

Full Length Research Paper

Antioxidant effects and phytochemical analysis of crude and chromatographic fractions obtained from *Eucalyptus globulus* bark

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Eucalyptus globulus is extremely used in traditional medicine in Algeria and its leaf extract is included as one of the antioxidant in the list of existing food additives in Japan. In the present study, the crude extract from bark of this plant and its fractions obtained by Sephadex LH-20 column were studied to quantify their levels of phenolic compounds and their antioxidant activity (ferric reducing power, 1,1-diphenyl-2-picryl hydroxyl (DPPH[•]) radical scavenging activity and scavenging of hydrogen peroxide). All extracts exhibited a reducing power effect higher than that of α -tocopherol. It was observed that crude extract displayed the highest DPPH[•] scavenging ability (IC₅₀ 115.40 μ g/ml) followed by α -tocopherol (IC₅₀ 233.78 μ g/ml). Crude extract also displayed the highest ability to neutralize hydrogen peroxide at 65 μ g/ml (75.55%), followed by α -tocopherol (20.07%) at the same concentration. The results show that fractions b and c exhibited lower antioxidant activities for the three antioxidant tests comparatively to that of crude extract. Fraction a exhibited the lowest antioxidant capacity.

Key words: *Eucalyptus globulus*, bark extract, phenolic compounds, antioxidant effect, Sephadex LH-20 column.

INTRODUCTION

Various medicinal properties have been ascribed to natural herbs. Medicinal plants constitute the main source of new pharmaceuticals and healthcare products. The preservative effect of many plant species and herbs suggests the presence of antioxidative constituents in their tissues (Djeridane et al., 2006).

Eucalyptus globulus grows under a wide range of climatic conditions and is widely distributed throughout Algeria. Because of its fast growth and good quality, it is being established in plantations in many countries, mainly for pulp and paper production. Moreover, its secondary

metabolites are now recognised as potential renewable natural resources for human health care (Kim et al., 2001). *Eucalyptus* species have been utilized for medicinal purposes, their leaves, roots, bark and fruits have been used as traditional remedies for treatment of various diseases such as pulmonary tuberculosis, influenza, diabetes, toothaches, snakebites, diarrhea and other complaints (Hou et al., 2000; Kim et al., 2001; Gallagher et al., 2003; Jouad et al., 2003; Sherry and Warnke, 2004; Hasegawa et al., 2008). These medicinal purposes have been attributed to their essential oils and phenolic contents (Atoui et al., 2005; Amakura et al., 2009).

Considering the developing nutraceutical industry and increasing demand for natural functional food additives, bark of *E. globulus* could be further assessed and utilized in view of their phenolics content which have been claimed to have health benefit effects. It has been reported that the natural food additive, "eucalyptus leaf extract", is included as one of the antioxidant in the list of Existing Food Additives [Notification No. 120 (16 April

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Abbreviations: BHT, Butylated hydroxytoluene; BHA, butylated hydroxyanisole; TBHQ, tertbutyl hydroquinone; DPPH, 1,1 diphenyl-2-picryl hydroxyl.

1996), Ministry of Health and Welfare, Japan] (Amakura et al., 2009).

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and tert-butyl hydroquinone (TBHQ), have been used for a long time. However, they are known to have not only toxic and carcinogenic effects on human, but also abnormal effects on enzyme systems (Göktürk et al., 2007). BHA and BHT have been suspected of being responsible for liver damage (Gülçin et al., 2005). Therefore, there is a growing interest in the natural secondary plant metabolites and their potential use as antioxidants in the food and pharmaceutical industries.

Up to now, no previous studies have been done in Algeria about phenolic compounds of *E. globulus*. Thus, the purpose of this study was to evaluate the phytochemical composition and the antioxidant activity of crude extract of the bark of this plant as well as its fractions obtained by fractionation by low pressure chromatography on Sephadex LH-20.

