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Phytochemical analysis and antioxidant activity of *Eucalyptus globulus*: A comparative study between fruits and leaves extracts

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Abstract: Eucalyptus leaf extract is a natural food additive which is included as one of the antioxidant in the list of existing food additives. Taking this into consideration, we compared the phytochemistry of fruits of *Eucalyptus globulus* to that of its leaves, by measuring the phenolic contents and the antioxidant effect of their crude acetonic extracts and their chromatographic fractions. Fraction extracts (Fa, Fb and Fc) were obtained by Sephadex LH-20 column chromatography using ethanol, methanol and acetone as elution solvents. Compared to extract of the leaves, crude extract of fruits contain the highest level of total phenolics and tannins, and exhibited the highest antioxidant effect. Crude extract of fruits has showed the best antioxidant activity compared to its fractions, but fractions (Fb and Fc) of leaves were most active than their crude extract. A positive and moderate correlation was found between phenolic contents and the antioxidant activity of both organs. This study demonstrates that the antioxidant power of crude extract of *E. globulus* fruits is higher than that of its leaves which are considered as a natural food additive. So, their use as preservative in food industry is of immense significance in view of the toxicological implications of the indiscriminate use of synthetic antioxidant.

Key words: *Eucalyptus globulus*; natural food additive, phenolic compounds; chromatographic fractions; antioxidant activity.

1.Introduction

Nowadays there is a significant consumer demand for minimally processed foods and free from synthetic chemical preservatives with the perception of being ''natural''. As a result the food industry is facing great challenges to produce naturally occurring food antimicrobials and antioxidants to reduce the use of synthetic chemical preservatives and still produce safe foods that are also regarded as healthy. Also, there are increasing trends to use herbal life style and dietary choices for human welfare and to improve the productivity and health of farm animals these natural products can help the whole body and improve the immunological status (Mahmoodi et al., 2011). In the other hand, Western society appears to be experiencing a trend of 'green' consumerism, desiring fewer synthetic food additives

and products with a smaller impact on the environment (Burt, 2004). Considering all this, fruits of *E. globulus* could be utilized as food preservative, as it was the case for natural food additive, "eucalyptus leaf extract", which is included as one of the antioxidant in the list of Existing Food Additives [Notification No. 120 (16 April 1996), Ministry of Health and Welfare, Japan] (Amakura et al., 2002; Amakura et al., 2009).

Eucalyptus globulus is native to Australia, it is represented by around 700 species is a genus of tall, evergreen and magnificent trees cultivated world over for its oil, gum, pulp, timber, medicine and aesthetic value (Batish et al., 2008). *E. globulus* is widely cultivated in subtropical and Mediterranean regions and was introduced in Algeria in 1854 by Ramel (Boulekbache-Makhlouf et al., 2010). There are 30000 ha plantations in 1990 and 39000 ha in 1995 (Mahmoudi, 1988). Its secondary metabolites are now being recognised as potential renewable natural resources for human health care (Kim et al., 2001). Its species have been utilized for medicinal purposes, their leaves, roots and fruits have been used as traditional remedies for treatment of various diseases such pulmonary tuberculosis (Sherry et Warnke, 2004) influenza (Ho et al., 2000; Hasegawa et al., 2008) and diabetes (Gallagher et al., 2003; Jouad et al., 2003). These medicinal purposes have been attributed to their essential oils and phenolic contents (Amakura et al., 2002; Atoui et al., 2005; Amakura et al., 2009).

The objective of the present work is to compare the phenolic composition and the antioxidant activity of crude acetonic extracts and the chromatographic fractions of fruits *E. globulus* to those of its leaves.

2. Materials and methods

2.1. Material

Fruits and mature leaves of *E. globulus*, Myrtaceae family, were obtained from field-grown plants. They were taken from 25-year-old trees (10 trees) randomly harvested from the arboretum of Derguinah; Bejaia in northeast Algeria (36.31' 13.56" N, 5.17' 18.43" E), in February 2008. Samples were cleaned with tap water, dried at 40 °C during 4 and 5 days for leaves and fruits, respectively. Finally, the dried samples reduced to thin powder using an electric grinder (Kika Labortechnik, Staufen, Germany).

