antioxidant with which it has affinity, whose concentration is proportional to the degree of color change (Huang *et al.*, 2005).

3.3.1. Total Antioxidant Activity (TAA)

In this assay, phosphomolybdenum (Mo^{6+}) present on the reagent solution is reduced to form a green phosphate/Mo^{5+} complex, by the antioxidant compounds present in the aqueous extract. Since it is a low specific reaction, this test measures all or at least the majority of compounds with antioxidant activity (Ganesan *et al.*, 2008).

TAA of aqueous extracts was estimated according to Prieto *et al.*, (1999). Briefly, an aliquot of 0.1 mL of sample solution was combined in an *Eppendorf* tube with 1 mL of reagent solution of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, previously prepared. The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples' cooling to room temperature (around 25º C), the absorbance was measured at 695 nm against a blank of water instead of the extracts, using a T70+ UV/Vis Spectrometer (PG Instruments Ltd, United Kingdom). Antioxidant activity was calculated based on the calibration curve of ascorbic acid and was expressed as mg ascorbic acid equivalent (AAE) per 100g dry weigh.

3.3.2. Total Phenolic Content (TPC)

This method is one of the most popular assays on food science research. Initially, it was used to analyze proteins (it binds to the phenol group of tyrosine's residue), but over the years it was been adapted (Huang *et al.*, 2005) and today the Folin–Ciocalteau reagent has been extensively used to determine the total phenols content of a varied number of matrixes (Oliveira *et al.*, 2009).

TPC of each aqueous extracts was determined following the method of Huang *et al.*, (2006). Each aqueous extract (0.1 mL) was mixed with 0.50 mL of Folin–

Ciocalteau's reagent (previously diluted in water in a proportion of 1:10) and 0.4 mL of saturated sodium carbonate solution (7.5%). After standing 30 minutes in the dark, the absorbance was read at 765 nm against a blank in a spectrophotometer. Content of phenols was calculated using calibration curves of gallic acid, and was expressed as mg gallic acid equivalent (GAE) per 100g dry weigh.

3.3.3. Total Flavonoids Content (TFC)

A well known method to study TFC is based on the formation of stable acids by aluminum chloride and specific groups on structure of flavonoids, which leads to a color change of the initial solution (Chang *et al.*, 2002).

In this study, TFC was measured as described in Huang *et al.*, (2006). A sample composed of 0.5 mL of aqueous extract solution and 1.0 mL of 2% methanolic aluminium chloride solution was left in the dark for 10 minutes. As with TAA and TPC, the absorbance of the sample was read in a spectrophotometer, in this specific case at 430 nm against a blank, and the flavonoids content was calculated based on the calibration curve of quercetin, expressed as mg quercetin equivalent (QE) per 100g dry weigh.

3.3.4. Total Carotenes

Total carotenes assay was performed adapted from the method of Bunea *et al.*, (2008). A direct measure from the different aqueous extracts was performed spectrophotometrically at 470 nm against a blank and the total carotenes concentration was calculated based on the calibration curve of β-carotene and expressed as mg carotene per 100g dry weigh.

3.3.5. Total Monomeric Anthocyanins (TMA)

Anthocyanins are natural pigments, considered the great responsible for flowers and fruit coloration (Stintzing & Carle, 2004), such as in raspberries for instance (Liu *et al.*, 2002). They also play important roles in plants physiological processes, and in human nutrition, mainly due to its higher radical scavenging capacities (Stintzing & Carle, 2004). These pigments may suffer reversible structural transformations with pH

changes, manifested by strikingly different absorbance spectra (figure 5). The pH-differential method is based on reaction from oxonium form at pH 1 to colorless form at pH 4.5, and permits accurate and rapid measurement of the TMA, even in the presence of polymerized degraded pigments and other interfering compounds (Giusti & Wrolstad, 2001).

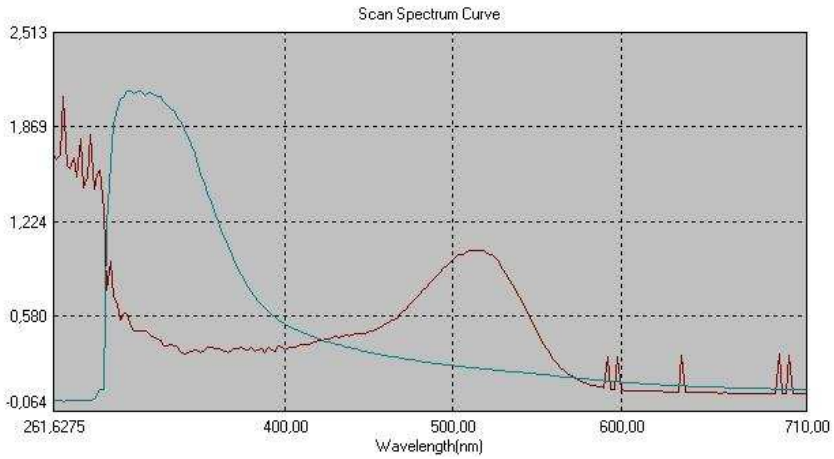

Fig. 5 - Spectral characteristics of anthocyanins in pH 1.0 (red) and pH 4.5 (blue) buffers

TMA was measured and determined according to Giusti & Wrolstad (2001). First, the appropriate dilution factor for the sample was determined by diluting the sample with 0,025M potassium chloride buffer (pH 1.0) for a total volume of 1 mL until the absorbance at wavelength with maximum absorbance ($\lambda_{vis\text{-}max}$) is less than 1.2. To determine the $\lambda_{vis\text{-}max}$ a spectrum of the sample (260-710 nm) was obtained and in order to not exceed the buffer's capacity, the sample should not exceed 20% of the total volume and the results showed that the maximum absorbance was in a wavelength of 310 nm. Two dilutions of the sample were prepared until total volume of 1 mL is reached, one with buffer pH 1.0, and the other with 0,4 M sodium acetate buffer (pH 4.5) diluting each by the previously determined dilution factor. These dilutions were left quietly for 45 minutes to reach equilibrium. A blank of distilled water was used to zero the spectrophotometer at all wavelengths that were used. The final absorbance of each dilution was measured at the 310 and at 700 nm.

The monomeric anthocyanin pigment concentration in the original sample was calculated using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/liter)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

where $A = (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 4.5}$,

MW is the molecular weight expressed in g,

DF is the dilution factor and

ε is the molar absorptivity

The equation presented above assumes a pathlength of 1 cm. When the sample composition is unknown, pigment content is expressed as cyanidin-3-glucoside, where MW = 449.2 and ε = 26 900.

3.3.6. Condensed Tannins (CT)

Although some methods to measure condensed and hydrolyzable tannins have been developed, their repeatability and sensitivity are sometimes problematic (Cai *et al.*, 2004). One of them is the vanillin assay, in which one molecule of vanillin reacts with one molecule of flavanol to produce a red chromophore (Tabart *et al.*, 2010). In this test it is important to use aqueous extracts instead of methanol, because methanol will inhibit the formation of the chromophore (Butler *et al.*, 1982).

CT were determined as described by Tabart *et al.*, (2010). A solution of 0.2 mL of extract, 0.5 mL of 1% vanillin solution and 0.5 mL HCl 9M was previously prepared. After standing 20 minutes at 37º C, the absorbance was read in a spectrophotometer at 500 nm against a blank. Content of condensed tannins was calculated based on the calibration curve of catechin, and was expressed as mg catechin equivalent (QE) per appropriate amount of sample.

3.4. *In vitro* Antioxidant Activity assays

3.4.1. Reducing Power assay

In Reducing Power (RP) assay, the presence of antioxidants in the extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reaction occurs at a pH a bit acidic, but closer to the neutral and leads to a colour change of the solution. Initially, the yellow solution turns out to various shades of green and blue (figure 6), depending to the reducing power capacity of the sample (Oliveira *et al.*, 2009).

The method used in this study to determine the RP of

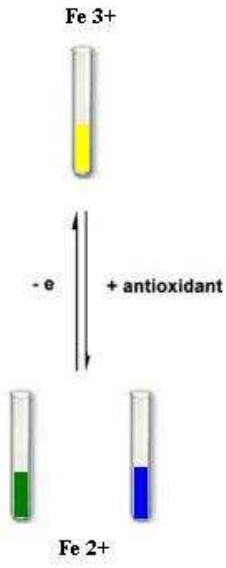

Fig. 6 – Representative scheme of RP assay reaction.

aqueous extracts was previously explained in Yen *et al.*, (2000). In this sense, 0.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) was mixed with 0.5 mL of potassium ferricyanide (1.0%) prepared daily (in order to don't lose the properties) and with 0.2 mL of extract. Afterword, the solution was incubated at 50 ºC for 20 minutes and 0.5 mL of trichloroacetic acid (10%) was added. This was followed by a 10-minutes centrifugation process at 650 g. The obtained supernatant (1.0 mL) was mixed with distilled water (1.0 mL) and ferric chloride (0.2 mL, 0.1%) daily prepared. The absorbance of the final solution was read spectrophotometrically at 700 nm against a blank, and the reducing power was calculated based on the calibration curves of Trolox, expressed as mg Trolox equivalent (TE) per 100g dry weigh.

3.4.2. Ferric-Reducing Antioxidant Power (FRAP) assay

The FRAP assay is based on the same process that Reducing Power assay, however it is more specific than the previous one. In this assay, the ferric salt Fe(III)(TPTZ)2Cl3 (TPTZ)2,4,6-tripyridyl-s-triazine is used as an oxidant to be reduced to the ferrous form (Fe'') at an acidic pH (Figure 7). This non-specific reaction produces a blue color that is more intense as more reduced TPTZ is (Benzie & Strain, 1996).

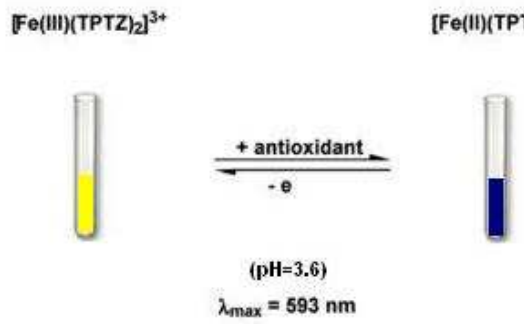

Fig. 7 – Representative scheme of the reaction measured in FRAP assay

FRAP was evaluated according to Thaipong *et al.*, (2006). Initially, three stocks solutions are prepared: a 300 mM acetate buffer, a 10 mM 2,4,6-tripyridyl-2-triazine (TPTZ) in 40 mM of HCl and a 20 mM ferric chloride solutions. Fresh FRAP working solution was prepared by mixing 25mL acetate buffer, 2.5mL TPTZ solution, and 2.5mL ferric chloride solution that was warmed at 37 ºC before using. A mixture of 0.1 mL of extract solution was added to 0.9 mL of the FRAP solution and left in the dark to react for 30 minutes. Finally, absorbance was read spectrophotometrically at 593 nm against a blank and FRAP was calculated based on calibration curves of Trolox, and was expressed as mg Trolox equivalent (TE) per 100g dry weigh.

3.4.3. Inhibition Rate Assays

Although the mechanisms of the chemic reactions are the same, these methods are carried out on different ways. Different dilutions are done to obtain different concentrations of the sample, and then dilutions are analyzed and inhibition rate is calculated to all of them in order to manage their evolution as the concentration of the sample increases.

3.4.3.1. DPPH Radical Scavenging Capacity

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a non naturally occurring radical, relatively stable compared to the highly reactive superoxide and hydroxyl species that played important roles for oxidative damage in biological systems (Heim *et al.*, 2002). This radical present high stability and tend to accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Prasad *et al.*, 2011), and so it is very used as a screening method for testing the antiradical activity of different plant materials such as leaves, stems, seeds, flowers and fruits, among others, being measured the ability of the sample to scavenge the radicals. DPPH present a characteristic absorption maximum between 515 and 517 nm, which is diminished in the presence of compounds able to reduce it by hydrogen/ electron donation to its hydrazine form (figure 8), such as the case of antioxidants (Oliveira *et al.*, 2009).

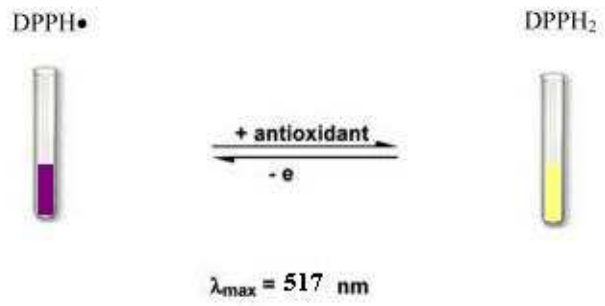

Fig. 8 - Representative scheme of the reaction measured in DPPH assay.

The DPPH Radical Scavenging capacity of extracts was determined adapting the method of Yen *et al.*, (2000). A previous methanolic solution of 0.16 mM DPPH was prepared dissolving 0.0099 g of DPPH in 100 mL of methanol. Later, 0.5 mL of DPPH solution was added to the test tube containing 0.5 mL aliquot of sample. The mixture was vortexed 1 minute and kept at room temperature (around 25º C) for 30 minutes in

the dark. The absorbance of the solution was read spectrophotometrically at 517 nm against a blank.