MATERIALS AND METHODS

Plant material

Bark of *E. globulus*, Myrtaceae family, was obtained from its field-grown plants. It was collected from the arboretum of Derguinah (36°31'13.56" N, 5°17' 18.43"), Bejaia, in the north east of Algeria, in February 2010. The plant was identified by Professor Max Henry of the University of Nancy 1, France. A voucher specimen was deposited in the 3BS laboratory of Bejaia University.

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). Other chemicals were purchased from Sigma (represented by Algerian Chemical Society, Sétif, Algeria).

Evaluation of moisture content of the sample

Before proceeding with the extraction of polyphenols, several preliminary steps have been performed; among them was the evaluation of the moisture content. This test was determined according to the method of Nwinuka et al. (2005). Ten gram (10 g) of bark of the studied plant were placed in an oven at 105°C for 3 h, the percentage moisture content was calculated by expressing the loss in weight on drying as a fraction of the initial weight of sample used and multiplied by 100. $MC (\%) = W_o/W_i \times 100$, where W_o correspond to the loss in weight (g) on drying and W_i correspond to the initial weight of sample (g).

Preparation of crude extract

Sample was cleaned with tap water, dried at 40°C and reduced to thin powder. Ten gram (10 g) of dried powder was extracted for a week with 1000 ml of acetone-water (700:300, v/v) containing 0.5% acetic acid to prevent oxidation. The extract was filtered (Whatman paper no. 4) and the acetone was evaporated under reduced

pressure in rotary evaporation at 40° C. The remaining aqueous phase was treated with hexane (25 mL x 3) to remove lipids, concentrated under reduced pressure, and lyophilized. The extraction yield efficiency was calculated as the percentage weight loss of the starting material.

Column chromatographic fractionation of extract

Sephadex LH-20 gel was used for fractionation by column chromatography. Crude extract was dissolved in aqueous ethanol (75%); after sonication for 20 min, the mixture was applied onto a chromatographic column (length 30 cm, internal diameter 1.6 cm) packed with Sephadex LH-20. Sephadex LH-20 was equilibrated with 95% (v/v) ethanol for 12 h and then the column was manually packed by elution with the same solvent. The column was exhaustively washed with 95% (v/v) ethanol then eluated with 99% (v/v) methanol and aqueous acetone 60% (v/v), at a flow rate of 1.7 ml/min. After evaporation of solvents under vacuum at 40°C, all the fractions were reconstituted with ethanol and then, their absorbance was measured at 280 nm. Based on the absorbance data, fractions with similar optical density were pooled; three subfractions (Fa, Fb and Fc) were obtained. Solvent was evaporated under vacuum at 40°C, and then each phenolic fraction was lyophilized.

Determination of total polyphenols

The amount of total phenolics in different extracts was determined with Folin-Ciocalteu reagent using the method of Othman et al. (2007); 200 µl of sample was mixed with 1.5 ml of the Folin-Ciocalteu reagent. The solution were mixed and then incubated at room temperature for 5 min. 1.5 ml of sodium carbonate NaCO₃ (7.5%) was added. After incubation for 90 min in the dark at room temperature, the absorbance was recorded at 750 nm. A standard curve was prepared with the same procedure using gallic acid and the concentration of total phenols was expressed in mg of gallic acid equivalent (GAE) per gram of extract.

Determination of flavonoids

The total flavonoid content in the samples was determined by the method of Djeridane et al. (2006). This method is based on the formation of a complex flavonoid-aluminium, having the absorbivity maximum at 430 nm. One milliliter (1 ml) of crude extract was added to 1 ml of 2 mg/ml of aluminium chloride methanolic solution. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 430 nm. Concentration of flavonoids was deduced from a standard curve and calculated in mg of quercetin equivalent (QE) per gram of extract.