2.2. Chemicals and reagents

Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Pure water Milli-Q- was delivered by water purification system Millipore (Bedford, MA). Acetone, ethanol, methanol, acetic acid, hexane, Sephadex LH-20 gel, gallic acid, quercetin, tannic acid, 2,2-diphenyl-1-pycryl hydrazyl (DPPH), Hydrogen peroxide (H_2O_2), Butylated hydroxyanisole (BHA) and α tocopherol were purchased from Sigma (represented by Algerian Chemical Society, Setif, Algeria).

2.3. Evaluation of moisture content of the sample

Ten grams of the two samples (fruits and leaves) were placed in oven at 105 °C for 3 hours, the percentage moisture content was calculated as described in Eq. (1).

$$MC(\%) = \frac{W_0}{W_i} \times 100 \quad (1)$$

Where W_0 correspond to the weight loss (g) on drying and W_i correspond to the initial weight of sample (g).

2.4. Preparation of crude extracts

Ten grams of dried powder was extracted for a week with l000 mL of 70% acetone containing 0.5% acetic acid to prevent oxidation (Boulekbache-Makhlouf et al., 2010). The extract was filtered through a Whatman filter paper no. 4 and concentrated to dryness under reduced pressure in rotary evaporation at 40 °C to yield dried extract. Residues were defatted with 25 mL of hexane to remove lipids, and procedure was repeated twice more, concentrated under reduced pressure, and lyophilized to dry powder. The extraction yield efficiency was calculated as the percentage weight loss of the starting material.

2.5. Chromatographic fractionation of extract

Sephadex LH-20 gel was used for fractionation by column chromatography. Crude extract was dissolved in ethanol 75%. After sonication during 20 min, the mixture was applied on to a chromatographic column (length 30 cm, internal diameter 1.6 cm) packed with Sephadex LH-20. Sephadex LH-20 was equilibrated with 95% ethanol for 12 h and then the column was manually packed by elution with the same solvent. The column was exhaustively washed with 95% ethanol then eluated with 99% methanol and aqueous acetone 60%, at a flow rate of 1.7 mL/min, at laboratory temperature (25 °C). After evaporation of solvents under vacuum at 40 °C, the dried fractions were solubilised in ethanol 75% and then, their absorbance was measured at 280 nm using Agilent Spectrophotometer (SpectroScan 50, United Kingdom). Based on the absorbance data, fractions with similar optical density were pooled determining three

subfractions (Fa, Fb and Fc). Solvent was evaporated under vacuum at 40 °C, and then each phenolic fraction was lyophilised (Boulekbache-Makhlouf et al., 2010).

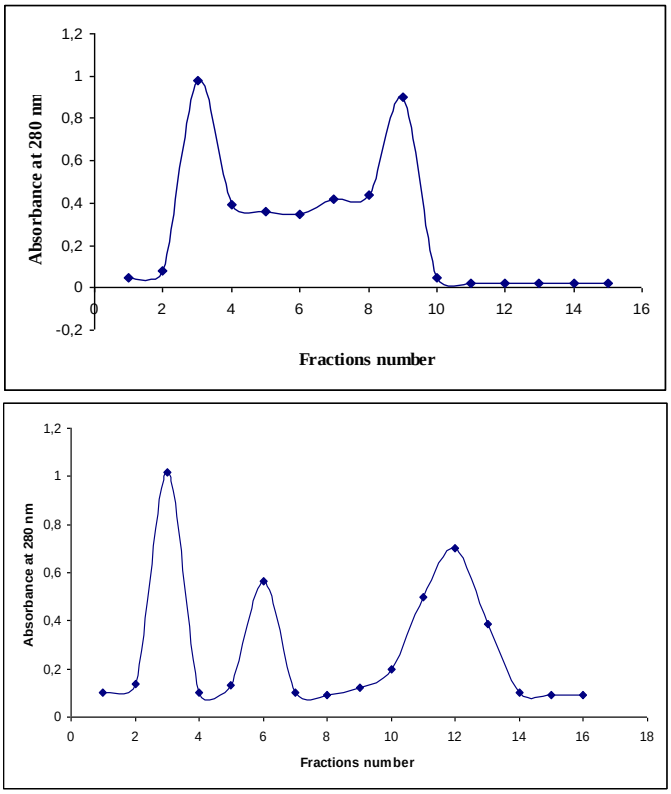

FIGURE 1: Column chromatographic fraction profiles at 280 nm: fruits (A) and leaves (B) of *E. globulus* plant.