Results are expressed by mean of an inhibitory concentration (IC_{50}). The percentage of inhibition of free radical for different extract concentrations was determined according to equation:

$$\%\, I = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is the control absorbance and

A_t is the sample absorbance after a certain time, in this case 30 minutes. The IC_{50} is determined by analysis of inhibition rates of extract concentrations.

3.4.3.2. ABTS Radical Scavenging Capacity assay

The mechanisms of the assay are very similar to the DPPH (Teow *et al.*, 2007), but since the blue/green color of ABTS differ from purple DPPH (Floegel *et al.*, 2011) the absorbance is read in a different wavelength. This method evaluates the hydrogen donating ability of the sample in the aqueous phase by measuring the 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) cation scavenging (Heim *et al.*, 2002), and is represented in figure 9. Its use for plant extracts is recommended because the interference of color on these extracts is eliminated due to the long wavelength absorption maximum of this assay (Awika *et al.*, 2003).

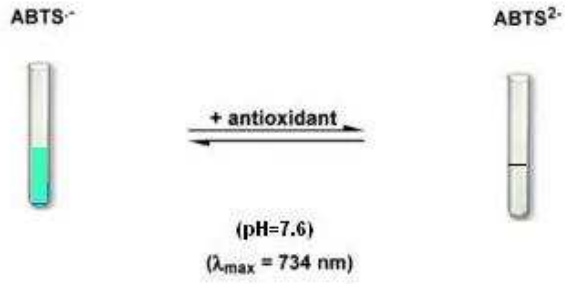

Fig. 9 – Representative scheme of the reaction measured in ABTS assay.

ABTS Radical Scavenging Capacity (ABTS) assay was done according to Zulueta *et al.*, (2009) method. A stock solution of ABTS was made by spiking 25 mL of ABTS (7 mM) with 440 µl of potassium persulfate $K_2S_2O_8$ (140 mM) and allowed to stand in darkness at room temperature for 12–16 h. Just before use, this solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. Then, 1 mL of diluted ABTS

was added to 0.05 mL of the extract and well mixed. After 2 hours, the reaction reached a plateau and the absorbance was read at 734 nm against a blank.

The final results are expressed by mean of inhibitory concentration (IC_{50}). The percentage of inhibition of free radical for different extract concentrations was determined according to the equation previously mentioned in 3.4.3.1., and the IC_{50} is determined by linear regression of % I and extract concentration.

3.4.4. DNA Nicking Assay

The most reactive radicals in biological systems, which are responsible for the oxidation of much biomoléculas, are hydroxyl radicals. These radicals are generated by the reaction of ferrous iron with hydrogen peroxide, on a process known as Fenton reaction and hydroxyl radical production and the concentration of copper or iron are directly related (Heim *et al.*, 2002). DNA nicking assay evaluates the ability of plant extracts to protect DNA from hydroxyl radicals produced by a Fenton reaction done *in vitro*.

On this study, DNA Nicking Assay was performed by the method of Lee *et al.*, (2002), with minor modifications. Competent cells from E-coli were transformed in order to incorporate the pBR32 plasmid DNA and plated. Then, some colonies of transformed cells were incubated in LB medium and ampiciline for 24 hours at 37ºC to grow up with the consequent extraction of DNA. 5 µL of this plasmid DNA was added to 5 µL of the water extracts of different parts of *Portulaca oleracea*, and after 10 minutes at the environmental temperature, it was added 10 µL of Fenton's reagent (30 mM H_2O_2, 50 µM ascorbic acid, and 80 µM $FeCl_3$) daily prepared, since time may degrade its properties. The mixture was then incubated for 30 min at 37 °C and the DNA was analyzed on a 1% agarose gel followed by ethidium bromide staining at 50 V.

3.5. Individual Analysis Assays

3.5.1 Quantification of flavonoids and phenolic acids by High Performance Liquid Chromatography (HPLC)

HPLC has been proposed to separate and identify phenolic compounds and flavonoids effectively (Yao *et al.*, 2004), especially Reverse Phase (RP) HPLC

(Proestos *et al.*, 2006). Generally HPLC uses different types of stationary phases, a pump that moves the mobile phase and analyzes through the column, and a detector to provide a characteristic retention time for the compound to identify. RP-HPLC is the most used in Liquid Chromatography and is faster and more efficient with more polar solvents. Thus, it is more convenient to use methanol extracts than water extracts. On the other hand, viscosity of water is bigger than of organic solvents, so the pressure on the machine is also bigger. Finally, the water extracts requires more cares since there are a small risk of formation of bacterial cultures on the circuit if not well washed with organic solvents at the end of the usage (Skoog *et al.*, 1998; Harris, 2003).

In this way, methanolic extracts were filtered through a 0.45 lm filter and HPLC analyses of flavonoids and phenolic acids were conducted with a Dionex liquid chromatography (USA) equipped with a model P580 solvent pump (USA), a ASI-100 autosampler (USA), a PDA-100 photodiode- array detector (USA) and Dionex Software. A Lochrospher 100 RP-18, with a reversed-phase column (25 cm _ 4 mm, 5 lm; Merck, Darmstadt, Germany), was used throughout this study. Phenolic acids and flavonoids were detected at 280 and 360 nm, with the exception of anthocyanins, which were only detected at 510 nm. The mobile phase was 5% formic acid and methanol in a linear gradient starting at 15% and reaching 35% in 15 min (then isocratic until 20 min), at a flow rate of 1 mL/min and an injection volume of 20 µl. Phenolic compounds were identified by comparison of their retention times with those of pure standards and quantified individually, based on standard curves of each flavonoid or phenolic acid type. Quantification was performed with the linear calibration curves of standard compounds according to Jaakola *et al.*, (2002) and Jaakola *et al.*, (2004).

3.6. Data Statistical Analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc, Chicago). The level of α was fixed at 0.05. All results are shown as mean ± S.D from three repetitions, except for IC_{50} values computed from linear regression of triplicates of each concentration tested. One-Way ANOVA, 2-Way ANOVA and Post hoc tests were applied to date in order to verify if there was any significant difference between location and/or plant parts.

4. Results

The following results related to TAA, TPC, TFC, RP, FRAP, TMA, carotenes and CT are characterized by some differences depending on the location site. Usually, location 2 presents higher values for the majority of the tests (TAA, TPC, FRAP) but in others, location 1 values may be very similar (TFC, carotenes, RP) or exceed those from location 2.

4.1. Total Content Assays

4.1.1. Total Antioxidant Activity (TAA)

Total antioxidant activity analyzed from *Portulaca oleracea* showed significant different results when comparing location 1 and 2 (P=0.000 F=905.57), with the last one presenting higher concentration values for all samples (leaf, stem and flower). The most relevant difference is found on leaves that present in average 148.17 mg/100g on location 1 and 770.20 mg/100g on location 2 (see table I in annexes and figure 10). Nonetheless, for both locations, stems showed the highest values of TAA (508.81 mg/100g and 982.33 mg/100g).

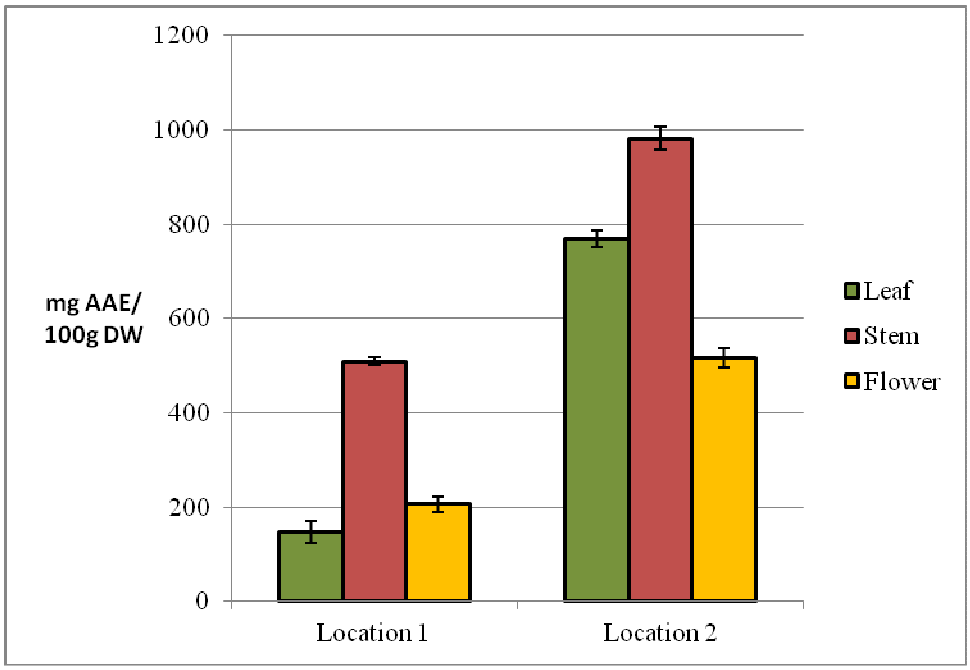

Fig. 10 – Quantification of Total Antioxidant Activity (TAA) for two different locations in three parts of the plant, expressed in mg of ascobic acid equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

Specifically, in the first location, flowers had more TAA than leaves, however, for location 2, flowers presented the lowest TAA values (517.29 mg/100g), almost half of stems value (982.33 mg/100g). Also for the part of the plant, results show significant differences (P=0.000 F=218.65) for all of them globally, and specifically on both locations (P=0.000 F=127.53 and P=0.000 F=124.76).

4.1.2. Total Phenolic Content (TPC)

As with TAA, results related to TPC showed significant differences between locations, with location 2 presenting the highest values of all samples (5 times higher than location 1 for leaves, 2 times higher for stems and 4 times higher for flowers) (Annexes table II; Figure 11). However, both locations showed different specific results. Location 1 is characterized by significant higher stem values (1008.57 mg/100g), followed by flowers (455.58 mg/100g) and leaves (441.81 mg/100g). Also, both leaf and flower values were approximately half of the stems value. Furthermore, location 2 presented higher values for leaves but the difference for stems is not significant as well (P=0.52 F=0.46).

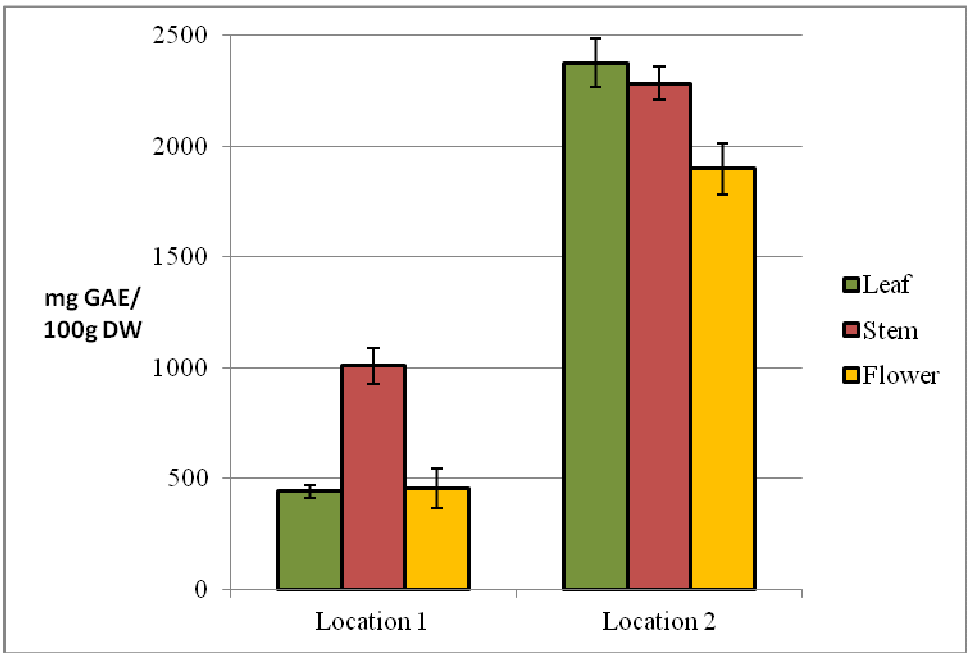

Fig.11 – Quantification of Total Phenolic Content (TPC) for two different locations in three parts of the plant, expressed in mg of gallic acid equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

However, globally the differences between part of the plant are significant (P=0.000 and F=158.06). Also, the biggest difference between sample locations seems to be related to leaves that presented in average 441.81 mg/100g for location 1 and 2377.49 mg/100g for location 2. Moreover, locations present significant differences between each other (P=0.000 and F=164.63).

4.1.3. Total Flavonoids Content (TFC)

In general, TFC values are lower than those from TAA and TPC tests but the trend related to higher location 2 values is not present here. Also it is not possible to say which sample presents higher total flavonoid content, since it depends on the location (Table III in annexes, figure 12). For location 1, in average leaves presented significant higher values (16.28 mg/100g), followed by stem (11.50 mg/100g). However, for the second location the highest TFC values are present in stem samples (17.31 mg/100g), followed by leaf (15.07 mg/100g). Despite this, both locations presented flowers as the sample with lower TFC concentration with 9.60 mg/100g and 10.50 mg/100g for location 1 and 2, respectively.