Determination of flavonols

The content of flavonols was determined in different extracts using the method of Abdel-Hameed (2009) with some modifications. This method was also based on the formation of complex with maximum absorption at 440 nm. About 1 ml of each plant extract was mixed with 1 ml aluminium trichloride (20 mg/ml) and 3 ml sodium acetate (50 mg/ml). The absorbance at 440 nm was read after 30 min. The amount of flavonols was expressed as mg of quercetin equivalent (QE) per gram of extract.

Determination of tannins

Tannins were estimated spectrophotometrically according to the

protocol developed by Hagerman and Butler (1978). The method is based upon the obtention of a coloured complex Fe^{2+} -phenols which can be measured at 510 nm. One milliliter (1 ml) of the sample was added to 2 ml of bovine serum albumin (BSA) (1 mg/ml) in methanol, the mixture was vortexed, incubated for 24 h at 4°C and centrifuged at 5000 t/min for 20 min. The obtained pellet was completely dissolved with 4 ml of buffer C (SDS/TEA) and 1 ml of the ferric chloride reagent (FeCl_3), the mixture was incubated for 15 min and then the absorbance was measured spectrophotometrically at 510 nm. Content of tannins was obtained in mg of tannic acid equivalent (TAE) per g of extract, from a standard curve.

Determination of antioxidant activity

Several methods have been developed to assay free radical scavenging capacity and total antioxidant activity of plant extracts. The most common and reliable method involves the determination of the disappearance of free radicals using a spectrophotometer. In our study, we used three methods: reducing power, scavenging capacity against the radical, DPPH and H_2O_2 . BHA and α -tocopherol were used as positive controls.

DPPH assay

The ability of the extracts to scavenge DPPH free radicals was determined by the method of Suja et al. (2005). One hundred microgram per milliliter (100 $\mu\text{g}/\text{ml}$) of various concentrations (25, 50, 75, 100 and 125 $\mu\text{g}/\text{ml}$) of the samples was mixed with 3 ml of DPPH in methanol (0.1 mM). After 30 min of incubation in the dark and ambient temperature, the resultant absorbance was recorded at 515 nm. The percentage scavenging was calculated according to the following equation:

$$\% \text{ scavenging} = [(A_c - A_e) / A_c] 100$$

Where, A_c is the absorbance of the control after 30 min and A_e is the absorbance of extract or standards after 30 min. IC_{50} was calculated as the concentration of extracts causing a 50% inhibition of DPPH radical.

Reducing power test

The reducing power of the extracts was evaluated according to the protocol of Hseu et al. (2008). One milliliter (1 ml) of different concentrations (25, 50, 75, 100 and 125 $\mu\text{g}/\text{ml}$) of the samples was mixed with phosphate buffer (1 ml, 0.2 M, pH= 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (1 ml, 1 g/100 ml). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (TCA) (1 ml, 10g/100 ml) was added to the solution which was then centrifuged for 10 min at 3000 x g. The supernatant was gathered and mixed with distilled water (1.5 ml) and FeCl_3 (150 μl , 0.1 g/100 ml), and the absorbance was measured at 700 nm and compared to the standards, increased absorbance of the reaction mixture indicated increased reducing power.

H_2O_2 assay

H_2O_2 scavenging ability of the different extracts was determined according to the method of Sfahlan et al. (2009). A 40 mM solution of hydrogen peroxide was prepared in phosphate buffer (pH=7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. Extracts (5, 20, 35, 50 and 65 $\mu\text{g}/\text{ml}$) in distilled water were added to 0.6 ml of hydrogen

peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentages of H_2O_2 scavenging of extracts and standards were calculated as:

$$\% \text{ Scavenged } [\text{H}_2\text{O}_2] = [(A_c - A_e) / A_c] 100$$

Where, A_c is the absorbance of the control and A_e is the absorbance in the presence of the extracts or standards.