2.6. Determination of phenolic contents

The amount of total phenolics in different extracts was determined using the method by Othman et al. (2007). The total phenolics were determined as mg gallic acid equivalent per g of extract (GAE/g of CE) comparing to gallic acid calibration curve prepared in the same conditions with different concentrations of gallic acid (50 to 200 µg/mL) using Eq. (2).

$$y = 6.7032 \cdot x - 0.0246 \left(R^2 = 0.9972\right) \quad (2)$$

The total flavonoid content in the samples was determined according to Djeridane et al. (2006) method, flavonols were determined using the method of Abdel-Hameed (2009). Concentration of flavonoids and flavonols were obtained from a standard curve prepared in the same conditions with different concentrations of quercetin (5 to 60 µg/mL for flavonoids and 10 to 120 µg/mL for flavonols) using Eq. (3) and (4) respectively.

$$y = 32.125 \cdot x - 0.0273 \left(R^2 = 0.9996\right) \quad (3)$$

$$y = 9.715 \cdot x + 0.0811 \left(R^2 = 0.9973\right) \quad (4)$$

Results are expressed as mg of quercetin equivalent per gram of extract (mg quercetin equivalent (QE)/g of CE).

Tannins were estimated spectrophotometrically according to the protocol developed by Hagerman and Butler (1978). Content of tannins was obtained in mg of tannic acid equivalent per g of extract (mg TAE/g of CE) from a standard curve prepared in the same conditions with different concentrations of tannic acid (100 to 1200 µg/mL) using Eq. (5)

$$y = 1.5078 \cdot x + 0.0529 \left(R^2 = 0.9991\right) \quad (5)$$

All analysis were made in triplicate and the mean value was calculated.

2.7. Antioxidant estimation

The diversity of nature and the complexity of phytochemical compounds of plant extracts impose the development of many methods to evaluate the antioxidant activity and to estimate the effectiveness of these substances. We used three methods for the antioxidant estimation of different extracts: scavenging capacity against DPPH radical, based on the procedure by Suja et al. (2005); reducing power was determined according to the method of Hseu et al. (2008) and H_2O_2 scavenging activity assayed according to the method of Sfahlan et al. (2009). Butylated hydroxyanisole (BHA) and α tocopherol were used as positive controls. Results were expressed as IC_{50} value which is defined as a concentration of extract needed to decrease the initial concentration of radicals by 50%. In the case of the reducing power assay, IC_{50} is a concentration of extract needed to gives an absorbance of 0.5. All analyses were done in triplicate and the mean value was calculated.

2.8. Statistical analysis

All experiments were conducted in triplicate and results are expressed as mean ± standard deviation (SD). One-way ANOVA procedure ($p < 0.05$) was performed to compare moisture and phenolic contents means; Tukey's multiple range test (HSD) ($p < 0.05$) was used to compare means of the calculated antioxidant activity

values using STATISTICA 5.5 Fr software. Correlation analysis of antioxidant activity versus the phenolic content was carried out using Microsoft Excel program.

3. Results and discussion

Eucalyptus leaf extract is a natural food additive which is used as food additives. So, in this study we were interested in comparing the phytochemistry of the fruits to that of the leaves for their possible use as natural food preservative in the food industry.

3.1. Moisture content of plant materials and extraction yield of crude extracts

Moisture content organs was significantly different ($p < 0.05$). Leaves present the lower level of water (51.5 ± 0.6%) comparatively to fruits (57.1 ± 0.1%). The extraction yield of crude extract of fruit was higher (30.83 ± 1.2%) than that of leaves (24.37 ± 2.4%).