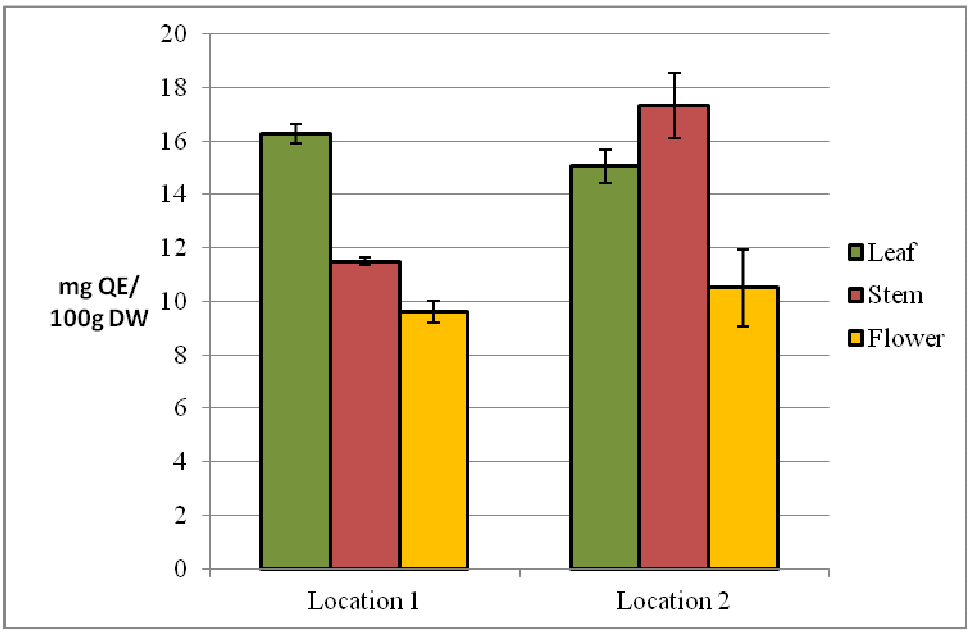

Fig. 12 – Quantification of Total Flavonoids Content (TFC) for two different locations in three parts of the plant, expressed in mg of quercetin equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

4.1.4. Total Carotenes

Once again, no pattern can be established with just one location presenting the highest values for all samples (table IV in annexes, figure 13). In this case, location 1 presented significant higher values than location 2 for leaves (26.92 mg/100g vs 15.64 mg/100g; P=0.000 and F=1148) and for flowers (7.04 mg/100g vs 5.76 mg/100g); (P=0.001 and F=74.2019). However, location 2 presented higher stem values (37.45 mg/100g – the highest of all carotene values) than location 1 (28.47 mg/100g) which were slightly different (P=0.000 and F=214.017). Specifically, location 1 presented approximated values for leaf (26.92 mg/100g) and stem (28.47 mg/100g), however, these were also significantly different (P=0.017 and F=15.526). Moreover, when comparing the results of location 2, stems presented higher total carotene values (37.45 mg/100g) than leaves (15.64 mg/100g). In both locations, as in TFC tests, flowers presented the lowest values of all. In this sense, it is possible to suggest that stem samples present higher carotene values that the rest of the samples, followed by leaves and flowers.

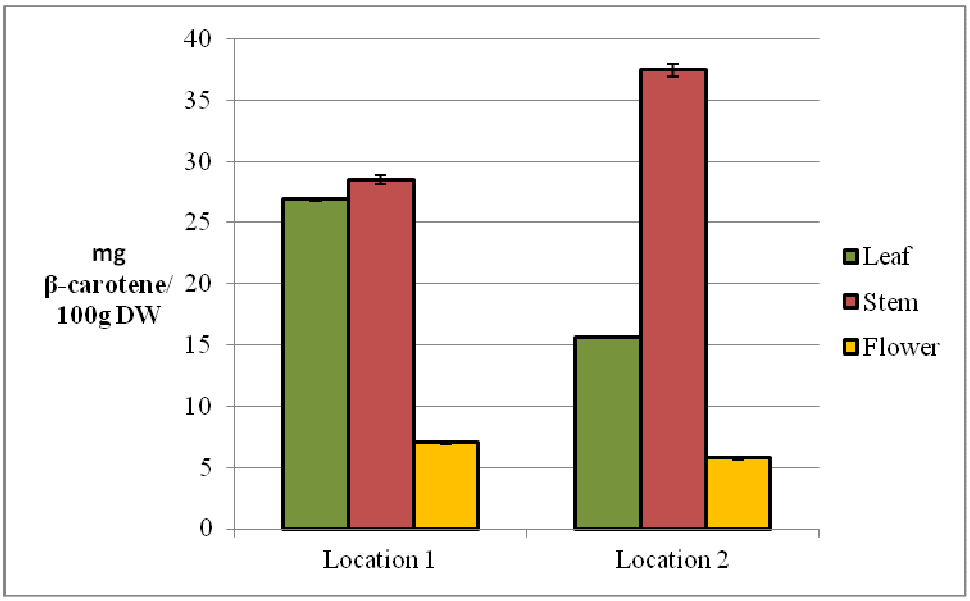

Fig. 13 – Quantification of Total Carotenes for two different locations in three parts of the plant, expressed in mg of β-carotene equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

All these results lead to a relationship between places where no pattern is present and only stems present significant differences (P=0.009 and F=22.41). However,

looking to the global values of post-hoc test for part plant, independent of the location, only leaves and stems are not significant different (P=0,320).

Within locations, on location 1 all the parts of the plant present significant different values from each other (P=0.000 and F=113.84), and on location 2 only leaves and stems values are not significant different (P=0.17 and F=2.690).

4.1.5. Total Monomeric Anthocyanins

Results for Total Monomeric Anthocyanins are the opposite of the study's general trend, since location 1 presents values significantly higher than location 2 (P=0.022 and F=6.86) (table V in annexes, figure 14). On location 1, flowers present higher values (90.15 mg/L) followed by stems and leaves (84.18 and 76.12 mg/L, respectively), however, differences are not significant (P=0.45 and F=0.941).

On the other hand, on location 2 significant differences were found between different plant part (P=0.034 and F=6.1899), specifically between flowers and stems (corresponding to the highest and lowest values, with 81.67 and 59.76 mg/L respectively). Nonetheless, taking into account the results of both location, differences between several plant part were not significant (P=0.073 and F=3.280).

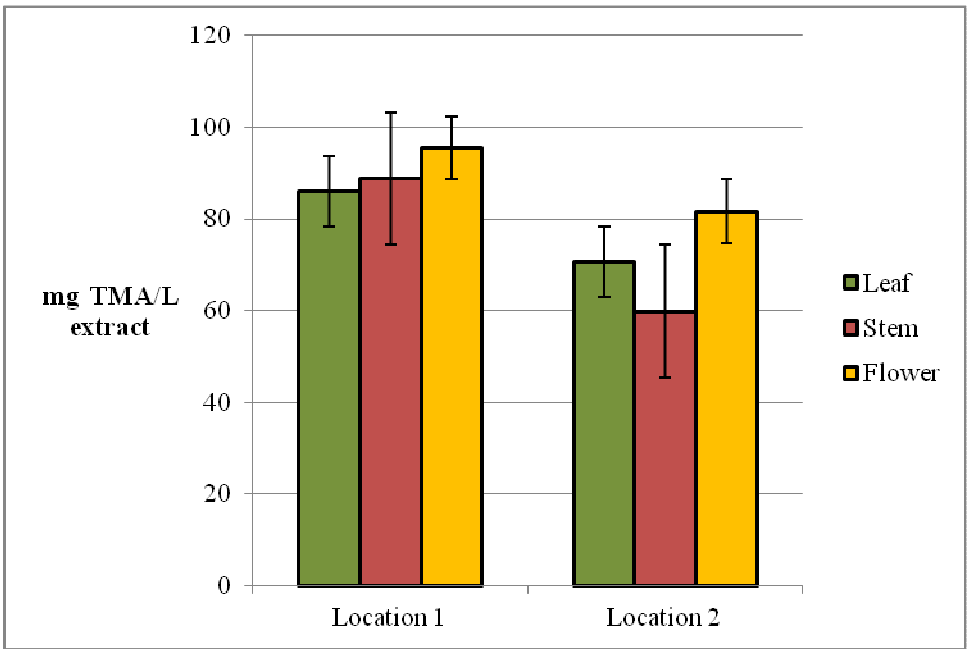

Fig. 14 – Quantification of Total Monomeric Anthocyanins (TMA) for two different locations in three parts of the plant, expressed in mg of anthocyanins equivelents by L of extract. Each value is expressed as mean ± standard error.

4.1.6. Condensed Tannins

Results for Condensed Tannins showed significant differences between different parts of the plant (P=0.003 and F=9.509), but not for different locations (P=0.667 and F=0.194) (table VI in annexes, figure 15).

In both locations stems present the highest values (700.97 in location 1 and 729.45 in location 2), followed by leaves (581.65 in location 1 and 620.65 in location 2) and flowers (562.80 in location 1 and 537.22 in location 2). However, in location 1 differences between leafs and flowers are not significant.

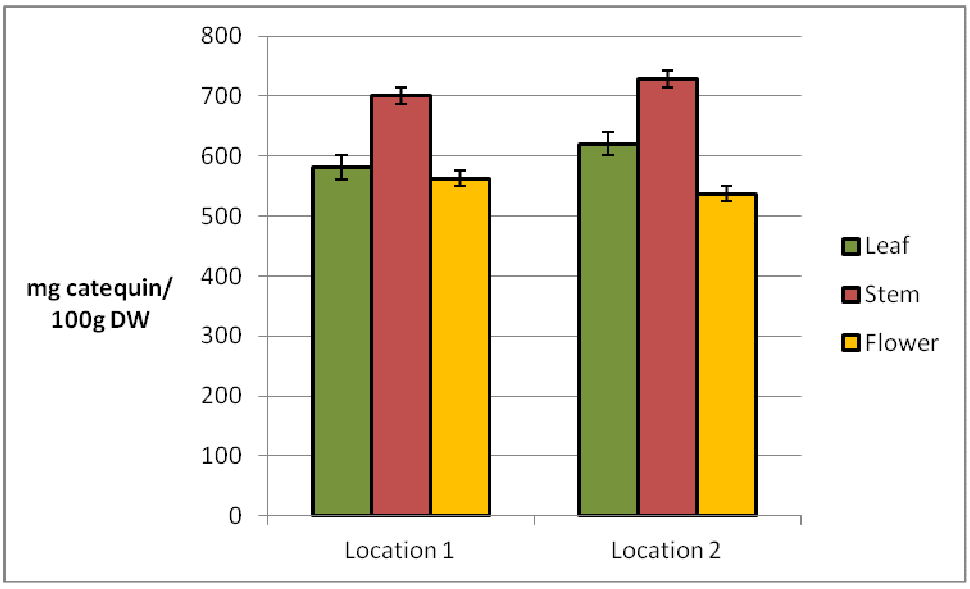

Fig. 15 – ABS values of Condensed tannins (CT) for two different locations in three parts of the plant. Each value is expressed as mean ± standard error.

4.2. *In vitro* Antioxidant Activity assays

4.2.1. Reducing Power (RP)

In the Reducing Power results, as with previous tests, generally, location 2 presents higher values than location 1 (table VII, figure 16). Stem samples showed for both locations the highest values of all with 151.59 mg/100g and 172.61 mg/100g for location 1 and location 2, respectively. Nonetheless, in both cases the differences related

to other samples were not significant (P=0.085 for flowers and P=0.102 for leaves). Once again, and regarding the non significant results there is no pattern present for specific samples with higher values since in location 1 stem samples (151.59 mg/100g) in average are followed by leaves (144.00 mg/100g) and flowers (142.38 mg/100g); whereas in location 2 stem samples (172.61 mg/100g) seemed to be followed by flowers (160.68 mg/100g) and leaves (160.02 mg/100g).

However, despite the non significant differences between the part of the plant, locations present significant differences between each other (P=0.000 and F=25.456).