Statistical analysis

All experiments were conducted in triplicates and results are expressed as mean \pm standard deviation (SD). Analysis of variance was performed by ANOVA procedure with one factor for the determination of phenolic contents. Statistical analysis of the antioxidant activity was performed by analysis of variance with two factors in the software STATISTICA 5.5 Fr. IC_{50} value were determined by regression analysis. Correlation analysis of antioxidant activity versus the phenolic content was carried out using the correlation and regression programme in the EXEL program. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Moisture content of plant material and extraction yield of crude extract

Moisture content of sample is 18.80% and the extraction yield is about 10.09% (Table 1). Phenolic compounds extraction in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size and storage time (Naczka and Shahidi, 2004). As reported in the literature, major compounds of *E. globulus* plant are hydrolyzable tannins and flavonol derivatives (Cadahia et al., 1997; Conde et al., 1997; Atoui et al., 2005). So, we used aqueous acetone (70%) as extraction solvent which is found to be better for the extraction of tannins (Cork and Krockenberger, 1991) and flavonoids (Cowan, 1999). Extraction with hexane is used as an additional step to remove non-phenolic substances such as waxes, fats, and terpenes (Robbins, 2003).

Sephadex LH-20 column chromatography of crude extract

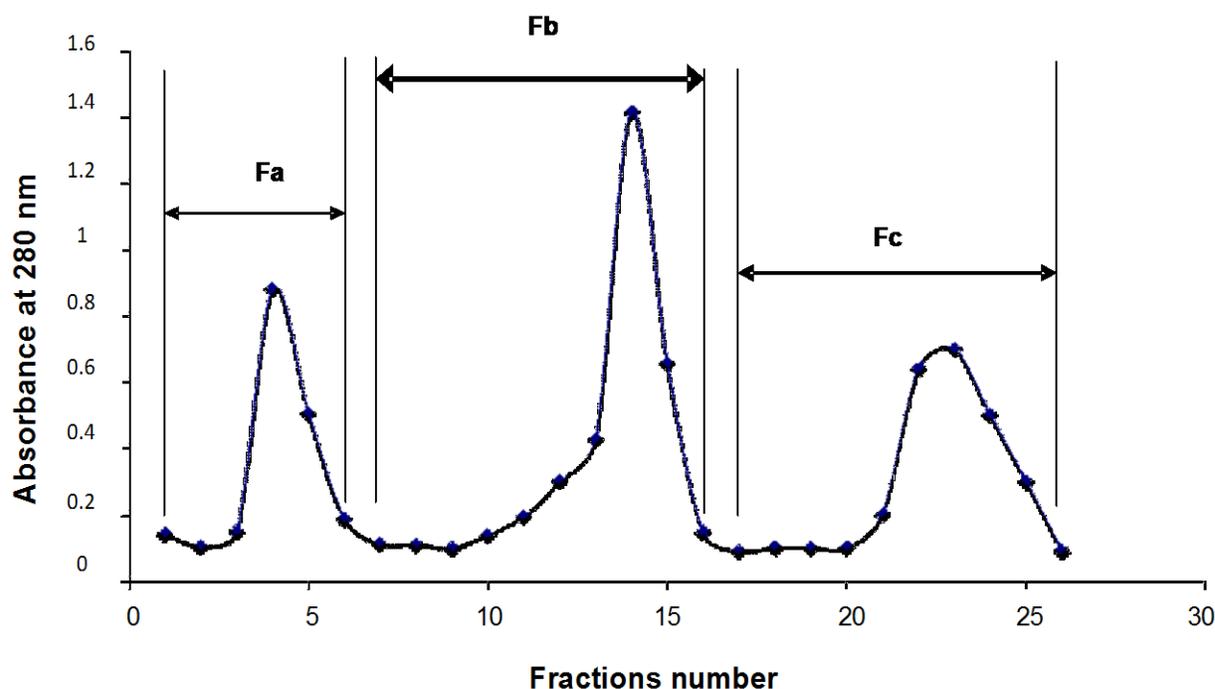
Fractionation of crude extract was performed by column chromatography on Sephadex LH-20. Elution with ethanol, methanol, and aqueous acetone, successively yielded twenty six fractions that were pooled in three fractions (labelled Fa, Fb and Fc), as shown in Figure 1, according to the absorbency readings at 280 nm.