Phenolic compounds extraction in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size and storage time (Naczk et Shahidi, 2004). Major compounds of *E. globulus* leaves are hydrolysable tannins and flavonol derivatives (Cadahia et al., 1997; Conde et al., 1997; Atoui et al., 2005). In the present study we used acetone 70% as an extraction solvent which is found, on one hand, to be better for the extraction of tannins (Cork et Krockenberger, 1991**)** and on the other hand excellent solvent for flavonoids extraction (Cowan, 1999). We have also used extraction with hexane as an additional step to remove non-phenolic substances such as waxes, fats, terpenes and chlorophylls (Robbins, 2003).

3.2 Phenolic contents of different crude extracts and their fractions

Crude extract of fruit had the highest phenolic, tannins and flavonols contents(Table 1), but the lowest amount of flavonoids compared to leaves extract. Total phenolic and tannin contents of both crude extracts was slightly higher than those of the sum of their fractions; this can be explained by the loss of some phenolic compounds during fractionation procedure (Babayi et al., 2004). Indeed, a brown layer has been observed at the beginning of the column after the fractionation procedure.

TABLE 1: Total phenols, flavonoids, flavonols and tannins contents of fruits extracts

Extracts	Polyphenols (mg EGA/g CE) **	Tannins (mg ETA/g CE) **	Flavonoïds mg EQ/g CE) **	Flavonols mg EQ/g CE) **
Crude extract	464.71 ± 1.52[a]	210.15 ± 3.55[a]	2.99 ± 0.01[c]	2.30 ± 0.22[b]
Fraction a	46.59 ± 0.23[c]	1.28 ± 0.,13[c]	ND	ND
Fraction b	178.20 ± 2.14[b]	86.12 ± 1.51[b]	ND	ND
Fraction c	22.53 ± 0.29[d]	1.19 ± 0.093[c]	ND	ND

*Separated on Sephadex LH-20 column. Values are averages±standard deviation of triplicate analyses; different letters in same column indicate significant difference ($p<0.05$). **Content was expressed per g of crude extract.

In the other hand, results reveals that the content of phenolics and tannins in different fractions of the leaves(Table 2) and fruits extracts were significantly different ($p < 0.05$). Indeed, Fb of fruit and leaves contained the most phenolic and tannins contents. Flavonoids and flavonols have not been detected in all fraction extracts (Fa, Fb, Fc).

TABLE 2: Total phenols, flavonoids, flavonols and tannins contents of leaves extracts

Extracts	Polyphenols (mg EGA/g CE) **	Tannins (mg ETA/g CE) **	Flavonoïds mg EQ/g CE) **	Flavonols mg EQ/g CE) **
Crude	432.63 ± 4.59[a]	105.39 ± 3.65[a]	30.65 ± 0.02[b]	0.33±0.07[c]
Fraction a	53.79± 0.42[d]	ND	ND	ND
Fraction b	206.41±2.81[b]	55.81± 0.96[b]	ND	ND
Fraction c	96.26± 2.33[c]	27.54 ± 0.41[c]	ND	ND

*Separated on Sephadex LH-20 column. Values are averages ± standard deviation of triplicate analyses; dif-

ferent letters in same column indicate significant difference (p<0.05). **Content was expressed per g of crude extract.

These results confirme those obtained in our previous study measuring the phenolic contents of fruits and leaves extracts of *E. globulus* plant (Boulekbache-Makhlouf et al., 2010; Boulekbache-Makhlouf et al., 2013) . Indeed, we have identified three groups of compounds in the two organs (leaves and fruits) of *E. globulus*, two major groups composed by gallic and ellagic acids derivatives, the third group is minor, which correspond to flavonols derivatives (Boulekbache-Makhlouf et al., 2010; Boulekbache-Makhlouf et al., 2013). Accordingly, phenolic compounds were found abundantly in all parts of the plant (Mohd Zin et al., 2006). In the present study, the variability of the distribution of this compound in this different organs may be due to the regulation of their synthesis and their accumulation is influenced by environmental factors (climate, soil) (Armando et al., 1997; Dugald et al., 2004). Indeed, the accumulation of phenolic compounds in grape berries is strongly affected by ‘‘terroir” factors (Li et al., 2011), and rainfall scarcity and long light exposure may be involved in the activation of phenol biosynthesis (Naczk and Shahidi, 2006). Light and water deficits and higher temperature differences between day time and night time could up-regulate the gene expression related to flavonoids metabolism, and thus significantly increase the contents of flavonoids. Infertile soil, rather than fertile ones, provides with more composite and content of inorganic ions, activating flavonoids synthesis (Brahmi et al., 2014).