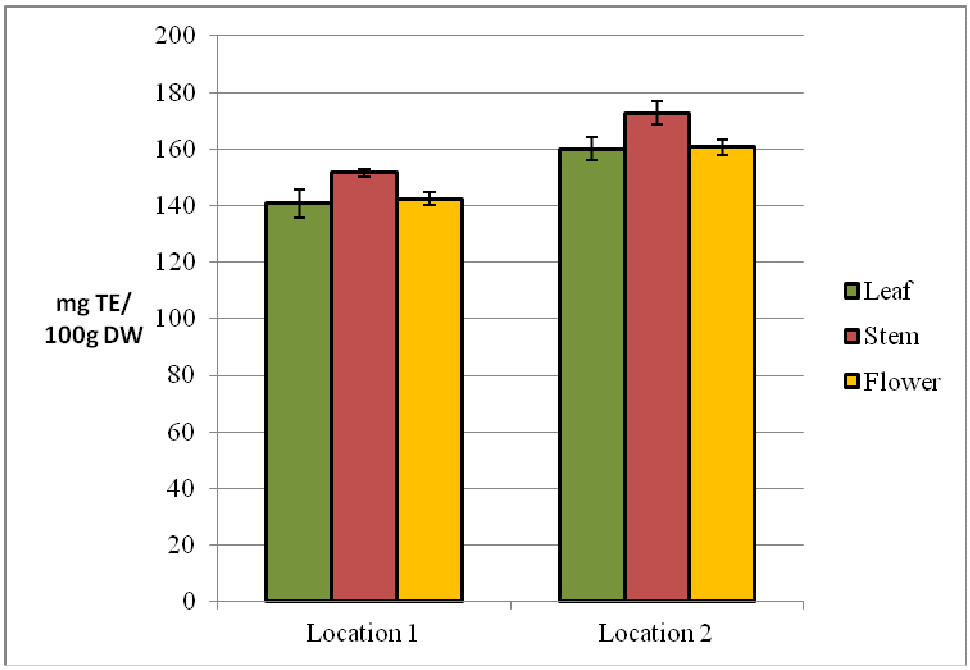

Fig.16 – Quantification of Reducing Power (RP) for two different locations in three parts of the plant, expressed in mg of trolox equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

4.2.2. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP assay revealed completely different results between locations (table VIII in annexes, figure 17), but with location 2 presenting once again the highest values of all, especially in the leaf samples (166.26 mg/100g) which in turn was the lowest sample value in location 1 (90.57 mg/100g). No pattern was observed in this test since location 1 presented in average higher flower concentrations (115.97 mg/100g), followed by stem (106.52 mg/100g) and leaves (90.57 mg/100g); presenting also the biggest variation in leaves and flowers. On the other hand, location 2 presented leaf

samples with higher values (166.26 mg/100g), then flower samples (164.60 mg/100g) and finally stem samples (157.53 mg/100g). However, leaf and flower values for location 2 were not significantly different (P=0.287 and F=1.5).

Globally, differences between locations are significant (P=0.000 and F=242.574), and differences between the part of plant (not taking in account the locations) are significant as well (P=0.019 and F=5.606).

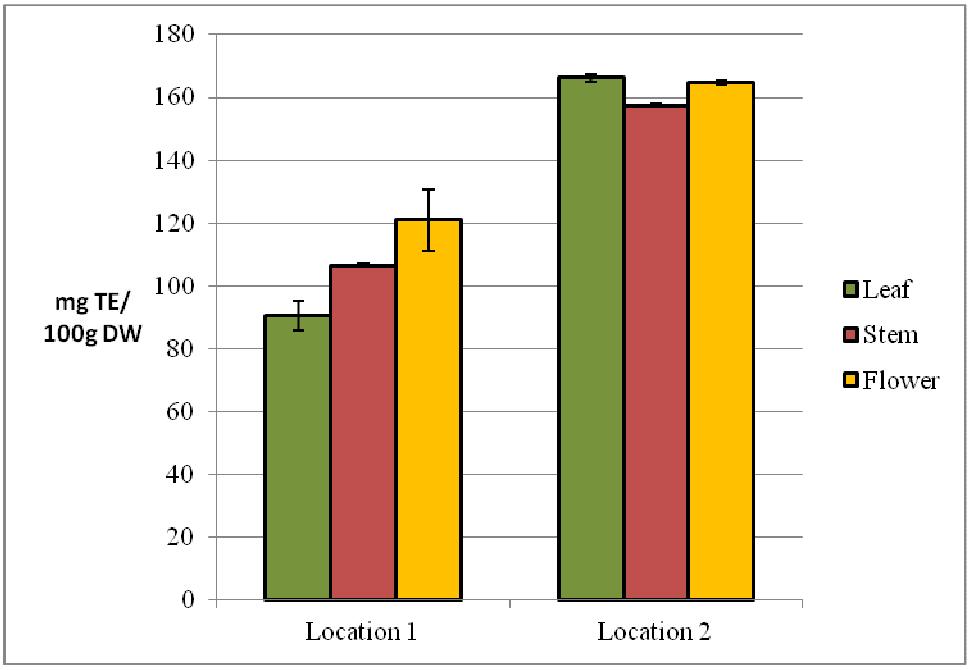

Fig.17 – Quantification of Ferric-Reducing Antioxidant Power (FRAP) for two different locations in three parts of the plant, expressed in mg of trolox equivelents by 100g of dry weight. Each value is expressed as mean ± standard error.

4.2.3. Inhibition Rate Assays

In order to simplify the analysis, results are presented separately for each location.

4.2.3.1. DPPH Radical Scavenging Capacity

On location1 (table IX in annexes, figure 18), stems present better IC_{50} values because smaller concentrations are needed to reach 50% of inhibition of DPPH radicals (between 2 and 4 mg of sample/mL of water), while leaves and flowers present similar values but higher (around 8 mg of sample/mL of water and between 6 and 8 mg of

sample/mL of water, for flowers and leaves, respectively). On location2 (table X in annexes, figure 19), despite the inhibition rate of stems reach the maximum value faster than leaves and flowers, where the increase is more gradual, differences between IC_{50} values for different parts of the plant are imperceptibles, with all parts presenting a value between 1 and 2 mg of sample/mL of water.

Comparing both locations, IC_{50} values are better on location 2 in all the parts of the plant, but especially on leaves and flowers, indicating than location 2 presents higher ability of scavenge the DPPH radicals at small concentrations.

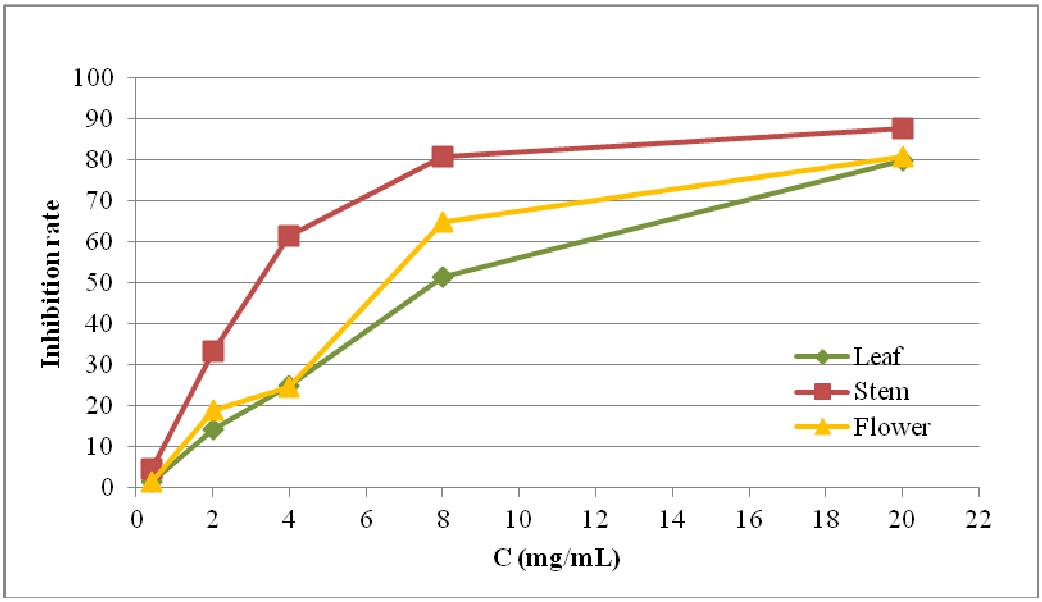

Fig.18 – Scavenging activity on DPPH radicals (%) of the extracts obtained with different plant parts of location 1. Each value is expressed as mean of three replicates.

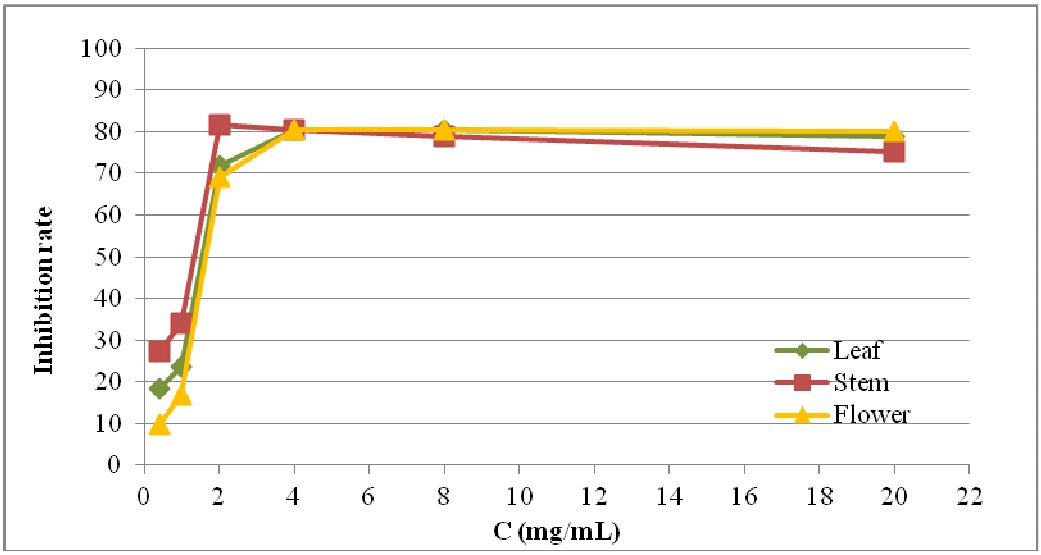

Fig.19 – Scavenging activity on DPPH radicals (%) of the extracts obtained with different plant parts of location 2. Each value is expressed as mean of three replicates.

4.2.3.2. ABTS Radical Scavenging Capacity

For ABTS assay such as for DPPH, stems from location1 present better IC_{50} values (table XI in annexes, figure 20), corresponding to a concentration of approximately 13 mg of sample/mL of water, while leaves and flowers present very similar values (around 30 mg of sample/mL of water for flowers and around 32 mg of sample/mL of water for leaves.

On the other hand, ABTS assay for location 2 presents more differences between the plant parts than the DPPH assay (table XII in annexes, figure 21).

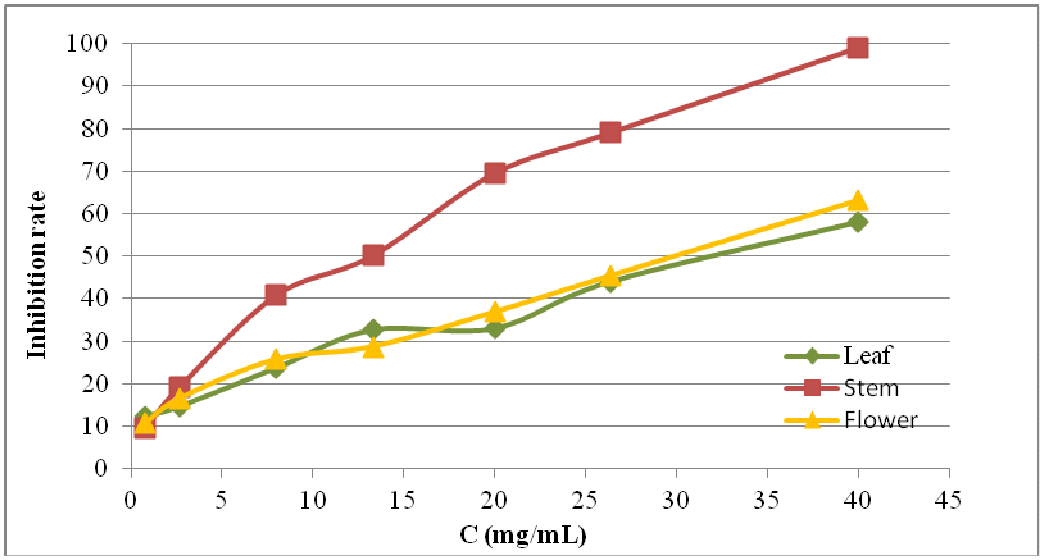

Fig. 20 – Scavenging activity on ABTS radicals (%) of the extracts obtained with different plant parts of location 1. Each value is expressed as mean of three replicates.