Phenolic contents of different extracts

As depicted in Table 2, phenolic contents and tannin of

Table 1. Moisture content and extraction yield of bark of *E. globulus*.

Sample	Moisture content (%)	Extraction yield (%)
Bark	18.8	10.09

**Figure 1.** Sephadex LH-20 chromatography profile of the extract of *E. globulus* bark.

crude extract was slightly higher than those of the sum of fractions; this can be explained by the loss of some compounds during fractionation procedure (Babayi et al., 2004). Indeed, we have observed a brown layer at the beginning of the column after the fractionation of crude extract. Concerning flavonoids and flavonols contents, all extracts were poor in these classes of phenolic.

DPPH assay

At different concentrations of eucalyptus extracts (25, 50, 75, 100, 125 $\mu\text{g/ml}$), both crude extract and its fractions showed a dose-dependent anti-radical activity by inhibiting DPPH radical (Figure 2). At 125 $\mu\text{g/ml}$, crude extract exhibited moderate scavenging activity (50.72 \pm 0, 61%).

IC₅₀ value is defined as a concentration of extract needed to decrease the initial concentration of DPPH by 50%. IC₅₀ value of crude extract is about 115.40 \pm 1.38 $\mu\text{g/ml}$, which is significantly lower ($p < 0.05$) than those of fraction b (158.61 \pm 1.68 $\mu\text{g/ml}$), fraction c (148.26 \pm 0.95 $\mu\text{g/ml}$) and the two tested standards BHA and α -tocopherol (134.26 \pm 1.55 and 233.78 \pm 1.00 $\mu\text{g/ml}$,

respectively). Fraction a presents the highest IC₅₀ value (2403.09 \pm 0.31 $\mu\text{g/ml}$).

Reducing power

Figure 3 depicts the reducing power of different concentration of extracts (25, 50, 75, 100, 125 $\mu\text{g/ml}$) compared with BHA and α -tocopherol. Apart from the fraction a, reducing power of all samples was found to be significant and dose dependent. All of the amounts of both eucalyptus extracts showed higher activities than that of α -tocopherol (0.53 \pm 0.01) and these differences were statistically significant ($p < 0.05$). With the exception of the fraction a, which showed poor reducing power (0.06 \pm 0.00), crude and fraction b as well as fraction c extracts, exhibited high reducing power (1.28 \pm 0.02, 1.27 \pm 0.05 and 1.04 \pm 0.02, respectively).

In the case of the reducing power assay, the reductive capacity (RC_{0.5}) is arbitrarily defined as the concentration of substrate that gives an absorbance of 0.5 at 700 nm. It is obtained from a linear regression curve (Bourgou et al., 2008). As shown in Table 3, RC_{0.5} value of crude extract is about 40.74 $\mu\text{g/ml}$, significantly lower ($p < 0.05$) than

Table 2. Total phenols, flavonoids, flavonols and tannins contents of bark extracts.

Extract	Polyphenol (mg GAE/g CE) *	Tannin (mg TAE/g CE) *	Flavonoïd (mg QE/g CE) *	Flavonol (mg QE/g CE) *
Crude extract	518.88 ± 4.02 ^a	332.05 ± 9.31 ^a	4.76 ± 0.02 ^a	4.57 ± 0.01 ^a
Fraction a	30.66 ± 1.14 ^d	3.54 ± 0.22 ^c	ND	ND
Fraction b	115.63 ± 0.79 ^c	117.86 ± 0.33 ^b	ND	ND
Fraction c	129.78 ± 0.15 ^b	111.41 ± 1.19 ^b	ND	ND

Values are averages ± standard deviation of triplicate analysis; different letters in same column indicate significant difference (p<0.05); *content was expressed per g of crude extract; ND: not identified.