3.3 Antioxidant activity of different crude extracts and their fractions

3.3.1. Free radical-scavenging capacity of different extracts

At different concentrations of eucalyptus extracts (25, 50, 75, 100, 125 µg/mL), both crude extracts and their fractions showed a dose-dependent anti-radical activity by inhibiting DPPH radical (Fig. 2). At 125 µg/mL of leaves extracts, Fb and Fc, showed high scavenging activity (67.27 ± 0.62%, 69.47 ± 0.21%, respectively) compared with that of crude extract (57.17%), standards BHA (26.98 ± 0.69%), α-tocopherol (17.17 ± 0.4%) and Fa (7.70 %).

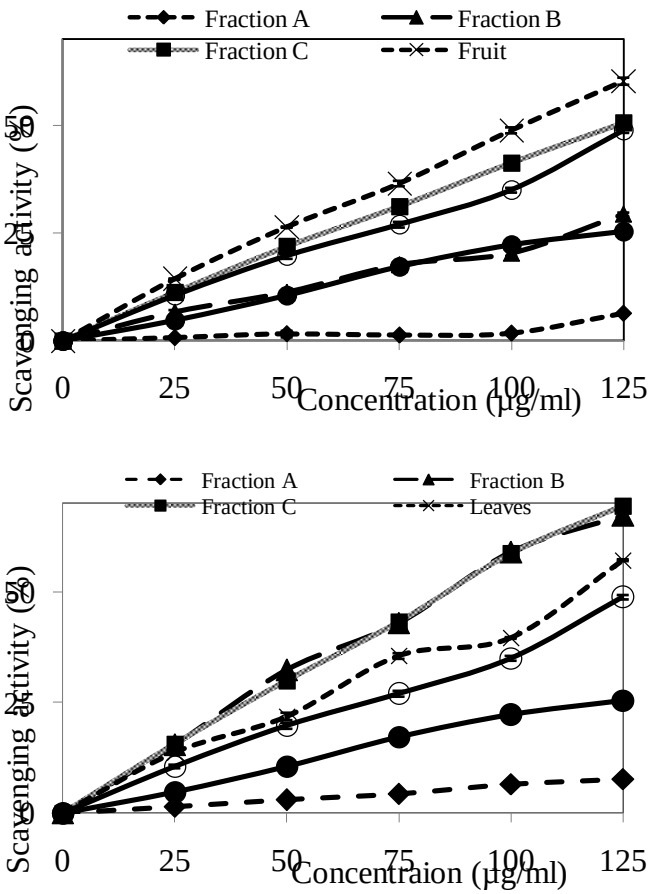

FIGURE 2: Antioxidant activity evaluated by scavenging effect of crude and fractions of fruits (A) and leaves (B) extracts on DPPH radical.

In the case of fruit extracts, crude extract shows the highest activity (60.3 ± 0.82%), but Fc exhibited just moderate activity against DPPH radical (50.62 ± 0, 81%), Fa and Fb have the lowest activities (6.36 ± 0.14%, 29.37 ± 0.43%, respectively).

IC_{50} value of Fb and Fc of leaves extracts which exhibited higher scavenging power against DPPH radical was about 87.77 ± 0.35µg/mL and 87.12 ± 0.51 µg/mL, respectively, significantly lower ($p < 0.05$) than those of BHA and α-tocopherol which were about 134.26 ± 1.55 µg/mL and 233.78 ± 1.00 µg/mL, respectively.

3.3.2. Reducing power

Fig. 3. depicts the reducing power of different concentrations of extracts (25, 50, 75, 100, 125 µg/mL) compared with BHA and α-tocopherol. A part from the Fa, reducing power of all samples was found to be significant and dose dependent. All extracts showed higher activities than that of α-tocopherol (0.53 ± 0.01) and these differences were statisticaly significant ($p < 0.05$). The best reducing power at 125 µg/mL is obtained from

fruits crude extract (1.37±0.03), followed by its Fc (1.18 ± 0.02). As to leaves crude extract, and its Fb and Fc show also effective reducing power (1.26 ± 0.03; 1.31 ± 0.06; 1.37 ± 0.03, respectively).