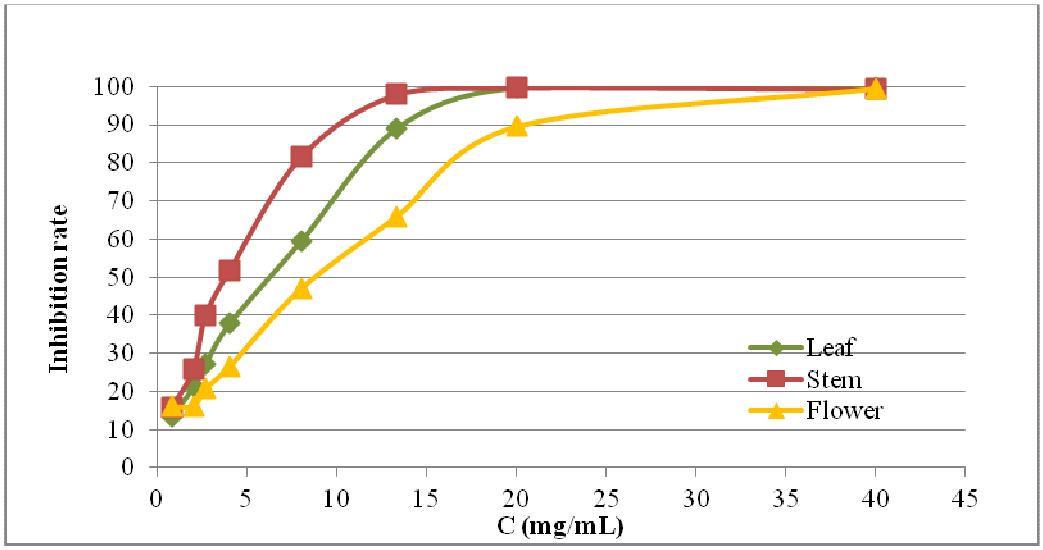

Fig.21 – Scavenging activity on ABTS radicals (%) of the extracts obtained with different plant parts of location 2. Each value is expressed as mean of three replicates.

Thus, stems present the best IC_{50} values, around 4 mg of sample/mL of water followed by leaves and flowers with values around 7 and 10 mg of sample/mL of water, respectively. In this sense, location 2 presents much better values than location 1, indicating than location 2 shows a higher ability to scavenge ABTS radicals than location 1 at small concentrations.

4.2.4. DNA nicking assay

Hydroxyl radicals generated by the Fenton reaction are known to cause oxidatively induced breaks in DNA chains (Prakash *et al.*, 2007). The effect of water extracts of different parts of the plant were studied on plasmid DNA damage (figure 22). As expected all the part of plant shows a protective effect of DNA, trough the inhibition of the Fenton reaction radicals. On lane 1 it is present the native form of pBR322, on lane 2 DNA and Fenton reagent, on lane 3, 4 and 5 only DNA, Fenton reagent and water extracts of leaves, flowers and stems of location1, respectively, and in lanes 6, 7 and 8 DNA, Fenton reagent and water extracts of leaves, flowers and stems of location 2.

(1) (2) (3) (4) (5) (6) (7) (8)

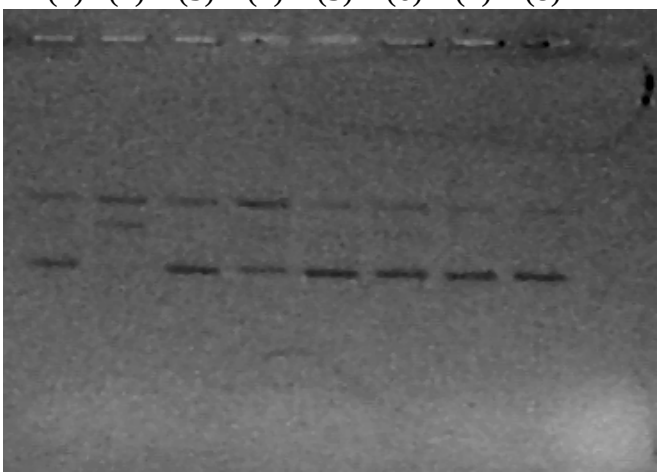

Fig.22 – Inhibitory effects of *Portulaca oleracea* extracts on DNA nicking caused by hidroxyl radicals.
Line 1: DNA plasmid pBR322
Line 2: DNA + Fenton reagent
Line 3 – 5: DNA + Fenton + Portulaca oleracea extracts, location 1 (leaves, stems and flowers, respectively).
Line 6 – 8: DNA + Fenton + Portulaca oleracea extracts, location 2 (leaves, stems and flowers, respectively).

4.3. Individual Analysis Assays

4.3.1. Quantification of flavonoids and phenolic acids by High Performance Liquid Chromatography (HPLC)

The individual analysis of samples from location 1 revealed 13 different compounds on leaves, 5 on stems and 15 on flowers (table II). On leaves, the major constituents are Gallic acid (269.50 µg/g of sample DW), Sinapic acid (71.29 µg/g of sample DW) and anthocyanins, specifically Pelargonidin 3,5 (247.46 µg/g of sample DW), Delphinidin 3 (168.29 µg/g of sample DW), Cyanidin 3 (234.66 µg/g of sample DW) and Pelargonidin 3 (83.53 µg/g of sample DW). Regarding stems, the identified components are present in relatively low concentrations compared with leaves/flowers and the majority is Gallic acid (26.37 µg/g of sample DW) and sinapic acid (30.57 µg/g of sample DW). Finally in flowers, a higher number of components was identified but, in general, in lower concentration than on leaves. The major compounds identified are Pelargonidin 3 (220.91 µg/g of sample DW), Delphinidin 3 (116.46 µg/g of sample DW), Cyanidin3,5 (52.18 µg/g of sample DW) and gallic acid (37.41 µg/g of sample DW).

On location 2, analysis revealed 11 compounds on leaves, 10 on stems and 15 on the flowers (table 15). On leaves the major constituents are gallic acid (110.21 µg/g of sample DW), Cyanidin 3 (111.54 µg/g of sample DW) and Pelargonidin 3 (88.81 µg/g of sample DW). On stems the most identified components are coumaric acid (45.53 µg/g of sample DW), Delphinidin 3,5 (51.18 µg/g of sample DW) and Cyanidin 3,5 (52.76 µg/g of sample DW). As in location 1, stems present lower concentrations than leaves and flowers, except for sinapic and coumaric acids. The majority of the compounds identified in flowers are Pelargonidin 3 (258.10 µg/g of sample DW), Delphinidin 3 (114.20 µg/g of sample DW), benzoic acid (69.44 µg/g of sample DW) and Delphinidin 3,5 and Cyanidin 3,5 (52.70 and 51.18 µg/g of sample DW, respectively).

Comparing both locations it is difficult to establish a relation between them since different parts of the plant present different behaviors. Thus, leaves differ in some components identified and for the same components location 1 presents generally higher concentrations than location 2. Stems, on the other side, present more compounds on location 2 and in relation to the same compounds, this location presents higher

concentrations of coumaric acid and lowers concentrations of Kaempferol and gallic acid. At last, flowers present almost the same concentrations between the two locations.

Table II – Content of phenolic acids and flavonoids in three parts of *Portulaca oleracea* from location1 and location 2. Nd means not detected.

		Location 1			Location 2		
		Concentration (µg/ g)			Concentration (µg/ g)		
	λ (nm)	Leaf	Stem	Flower	Leaf	Stem	Flower
Phenolic acids							
Gallic acid	310	269.49	26.37	37.41	110.21	22.45	29.38
Gentisic acid	310	Nd	Nd	2.05	0.77	1.613	3.90
Benzoic acid	310	3.29	Nd	Nd	3.08	Nd	69.44
Anisic acid	310	6.44	10.07	Nd	2.68	Nd	Nd
Hydroxycinnamic acids							
Chorogenic acid	310	Nd	Nd	2.52	2.09	0.749	1.63
Coumaric acid	310	5.69	13.58	7.85	27.32	45.53	10.52
Sinapic acid	310	71.28	30.57	7.99	18.38	30.57	8.50
Caffeic acid	326	1.23	Nd	0.81	Nd	Nd	0.81
Flavanols							
Catechin	278	4.23	Nd	5.00	Nd	2.18	3.87
Epicatechin	278	6.83	Nd	0.99	Nd	1.82	0.97
Flavonols							
Kaempferol	348	7.27	7.87	0.81	1.80	0.77	0.82
Myricetin	354	Nd	Nd	0.82	Nd	Nd	Nd
Quercetin	354	0.77	Nd	Nd	Nd	Nd	Nd
Anthocyanins							
Delphinidin 3.5	510	Nd	Nd	Nd	53.18	51.18	52.70
Cyanidin 3.5	510	Nd	Nd	52.18	Nd	52.76	51.18
Pelargonidin 3.5	510	247.46	Nd	24.80	Nd	Nd	27.51
Delphinidin 3	510	168.29	Nd	116.46	Nd	Nd	114.20
Cyanidin 3	510	234.66	Nd	67.61	111.54	Nd	Nd
Pelargonidin 3	510	83.53	Nd	220.90	88.81	Nd	258.10

5. Discussion

It is well-known that plant phenolics are, in general, the highest effective free radical scavengers and the best antioxidants (Oliveira *et al.*, 2009) and it has also been recognized the evidence that consumption of some fruits and vegetables, and morbidity and mortality from degenerative diseases are inversed associated, which could be partly attributed to their antioxidant activity (Li *et al.*, 2008). Specifically, it was reported that *Portulaca oleracea* is rich in omega-3 fatty acids (Teixeira & Carvalho, 2009) and may be a good source of antioxidants because it presents some flavonoids and phenolic compounds (Lim & Quah, 2007; Liu *et al.*, 2000).

On this study all the antioxidant profiles were measured based on several different methodologies since antioxidants may act in several different ways, and they may be recognized by one method but not by another one (Dudonné *et al.*, 2009).

5.1 Total Content Assay

5.1.1 Total Antioxidant Activity

Several studies had highlighted the positive relationship between total antioxidant activity and total phenolic content (Luo *et al.*, 2010; Shin *et al.*, 2008; Li *et al.*. 2008; Liu *et al.*, 2002). This is also showed here since the significant differences found in TAA values (between locations or different parts of the plant) are also present in TPC values (see figure 23, as well). For instance, leaves' TPC values for both locations are significantly different and the same occurs for TAA values.

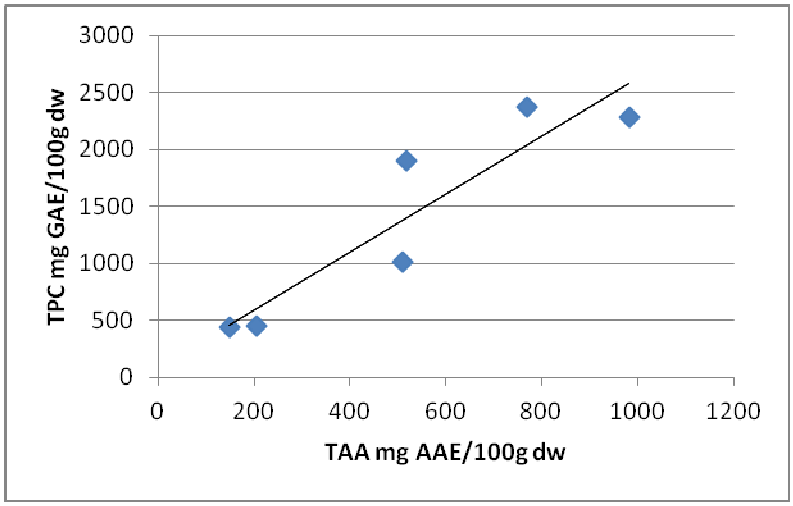

Fig. 23 – Correlation between TAA and TPC (Pearson coefficient = 0.91)

Moreover, within locations, the part of the plant that presents higher values of TPC also presents higher values of TAA. This evidence suggest than in *Portulaca oleracea* the total antioxidant activity is mainly due to the total phenol content and less to the flavonoid content, since this not always happens with TFC values and the pearson coefficient between them is not good. (figure 24).

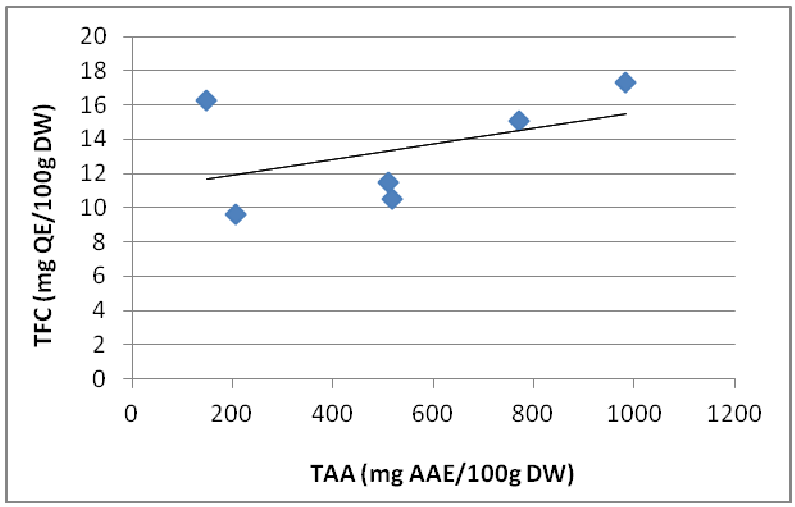

Fig.24 – Correlation between TAA and TFC (Pearson Coefficient = 0.50)

Total antioxidant activity provides a very good understanding about global contributions of the compounds because some of them may have synergistic (Shin *et al.*, 2007; Trabelsi *et al.*, 2010), additive or antagonist interactions with other compounds or even with the medium (Lim & Quah, 2007). Using the same method as in this study but with a different plant, Carvalho *et al.*, (2011) showed that artemisia herb presents lower values of TAA than *Portulaca oleracea*. It is extremely difficult to know if the values reported here are in accordance to what is theoretically expected since little information is available and no published study reporting measurement of total antioxidant activity in *Portulaca oleracea* by this method was found. To this purpose, authors used different methodologies such as DPPH or ABTS assay, which will be discussed later (Lim & Quah, 2007; Ismail *et al.*, 2010; Dudonné *et al.*, 2009).