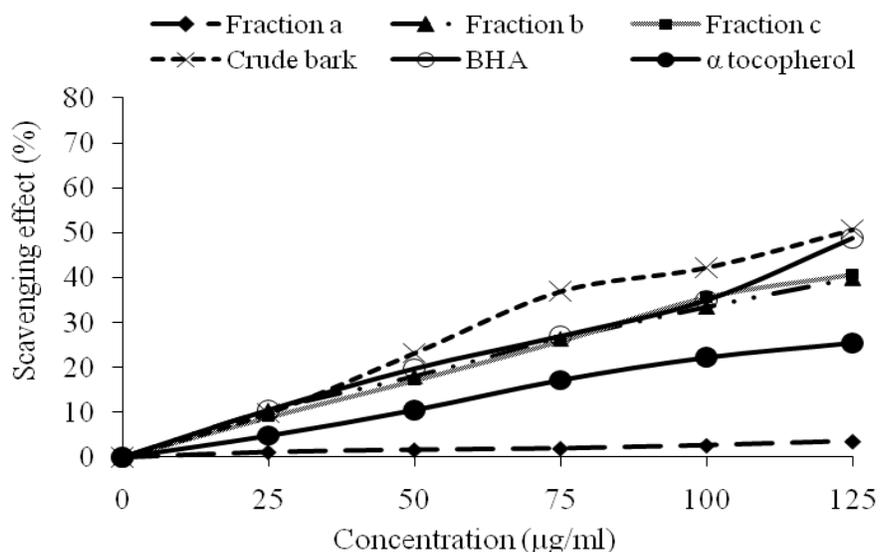


Figure 2. Scavenging effect of extracts, BHA and α tocopherol on DPPH radical.

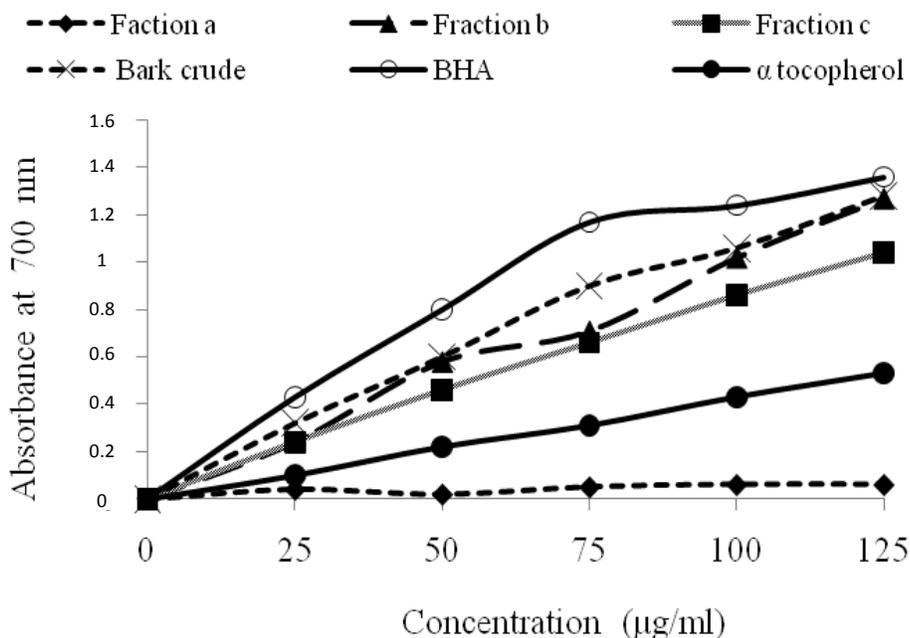
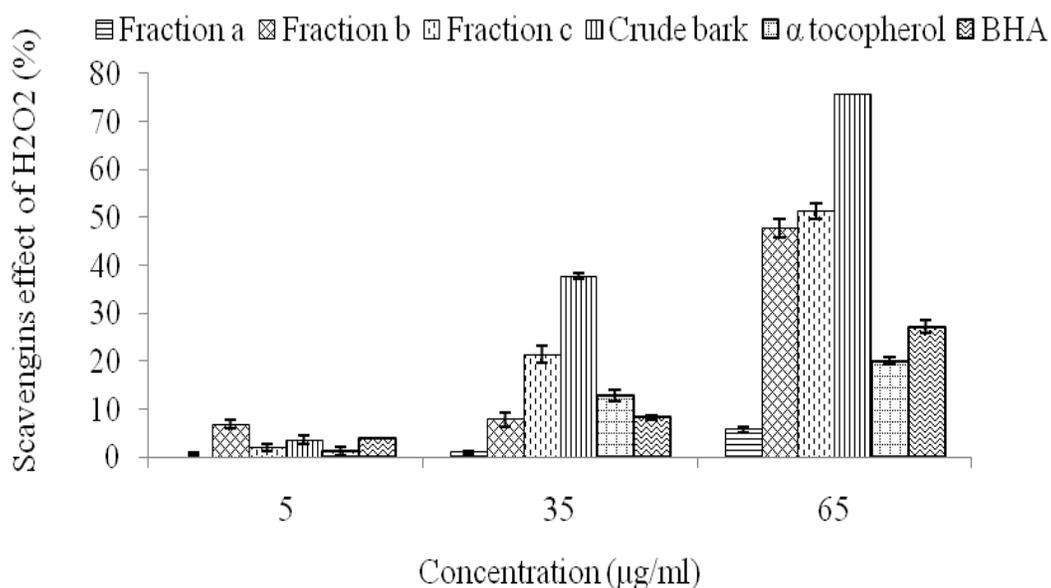