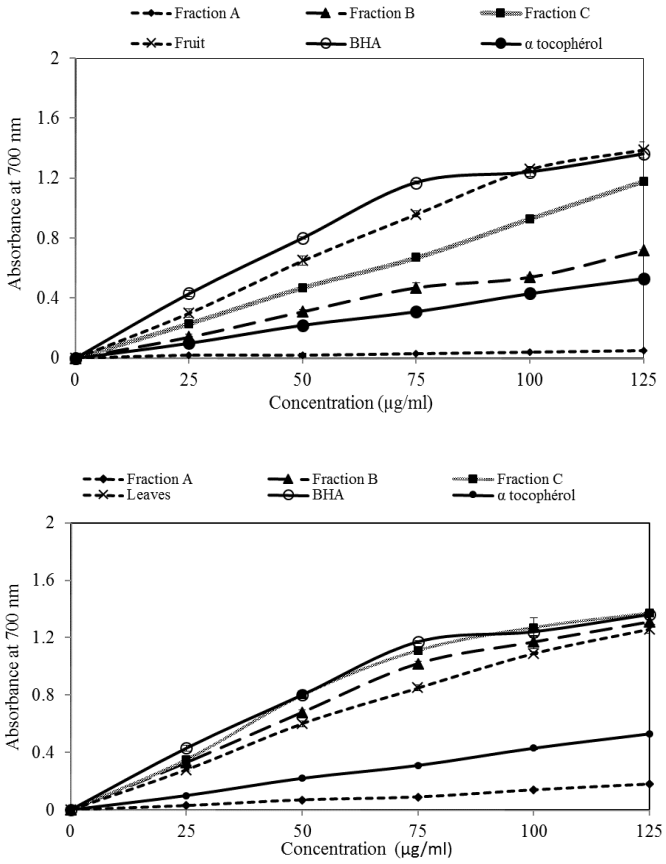

FIGURE 3: Antioxidant activity evaluated by reductive potential of crude and fractions of fruits (A) and leaves (B) extracts on DPPH radical.

IC_{50} values of fruit crude extract and Fc of leaves extract exhibited high reducing power about 40.74 and 33.41 µg/mL, respectively, and were significantly lower ($p < 0.05$) than that of the standard α-tocopherol (117.76 µg/mL).

3.3.3. H_2O_2 scavenging activity

The scavenging activity against H_2O_2 substrate of *E. globulus* plant extracts increased with increasing amounts of sample (Fig. 4). At a concentration of 65 µg/mL, crude extract of fruit and Fc of leaves shows also moderate scavenging activity against H_2O_2 (50.74 ± 0.41; 43.20 ± 0.00%, respectively). A part from Fa of the two parts of the studied plant, scavenging activity of all extracts was higher than of those of the controls BHA (27.25 ± 1.36%) and α-tocopherol (20.07 ± 0.77%). Crude extract of fruit showed the best antioxidant activity by comparison with that of its fractions but in the case of leaves extracts the best activity was exhibited by Fb and Fc.