Differences between locations may be explained by the influence of growth conditions on the concentration of antioxidants compounds which, as it was early referred, including temperature, humidity or drought and characteristics of the soil, salinity, among others (Meot-Duros & Magné, 2009; Aberoumand & Deokule, 2008; Wang & Zheng, 2001; Howard *et al.*, 2002). It is commonly known that countryside

regions such as Alentejo (countryside of Portugal where location 2 is) are subjected to higher temperatures and drought than coastal regions as Algarve, where the proximity to the sea allows higher humidity rates. All this may interfere on soil conditions and on other plant growth conditions.

5.1.2 Total Phenolic Content

Excluding the fact that it is difficult to compare results which result from different analysis methods or due to possible differences on the extraction yield (Spigno *et al.*, 2007) and also from different environmental conditions, the results from this study are now compared with other results from Lim & Quah, (2007). These authors concluded that *Portulaca oleracea* leaves present high values of TPC (around 300 mg GAE/100g DW) and, as present above, the results of this study show even higher values for leaves of both locations. Different environmental conditions between Malaysia and Portugal may be the explanation for these differences since both countries show different climates: Malaysia is from a tropical zone whereas Portugal is from a temperate one. However this evidence seems to be against what is reported in literature. Wang & Zheng, (2001), Howard *et al.*, (2002) and Meot-Duros & Magné, (2009) proposed that higher temperatures increase phenolic contents, but in this study results may indicate that not only temperature must be taken into account, but it is also important to analyze the specific influence of other factors such as humidity and rainfall, which seems to play a role on TPC values.

Variability between locations is also showed here where location 1 presents TPC values around 500 mg GAE/100g DW and location 2 presents around 2300 mg GAE/100g DW, despite the harvest had occurred during the same season. Differences between locations may be explained by the influence of growth conditions on the concentration of polyphenols, as well.

In another study, Cai *et al.*, (2004) reported leaf TPC values around 600 mg GAE/100g but the authors do not refer the sampling period, which could be of extreme importance when comparing results. It is crucial to note that the plants used in this study were harvested at the end of September and beginning of October which coincides with an advanced state of maturation. Recent studies concluded that midmature plants present higher TPC, TFC and TAA values than immature or mature

plants (Pandjaitan *et al.*, 2005), because in this stage they are metabolically more active and require higher concentrations of essential compounds. Then, due to oxidative stress or to their concentration, as the leaf grows polyphenolic levels tend to decrease (Lim & Quah, 2007).

Also, Li *et al.*, (2008) compare some antioxidant properties of 45 Chinese medicinal plants, including *Portulaca oleracea*, presenting a total content of phenolic compounds around 900 mg GAE/100g. Once again, is not correct to compare this value with these presented here by two main reasons: first Li *et al.*, (2008) do not specify which part of the plant they analyzed, and second they used methanol extracts instead of water extracts. This alcohol presents different characteristics and significant differences on the TPC values of extracts from different solvents were reported (Oliveira *et al*., 2009), probably because of its different polarity (Trabelsi *et al.*, 2010). Some published studies refer methanol as the most indicated solvent for phenolic compounds (Siddhuraju & Becker, 2003; Spigno *et al.*, 2007; Trabelsi *et al.*, 2010; Ganesan *et al.*, 2008; Lim & Quah, 2007), due to its ability to inhibit the action of polyphenol oxidase that causes the oxidation of polyphenols (Lim & Quah, 2007; Yao *et al.*, 2004). However, when it is desired to study these properties in food it is better to use aqueous extracts, which are nutritionally more relevant and much more advantageous at the safety level than methanolic ones (Wong *et al.*, 2006), as this study requires. Furthermore, there is one case in purslane where water extraction presented higher phenolic content comparing to methanol extraction (Cai *et al.*, 2004), which indicates than the efficiency of the extraction is much more dependent on the plants compounds to be extracted than on the solvent characteristics. Moreover, Folin–Ciocalteau also reacts with other non-phenolic reducing compounds such as sugars, amino acids and ascorbic acids that are quantified as phenols (Oliveira *et al.*, 2009), and different locations may present different amounts of these compounds, which are not phenols. Regarding the differences between different parts of the plant, this study is contradictory with the one reported by Samec *et al.*, (2010) where flowers present higher TPC values than leaves, and by Ismail *et al.*, (2010) where leaves present higher phenol content than stems. However, in such cases, the plant analyzed is not *Portulaca oleracea* and the quantity and type of phenolic compounds may vary from plant to plant (Li *et al.*, 2008; Cai *et al.*, 2004).

5.1.3 Total Flavonoids Content

As previously mentioned, flavonoids are an important group of phenolic compounds, but the results of TFC not always agree with TPC. Stems are the only part of the plant where the difference between locations is significant. This fact may indicate not only that different part of plant present different components and at different concentrations but also that purslane present other phenolic compounds besides flavonoids, since the differences on TPC are significant even where there are not in TFC values. There is only a significant difference between TFC values from leaves and stems at location 1, and not at location 2. Nonetheless, this is consistent with results reported by Ismail *et al.*, (2010) where leaves show higher concentration of flavonoids than stems. Such as in total antioxidant activity, it is extremely difficult to know if the values reported here are in accordance to what is theoretically expected since little information is available and no published study reporting spetrophotometrical measurement of flavonoids in *Portulaca oleracea* was found. TFC values discussed here are about half of those reported by Lee *et al.*, (2003) for black tea, which plays a key role in the prevention and treatment of several disorders. Other Portuguese herbs, including *Erica sp, Pterospartum tridentatum* and *Cytisus Scoparius,* were reported to have higher values of TFC than the present results for purslane (Luís *et al.* 2009), but *Portulaca oleracea* also presented much higher values than artemisia species reported by Carvalho *et al.* (2011).

5.1.4. Total Carotenes

Compounds such as carotenes, tannins and anthocyanins usually are analyzed by High Performance Liquid Chromatography (HPLC) however, recent studies are measuring some of them by spectrophotometry. In this study there was an attempt to implement these techniques and the measurements of the previously indicated compounds were carried out. However, despite all the advantages this method presents it is very unusual to find published papers to compare results.

Tlili *et al.*, (2010) used HPLC to quantify the total carotenes content in *Capparis spinosa*, a shrub from Mediterranean region very used in traditional cuisine, and reported values around 18.52 and 4.83 mg/100g FW for leaves and flowers, respectively. However, no published study reporting *Portulaca oleracea* was found and

moreover, the values from both studies should not be compared since they are expressed in different units. The significant differences between different parts of the plant seem to support the idea of stems presenting higher content of antioxidant compounds and differences between locations may also be explained based on the geographical different characteristics of the locations (Tlili *et al.*, 2010). On the other hand, joining values of stems and leaves for both locations, they are almost the same. It brings the doubt if in purslane it is possible to separate effectively leaves from stems without traces of the other part of the plant.

According to the obtained results on this study, the concentration of carotenes seems to be related to total flavonoids content since it was presented a good correlation between both parameters (figure 25).

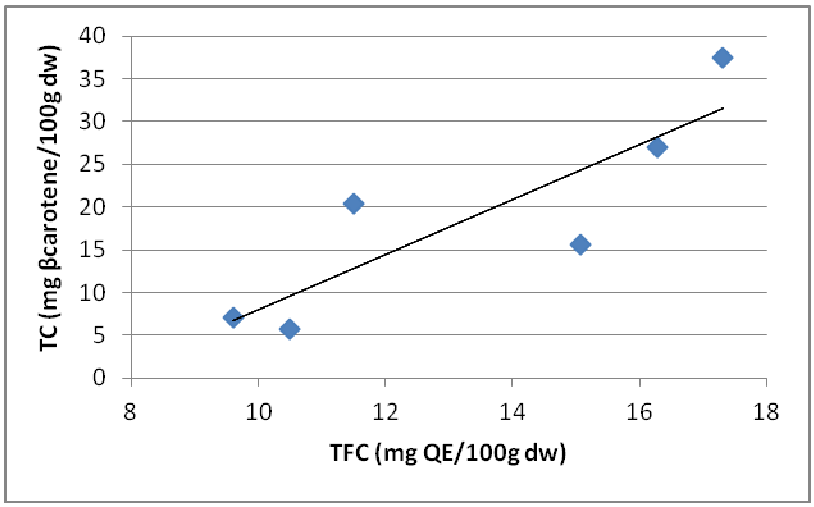

Fig.25 – Correlation between TFC and TC (Pearson coefficient=0.75)

5.1.5. Total Monomeric Anthocyanins (TMA)

Anthocyanins are especially studied on fruits and so, as with carotenes, no papers were found regarding *Portulaca oleracea*, since these plants still have some unknown properties and some studies are missing. The same method by which anthocyanins were analyzed was used by Lee *et al.*, (2005) to determine its content in some fruit juices with values ranging from 13.6 to 3006.8 mg/L of fruit juice. Here, values are compressed between 59.75 and 90.15 mg/L and present approximate values to strawberry juice (63.6 mg/L) and values that are higher than cranberry juice cocktail (13.6 mg/L). Contrarily to previous tests, total anthocyanins content is lower in location 2 than in location 1, and stems represent the part of the plant with lower values for both

locations. This leads to an unexpected inverse relation of total anthocyanins content and total flavonoids content, as well as with total phenol content and total antioxidant activity. On the other hand, it is common accepted that leaves and flowers present higher values of this compound, since it is the main responsible for colored pigmentation on these parts of the plant (Konczak & Zhang, 2004). Obtained results may lead us think that in purslane, anthocyanins are not part of the major responsible for antioxidant activity as it was proposed by Liu *et al.*, (2002) for raspberries.

5.1.6. Condensed Tannins

Information about condensed tannins measured by spectrophotometry was also extremely difficult to find like in the cases of carotenes and anthocyanins. Moreover, due to the problems with calibration curves, it was not possible to express these values in comparable units. However, significant differences were found between different plant parts. Eyob *et al.*, (2008) explained the possible existence of these differences using the interference of the plant part properties on biosynthesis of condensed tannins. Thus, also in purslane the biosynthesis of these compounds may be different on stems, leaves and flowers. Significant differences were found between locations as well, and as the case of phenolic compounds it can be attributed to geographic differences of the locations.

5.2. *In vitro* Antioxidant Activity assay

5.2.1. Reducing Power Assay

Another potential significant indicator of the antioxidant activity of the extract sample is their reducing capacity (Oliveira *et al.*, 2009). This capacity might be due to hydrogen-donating ability and it is generally associated with the presence of reductones (Chen *et al.*, 2007). In this study, significant differences between locations were reported but the same did not happen for different parts of the plant (table 8, figure 16). Once again, this seems to be related not the specificity of each part of the plant compound but with the environmental condition from where the plants are from. Andarwulan *et al.*, (2010) reported that purslane show a reducing power effect of 24.6

µmol TE/g of fresh weigh and it is not correct to compare with the present results since all the measurements were evaluated in dry weight.

Purslane is highly composed of water, which seems to be responsible by high variations in both dimension and weigh from fresh to dry plant. However, these good results obtained allow saying that purslane extracts act as an electron donor and could react with free radicals, converting them to more stable products, and are also able to block the radical chain reaction. No significant differences between parts of the plant may indicate a kind of homogeneity of antioxidant compounds specifically able to donate electrons while the other ones develop other tasks. Phenols are known to play a role on the reducing power, reason why Huang *et al.*, (2005) refer that Folin–Ciocalteau is used by some authors to measure the RP, which is also verified in this study (figure 26).

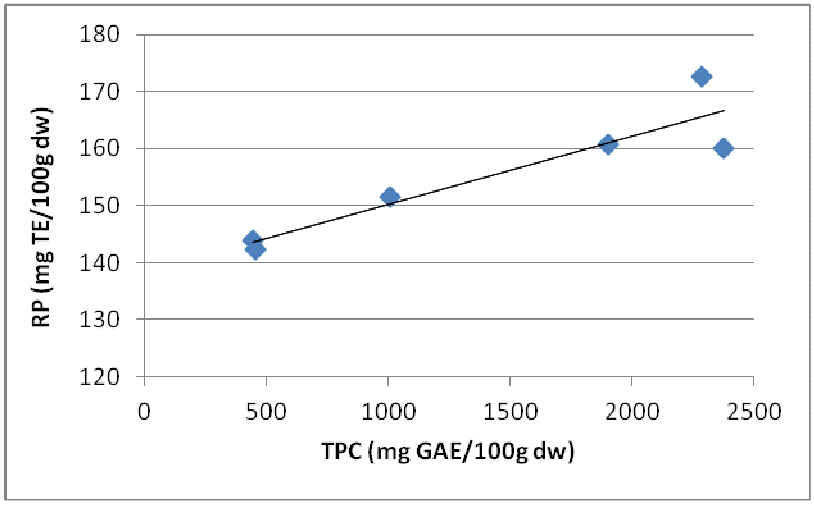

Fig.26 – Correlation between TPC and RP (Pearson coefficient=0.92)

Differences between values of reducing power and total phenolic compounds may be related to the multiple functions played by phenols, and/or with the high number of false positives of the test, early pointed. Also, pH has some influence in this case since acidic, neutral or basic conditions affect the reducing capacity of antioxidants. Basic pH of total phenol content leads to proton dissociation on phenolic compounds and consequently enhance of sample's reducing capacity compared with the neutral pH of the reducing power (Huang *et al.*, 2005).

5.2.2 Ferric-Reducing Antioxidant Power (FRAP) assay

There is still little information about differences between RP and FRAP assays (correlation on figure 27), however the major and more important difference seems to be related with the pH of the reaction atmosphere.

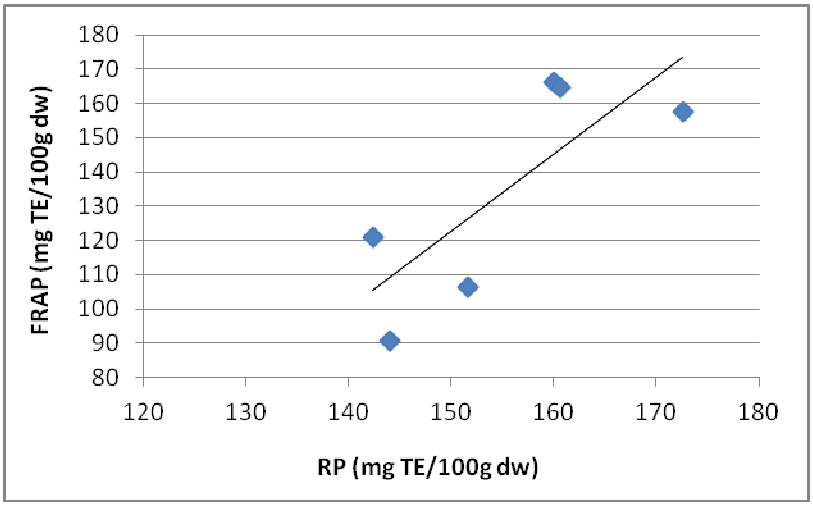

Fig.27 – Correlation between RP and FRAP (Pearson coefficient=0.79)

While RP occurs at a neutral environment, FRAP occur at acidic conditions, which may suppress the reducing capacity due to protonation on antioxidant compounds (Huang *et al.*, 2005). Lim & Quah, (2007) presented results from leaves of 5 different samples of *Portulaca oleracea*, with values ranged from 93 to 510 mg GAE/100g DW. Here, the reported results are among those values for both locations, even taking into account that these results are expressed in mg TE/100g DW and so it is not so linear to compare it. Also Li *et al.*, (2008) reported FRAP values for purslane of 302.233 mg Fe(II)/100g DW, but the same problem of using different units is present. Differences in values of RP and FRAP assays are probably due to the enhanced specificity of reaction, different pH of reaction, as it was early mentioned. However, these variations are not equal for both locations, since differences in both methods on location 2 are not significant (table 9, figure 17). This may indicate that compounds present in location 2 plants are more resistant to pH variations than the other ones. On the other hand, on FRAP assay significant differences were found between different parts of the plant which are consistent with the idea of different parts of the plant presenting significant differences on antioxidant activity, as it was supported by the other results of this study early described. Furthermore, as expected, FRAP and RP assays present a good

correlation, which means that RP may be used as a surrogate method to FRAP assay, since they present slightly the same results. Both RP (figure 26) and FRAP (figure 28) present good correlations with total phenolic content, which suggest that in purslane, the main responsible for the antioxidant activity are the phenolic compounds.

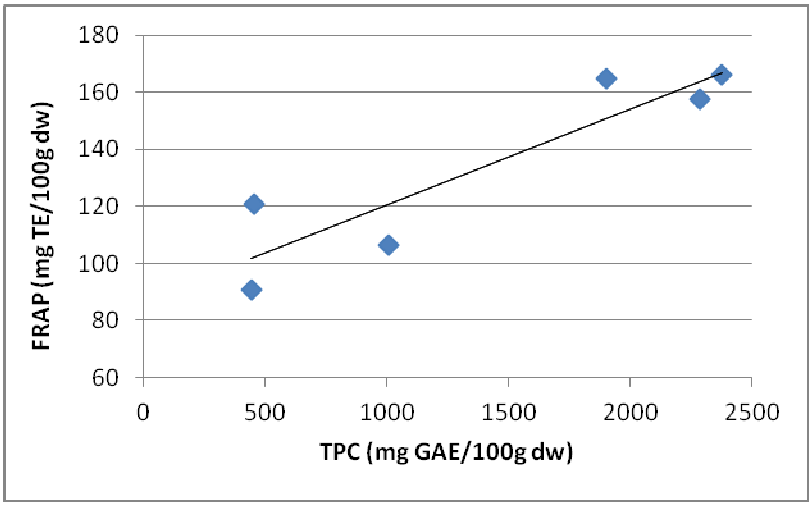

Fig.28 – Correlation between TPC and FRAP (Pearson coefficient=0.91)

5.2.3. Inhibition Rate Assays

5.2.3.1 DPPH Radical Scavenging Capacity

Based on the high efficiency of plant antioxidants to scavenge free radicals, two assays with the most reproducible results and high yield (Dudonné *et al.*, 2009) were done in this study: DPPH and ABTS. The fact that data are expressed in IC_{50} values makes it difficult to compare meanings and calculate possible significant differences however, differences between plant parts and locations are shown in both methods. Once again, location 2 presents more efficient results than location 1, which translates a higher capacity to scavenge radicals and is also consistent with the previously discussed data. The same occurs in plant parts, where generally stems seem to have more antioxidant activity than other parts. Specifically on DPPH, Lim & Quah, (2007) reported leaves values of IC_{50} ranging from 0.89 to 3.41 mg/mL for a range of several seasons, which are consistent with location 2 results, when plants were harvested in Autumn (between 1-2 mg/mL in this study, and values near 1.3 mg/mL in Lim & Quah, (2007)). However, leaves of location1 present worst results than those mentioned above (around 8 mg/mL) since it needs a higher concentration to inhibit 50 percent of DPPH.

It is still important to note that on location 2, all parts of the plant showed very close values of IC_{50}, which may indicate that specific compounds to scavenge DPPH radical are present in all of them, and specifically on location 2 in a much higher concentration than on location 1, leading to the strongest effect previously verified.

5.2.3.2. ABTS Radical Scavenging Capacity assay

In relation to ABTS assay, there are different values on leaves published by several authors such as Cai *et al.*, (2004) (141 µmol Trolox/100 g DW), Li *et al.*, (2008) (272.8 µmol Trolox/100g DW) and Andarwulan *et al.*, (2010) (567µmol Trolox/100g FW). However, none of them are expressed in inhibitory concentration, and so it is difficult to report which are the best results and do some comparisons. It is not common to find papers that expressed results of ABTS test by inhibitory concentration instead of trolox equivalents (reason why this assay is also known as TEAC). Nonetheless, this method was chosen for 4 main reasons: first, because it is possible to compare the inhibition of DPPH and ABTS. Second, because there is a need to reproduce as faithfully as possible the way people use this plant at home. The third reason is to give some highlights of how to increase the benefits of purslane and for last, because using concentrations in grams is easier to understand and to reproduce in the daily routine than TEAC assay.

Comparing both tests, these results are according with the Andarwulan *et al.*, (2010) data since in purslane DPPH radical inhibition is more effective that the inhibition of ABTS. This may be explained because different plant compounds may have more affinity to DPPH rather than to ABTS. Previous results are also consistent with what was proposed by different studies which report that phenolic content is strongly related with antiradical activity (Trabelsi *et al.*, 2010; Meot-Duros & Magné, 2009), in this case, especially with DPPH. As it was previously said methanol is more efficient to extract polyphenol compounds, however, some studies reported that aqueous extraction presented better results than extraction with organic solvents for both antiradical activity tests: DPPH (Luís *et al.*, 2009) and ABTS (Cai *et al.*, 2004).

5.2.4. DNA nicking assay

Results of DNA nicking assay (figure 22) show an effective protector effect of DNA chains by *Portulaca oleracea* extracts. Looking at lane 1 and lane 2 it is clear that

pBR322 DNA is different in the absence and in the presence of Fenton reagent. Hydroxyl radicals from Fenton reagent induce oxidative breaks in DNA strands, forming a linear (Prakash *et al.*, 2007) and single-stranded nicked form (Lee *et al.*, 2002). The lanes where *Portulaca oleracea* extracts were added are according to the first form, which means that no damage was done by the hydroxyl free radicals, indicating that these extracts present, in fact, a protective role in DNA against oxidative stress caused by free radicals. In biological systems, this protective role is also extended to other biomolecules, such as lipids and proteins (Lee *et al.*, 2002). Lane 4, which corresponds to flowers of location 1 seems to present more DNA on nicked form than the other lanes, which may suggest that these extracts do not present such a strong activity as the other ones. For future studies it will be important and extremely interesting to do this assay with different concentrations of extract, in order to determine the minimal concentration that inhibits the hydroxyl radical activity.

5.3 Individual Analysis Assay

5.3.1 Quantification of Flavonoids and Phenolic acids by High Performance Liquid Chromatography (HPLC)

Analysis and quantification of phenolic acids and flavonoids were taken by HPLC and the results seem contrary of the results from total phenolic content and total flavonoids content assay. When comparing different locations, location 1 seems to present higher concentrations than location 2, and looking to different parts of the plant, stems, which demonstrated high antioxidant compounds on spetrophotometric assays, present the lowest number of compounds and in lower concentrations. Possibly this is mainly due to different extraction solvents: water for spetrophotometric assays extracts and methanol for extracts analyzed by HPLC.

It was early described that solvents with different polarities present different extraction efficiency. Generally the extraction efficacy is higher with polar solvents (Trabelsi *et al.*, 2010), especially methanol (Oliveira *et al.*, 2009), however it needs to be taken into account the organic nature of the solvent, because some hydrophilic compounds are more easily extracted with water than with an organic solvent, and the inverse occurs with hydrophobic ones. These results may highlight that most of the phenolic compounds present on purslane samples, especially on location 2 are more

hydrophilic than hydrophobic and the same may be supposed for the stems of location 1. To minimize these problems Spigno *et al.*, (2007) proposed a mixture of alcohols and water as an efficient solvent to extract phenolic and other antioxidant compounds.

Xu *et al.*, (2006) reported that the major flavonoids in *Portulaca oleracea* are kaempferol, apigenin, myricetin, quercetin and luteolin, however from these ones only kaempferol, associated with reduced risk of ovarian cancer (Andarwulan *et al.*, 2010), was detected at all the samples and for both locations. Myricetin and quercetin are only present in negligible concentration in location 1 flowers and leaves, respectively and apigenin and luteolin were not even detected.

Also, Oliveira *et al.*, (2009) demonstrated the presence of aconitic, citric, fumaric, malic, oxalic and caffeic acids in purslane leaves and stems. Once again, most of all compounds were not detected, except for caffeic acid which was present at very low concentrations. Those results also differ from these present before regarding the relation between the different parts of the plant, since in Oliveira *et al.*, (2009) study stems present higher concentrations than leaves, and here it is the opposite. It is also important to mention that Oliveira *et al.*, (2009) harvested purslane samples at the northeast of Portugal whereas this study harvested on the centre and south regions.

All these tests showed different compounds between locations, which is highly believed to be related to environmental conditions, since no overlapping of compounds was present, or in the case of presence of caffeic acid in a little concentration. However, this study identifies several compounds not detected yet, at least in consulted literature, such as some anthocyanins and gallic, coumaric and sinapic acids, among others. It is important to note that despite these anthocyanins are polymeric, their content is generally consistent with the results of total monomeric anthocyanins, since the concentration of these compounds are higher on location 1 than on location 2, as well as it is lower on stems than in the leaves, for both locations.

The main compounds identified both in location 1 and location 2 present a high range of therapeutical applications. Phenolic acids, for instance, have been reported to possess antibacterial, antiviral, anti-inflammatory and vasodilatory actions (Aberoumand & Deokule, 2008). Also coumaric acid, generally present in olives, apples, peas, beans and potatoes (Zang *et al.*, 2000) may be associated to the reduced risk of coronary heart disease showed by olive oil (Tuck & Hayball, 2002) and sinapic acid is reported to take part of anti-inflammatory processes (Yun *et al.*, 2008). Moreover, catechin present in both locations was proposed to present beneficial effects

besides the antioxidant and scavenger properties, such as protection of blood vessels, reduction of cholesterol levels, and prevention of arteriosclerosis (Lin *et al.*, 1998). According to the same author, the amount and proportion of catechin depends on the plant age/maturation, which suggests that plants harvested in early states of maturation may present different results. Boyer & Liu, (2004), still defend that high intakes of catechin were strongly inversely related with deaths by coronary heart disease. Also found in this study but in small concentrations, kaempferol was suggested to inhibit heart, spinal cord, and brain disease (Lau, 2008).