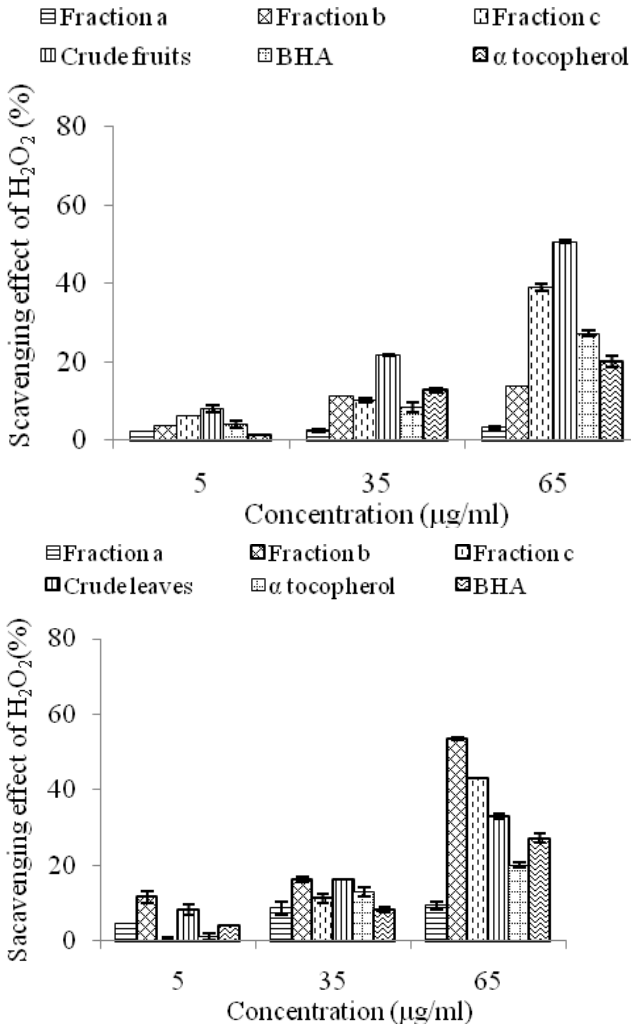

FIGURE 4: Antioxidant activity evaluated by scavenging effect of crude and fractions of fruits (A) and leaves (C) extracts on H_2O_2.

As depicts in Table 3 which shows the classification of the antioxidant activity of extracts of *E. globulus* plant in decreasing order; we note that for leaves extracts, Fb and Fc are more active than their crude extract, as for fruit extracts, crude one showed the highest activity compared to its fractions. As it was mentioned in Section 3.3, the low activity of the fractions of the fruit may be due to the loss of some phenolic compounds during fractionation procedure (Babayi et al., 2004). Indeed, we have observed a brown layer at the head of the chromatographic column after fractionation of our extracts, this layer was much thicker in the case of fruit extract compared to that of leaves one.

TABLE 3: Classification of the antioxidant activity of different extracts

Extracts	DPPH scavenging activity (IC50 µg/mL)	Reducing power (IC50 µg/mL)	H_2O_2 scavenging activity (%)
Crudes	Fruit>Leaves> BHA> α-tocopherol	BHA>Fruit>Leaves>α-tocopherol	Fruit>Leaves> BHA>α-tocopherol
Leaves	Fc=Fb>Crude> BHA> α-tocopherol>Fa	BHA>Fc>Fb>Crude>α-tocopherol>Fa	Fb>Fc>Crude> BHA>α-tocopherol>Fa
Fruit	Crude>Fc>BHA>Fb=α-tocopherol>Fa	BHA>Crude>Fc>Fb>α-tocopherol>Fa	Crude>Fc>BHA>Fb>α-tocopherol>Fa

We have evaluated the correlation coefficient between phenolics compounds and the antioxidant capacity of all extracts in order to determine the compounds which contribute to this activity. We have found a moderate correlation ($p < 0.05$) between total phenolic contents, scavenging of DPPH radical (r = 0.58) and reducing power (r = 0.61). Good correlations were found ($p < 0.05$) between the scavenger effect of hydrogen peroxide and total polyphenols (r = 0.65) as well as tannin contents (r = 0.74).

According to these results, phenolic extracts of *E. globulus* contribute moderately to the antioxidant activity of the studied plant, with the fruits one being the most active, and non phenolic compounds may contribute to the overall its antioxidant activity. This activity is probably due to the presence of hydrolysable tannins in both organs with their predominance in fruits. Our results are in agreement with those reported by Eyles et al. (2004), these authors have estimated the superoxide dismutase- like activity of crude *E. globulus* extracts obtained from wounded wood using the water-soluble tetrazolium salt assay, they concluded that the fraction which contains hydrolysable tannins including pedunculagin, di-, tri- and tetragalloylglucose as well as the standard compounds pentagalloyl glucose and pedunculagin showed the highest levels of antioxidant activity. In addition, it has been reported that the antioxidant activity of gallotannins increased with increasing number of galloyl groups (Tian et al., 2009). Thus, the higher antioxidant activity of the Fc of fruits extract was due to presence of gallotannins with *O*-dihydroxyl groups such as tetra and pentagalloylglucose, which are previously identified in Fc of both parts of the studied plant (Boulekbache-Makhlouf et al., 2010; Boulekbache-Makhlouf et al., 2013). The activity of Fb of fruit and leaves extracts eluted from gel by ethanol/methanol, is due to its contain on lower molecular weight (gallotannins, ellagitannins, ellagic acid derivatives and flavonols (Boulekbache-Makhlouf et al., 2010; Boulekbache-Makhlouf et al., 2013).

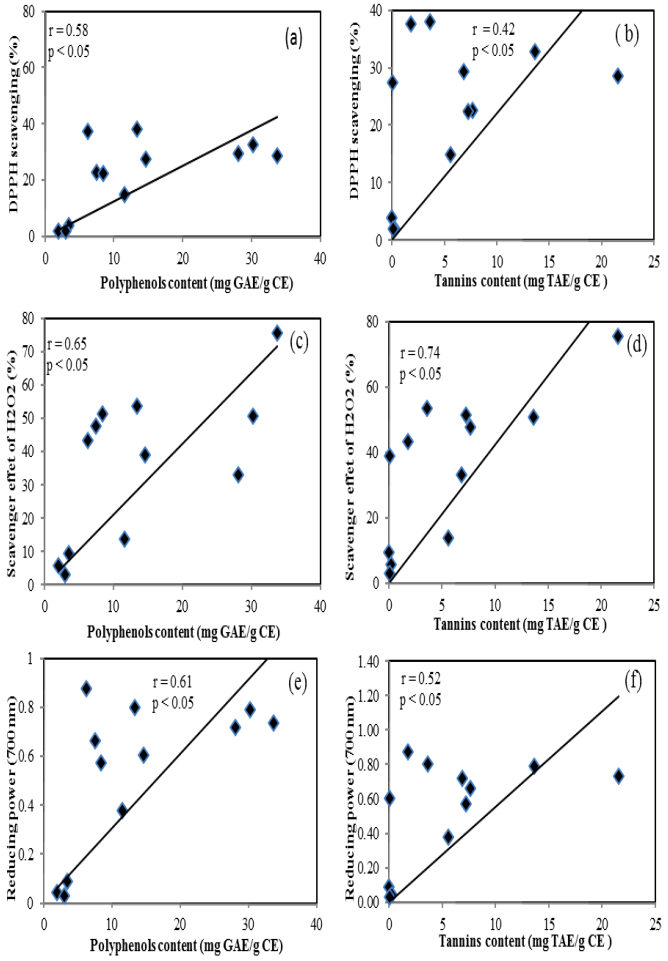

FIGURE 5: Correlation between total phenolic content and antioxidant activities of *E. globulus* extracts: (a) Correlation between total phenolic content and DPPH radical scavenging activity. (c) Correlation between total phenolic content and reducing power activity. (e) Correlation between total phenolic content and scavenging activity of H_2O_2.

Correlation between tannin content and antioxidant activities of *E. globulus* extracts: (b) Correlation between tannin content and DPPH radical scavenging activity. (d) Correlation between tannin content and reducing power activity. (f) Correlation between tannin content and scavenging activity of H_2O_2.

Extract from fruits of *E. globulus* can be used in synergy with that of leaves or other compounds as food additive. Several studies report direct addition of aromatic plants essential oils and extracts to foodstuffs to exert an antimicrobial or antioxidant effect (Carvalho Costa et al., 2015). Extract from fruits of *E. globulus* plants can be incorporated in polymeric matrices, in order to originate active packaging with natural molecules. It is possible to incorporate antioxidants into food packaging, with several advantages, including the delay of both lipid oxidation and protein denaturation (Sanches-Silva et al., 2014).

Conclusion

The fractionation of crude extract by Sephadex LH-20 column chromatography is a useful method for phenolic separation from *E. globulus* plant. This method allowed separation of three fractions with different phenolic contents and antioxidant activity. Fractions b and c of leaves exhibited the highest activity compared to their crude extract, but fruit fractions showed the lowest antioxidant activity compared to that of their fractions Fb and Fc. Our results showed also that fruits of *E. globulus* are a good source of polyphenols and high antioxidant activity compared to that of leaves which were previously used as potential food additives.

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