Regarding anthocyanins results, it was reported that some of these compounds, such as pelargonidin, cyanidin and delphinidin 3 have inhibitory effects in some enzymes, as well as vasorelaxant activities (Mazza, 2007). Mazza (2007) also refer the cyanidin as the most commonly occurring anthocyanin in nature.

5.4. *Portulaca oleracea* and health effects

According to what was previously mentioned about the therapeutic potential of tested compounds, this study proves that purslane may benefit health in several areas. High concentrations of phenolic compounds may be involved in several activities such as anticancer (Parr & Bolwell, 2000), anti-thrombotic, cardioprotective, vasodilatory (Trabelsi *et al.*, 2010, Siddhuraju, 2007), antibacterial, antiviral and anti-inflammatory (Aberoumand & Deokule, 2008), apart from their potent antioxidant action and consequent role in neurological and age-related diseases, such as Alzheimer and Parkinson. In this way, purslane may have a crucial role in several areas and not just in a specific one, and all these potential activities mentioned above may end up in different ways. *Portulaca oleracea* may be part of anti-inflammatory lotions or its antibacterial activity may be explored for creating cleaning gels.

Also, flavonoids present in this plant show the major part of these properties and inhibit lipid peroxidation and some enzymatic systems (Cook & Samman, 1996) and pathways (Halliwell *et al.*, 2005). They still present an important role on reducing the risk of coronary health disease (Heim *et al.*, 2002). Besides all these potential effects, purslane may also act in diseases related to urinary tract and diabetes since this plant presents anthocyanins and these compounds are known to have specific functions related to this type of diseases (Mazza, 2007). Nonetheless, they may act in protection of gastric tract, cholesterol lowering and anti-ulcerogenic activities by the action of

tannins (Dykes & Rooney, 2007), and so it is important to highlight here all these different good consequences that arise from the use of *Portulaca oleracea* in daily diets.

Concentrations of the previously referred compounds may vary between locations and parts of the plant making it difficult to say which location presents more beneficial effects for health. On the other hand, general antioxidant activity measured by FRAP, DPPH and ABTS assays seems to be higher in location 2 compared to location 1 and there are also interesting results for the part of the plant. Usually, people use purslane leaves in infusions like teas or soups in daily diets, but this study showed that leaves do not present the highest antioxidant activity values. Often, one part of the plant that is rejected as consumable is the stem, but stems presented higher values than the rest of the samples and people should consider including them on diets, at least with regard to their antioxidant activity. However, the best option is to include as much different parts of the plant as possible to enhance its potential benefits for human health and have a bigger range of compounds present in daily diets.

6. Conclusion

Antioxidants subject is a very complex topic, with several different methodologies and it seems that there is no consensus about which method is more effective (Huang *et al.*, 2005). In this sense, the present study used several different methods in order to obtain results as reliable as possible. Based on previously presented results, water extracts of location 2 seem to have higher values of antioxidant activity, but some compounds tend to present contrasting results, such as the case of flavonoids, carotenes and anthocyanins. The presented results may probably indicate that on purslane these compounds do not have such a crucial role in antioxidant properties as other phenolic compounds, for instance.

Both TAA and TPC present significant higher values in location 2 than in location 1, but for TFC only stems present these significant differences. This may suggest that samples of location 2 present high concentrations of other phenolic compounds besides flavonoids. Regarding natural pigments, both monomeric anthocyanins and carotenes seem to be present in high concentrations on samples of location 1, which hypothesize that on purslane these compounds are not so related with antioxidant potential as they are in other plants and fruits. Also, *in vitro* antioxidant activity assays presented significant higher values for location 2, indicating a biggest capacity of these samples to reduce and scavenge free radicals. Both RP and FRAP are strongly correlated to TPC, which may indicate that the main responsible for the reducing capacity of purslane are, undoubtedly, phenolic compounds.

Infusions of *Portulaca oleracea* still demonstrated high capacity in scavenging DPPH and ABTS free radicals. In both cases, location 2 results showed that a lower concentration is needed to inhibit the same rate of radicals when comparing to location 1. Generally, for all these tests, stems presented better and more efficient results than the other parts of the plan.

Moreover, DNA nicking assay proved that antioxidant compounds present in purslane infusions can, effectively, protect DNA strains from the oxidative damage of free radicals, in this case from hydroxyl radicals formed by Fenton reaction. Despite what was mentioned, phenolic acids and flavonoids individual analysis showed higher concentrations in location 1 than in location 2, such as anthocyanins and gallic acid among others. This analysis also showed higher concentrations on leaves than on stems, which is not according to the global trend of the study. However, due to some specific

method features, extracts used on these tests were obtained with several solvents of different polarities that may have influence with the type and concentration of extracted compounds (Trabelsi *et al.*, 2010).

Taking into account all the realized tests, the high antioxidant activity and the variety of compounds present in high concentrations, it is possible to affirm that purslane may present several important roles in preventing and decreasing effects of a wide range of diseases such as Alzheimer, Parkinson and other neurological diseases, coronary heart disease, myocardial infarction, cholesterol, but also in anti-cancer, anti-inflammatory and anti-viral mechanisms.

This study was able to answer all the questions that lead to the main aims of this work since both location and plant parts have a significant interference on antioxidant activity and also because *Portulaca oleracea* proved to be a rich source of antioxidants and other compounds with many potential benefits for human health. Once this was a recent study and few experiments were done in this area regarding this plant, it will be very interesting to keep going on studying this subject, which would complement and clarify some aspects that were not possible to achieve in this experiment. For instance, it will be very interesting to continue the DNA nicking assay, not only with extracts of different parts of the plant as it was done here, but also with different extracts concentrations, in order to find the minimal concentration that protects effectively DNA from hydroxyl radicals. Also, it would be interesting to study the influence of the harvested season/maturation of the plant on the antioxidant properties that this study confirms. Moreover, it would be good to complement the antioxidant profile traced here with the nutritional composition of purslane, such as the amino acid composition, and conjugate all these data in order to know the ideal dose to consume in a daily diet. Another potential work could be related with controlling growth plant conditions such as temperature, humidity rate and pH of soil, among others. In this sense, it would be possible to understand in what extent these factors interfere with concentration and presence/absence of individuals compounds, such as phenolic acids and anthocyanins in purslane, which allow to establish optimal growth conditions to increase the concentration of compounds with beneficial effects on health. All these future studies would be extremely important and would surely help people to understand how to take more advantage from such a wide dispersed plant.

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Annexes

Table I – Total Antioxidant Activity (TAA) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	AAE (mg/100g)			Average	Std. error
1	Leaf	191.34	139.54	113.64	148.17 [a]	22.84
	Stem	496.05	524.18	506.18	508.81 [b]	8.22
	Flower	190.41	240.01	187.03	205.82 [c]	17.12
2	Leaf	748.08	805.46	757.08	770.20 [d]	17.81
	Stem	935.78	1016.86	994.34	982.33 [e]	24.16
	Flower	497.81	557.38	496.68	517.29 [b]	20.05

Table II – Total Phenolic content (TPC) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	GAE (mg/100g)			Average	Std. error
1	Leaf	434.32	391.86	499.25	441.81[a]	31.22
	Stem	977.79	1164.92	882.98	1008.57 [b]	82.82
	Flower	632.25	389.75	344.75	455.58 [a]	89.28
2	Leaf	2584.58	2205.34	2342.56	2377.49 [c]	110.86
	Stem	2132.37	2382.12	2342.16	2285.55 [c]	77.45
	Flower	1729.56	2120.89	1846.71	1899.05 [d]	115.96

Table III – Total Flavonoids Content (TFC) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	QE (mg/100g)			Average	Std. error
1	Leaf	16.02	16.95	15.86	16.28 [a]	0.33
	Stem	11.34	11.81	11.34	11.50 [b]	0.15
	Flower	9.18	9.18	10.43	9.60 [c]	0.41
2	Leaf	16.31	14.45	14.45	15.07 [a]	0.62
	Stem	16.17	16.01	19.75	17.31 [a]	1.21
	Flower	8.84	9.31	13.35	10.50 [c]	1.43

Table IV – Total Carotenes of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	Concentration (mg/100g)			Average	Std. error
	Leaf	27.03	27.03	26.71	26.92 [a]	0.10
1	Stem	29.21	28.26	27.95	28.47 [b]	0.38
	Flower	7.14	7.14	6.83	7.04 [c]	0.10
	Leaf	15.64	15.64	15.64	15.64 [d]	1.26E-15
2	Stem	38.39	37.13	36.81	37.45 [e]	0.48
	Flower	5.86	5.86	5.56	5.76 [f]	0.10

Table V – Total Monomeric Anthocyanins (TMA) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	TMA mg/L			Average	Std.error
	Leaf	56.44	87.77	84.16	76.12 [a]	9.89
1	Stem	74.94	91.04	86.57	84.18 [a]	4.79
	Flower	79.35	100.39	90.71	90.15 [a]	6.08
	Leaf	63.19	69.80	78.62	70.54 [b]	4.47
2	Stem	51.23	62.25	65.79	59.76 [c]	4.38
	Flower	77.55	77.08	90.37	81.67 [b]	4.35

Table VI – Condensed tannins (CT) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	CT (mg/100g)			Average	Std.error
	Leaf	680,42	417,03	647,50	581,65[a]	82,85
1	Stem	733,83	657,16	711,92	700,97[b]	22,79
	Flower	570,11	570,10	548,18	562,80[a]	15,91
	Leaf	646,20	624,30	591,44	620,65[a]	15,91
2	Stem	711,21	733,09	744,04	729,45[b]	9,64
	Flower	602,99	526,25	482,40	537,21[a]	35,24

Table VII – Reducing Power (RP) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	TE (mg/100g)			Average	Std. error
	Leaf	151.00	136.03	134.97	140.67 [a]	5.17
1	Stem	149.95	150.65	154.16	151.59 [a]	1.30
	Flower	143.91	145.32	137.92	142.38 [a]	2.26
	Leaf	160.14	152,76	167,17	160,02 [b]	4,15
2	Stem	165.22	173,31	179,29	172,61 [b]	4,07
	Flower	157.17	158,92	165,95	160,68 [b]	2,68

Table VIII – Ferric-Reducing Antioxidant Power (FRAP) of *Portulaca oleracea* for two different locations in three parts of the plant (three replicates for each one). Different letters means significant differences

Location	Sample	TE (mg/100g)			Average	Std.error
	Leaf	84.91	99.89	86.91	90.57 [a]	4.69
1	Stem	106.77	105.77	107.01	106.52 [b]	0.38
	Flower	114.22	125.20	108.48	115.97 [c]	9.75
	Leaf	163.85	167.09	167.84	166.26 [d]	1.22
2	Stem	157.53	156.78	158.27	157.53 [e]	0.43
	Flower	164.43	165.93	163.44	164.60 [d]	0.72

Table IX – DPPH scavenging activity of *Portulaca oleracea* for three parts of the plant of location 1 at different concentrations. Values presented are the average of three replicates.

		Inhibition Rate				
Location	Sample	20 mg/mL	8 mg/mL	4 mg/mL	2 mg/mL	0.4 mg/mL
	Leaf	79.99	51.39	24.91	14.30	1.39
1	Stem	87.72	80.89	61.64	33.35	4.74
	Flower	80.93	64.95	24.69	18.91	1.69

Table X – DPPH scavenging activity of *Portulaca oleracea* for three parts of the plant of location 1 at different concentrations. Values presented are the average of three replicates.

		Inhibition rate					
Location	Sample	20mg/mL	8mg/mL	4mg/mL	2mg/mL	1mg/mL	0.4mg/mL
2	Leaf	78.95	80.31	80.57	71.86	23.72	18.32
	Stem	75.22	78.76	80.31	81.55	34.14	27.38
	Flower	80.17	80.52	80.41	68.92	16.98	9.91

Table XI – ABTS scavenging activity of *Portulaca oleracea* for three parts of the plant of location 1 at different concentrations. Values presented are the average of three replicates.

		Inhibition rate %						
Location	Sample	40mg/mL	26.4mg/mL	20mg/mL	13.3mg/mL	8mg/mL	2.66mg/mL	0.8mg/mL
1	Leaf	57.94	44.02	33.05	32.72	23.80	14.80	12.43
	Stem	99.10	79.15	69.51	50.17	40.87	19.15	9.66
	Flower	63.06	45.39	36.84	28.70	25.77	16.50	10.71

Table XII – ABTS scavenging activity of *Portulaca oleracea* for three parts of the plant of location 1 at different concentrations. Values presented are the average of three replicates.

		Inhibition rate %						
Location	Sample	40mg/mL	20mg/mL	13.3mg/mL	8mg/mL	4mg/mL	2.66mg/mL	2mg/mL
2	Leaf	99.57	99.57	88.89	59.51	37.83	27.19	21.33
	Stem	99.34	99.72	97.92	81.60	51.76	39.91	25.87
	Flower	99.53	89.57	65.91	47.08	26.54	20.76	16.26