Figure 3. Reductive potential of extracts, BHA and α tocopherol on reducing power.

Table 3. Reductive capacity ($RC_{0.5}$) values of bark extracts.

Extract	Polyphenol (mg GAE/g CE) *	Tannin (mg TAE/g CE) *	Flavonoïd mg QE/g CE) *	Flavonol mg QE/g CE) *
Crude extract	518.88 ± 4.02 ^a	332.05 ± 9.31 ^a	4.76 ± 0.02 ^a	4.57 ± 0.01 ^a
Fraction a	30.66 ± 1.14 ^d	3.54 ± 0.22 ^c	ND	ND
Fraction b	115.63 ± 0.79 ^c	117.86 ± 0.33 ^b	ND	ND
Fraction c	129.78 ± 0.15 ^b	111.41 ± 1.19 ^b	ND	ND

Values are averages ± standard deviation of triplicate analysis; different letters in same column indicate significant difference ($p < 0.05$); *content was expressed per g of crude extract; ND: not identified.

**Figure 4.** Scavenging effect of extracts, BHA and α -tocopherol on H_2O_2 substrate.

that of the standard α -tocopherol (117.76 μ g/ml). There is no difference between $RC_{0.5}$ value of fraction b (49.27 μ g/ml) as well as that of the crude extract (40.74 μ g/ml) and that of fraction c (55.82 μ g/ml).

H_2O_2 assay

The scavenging activity against H_2O_2 substrate of *E. globulus* plant extracts increased with increasing amounts of sample (Figure 4). At a concentration of 65 μ g/ml, crude extract showed high scavenging capacity against H_2O_2 (75.55 ± 0.07%), while that of fractions b and c showed moderate activity (47.74 ± 1.87; 51.45 ± 1.55%, respectively). Crude extract showed the best antioxidant activity by comparison with that of its fractions.

For the three assays, crude extract displayed the strongest antioxidative activity that was significantly ($p < 0.05$) different from those of its fractions and the tested standards. Activities of all extracts are probably

due to the presence of polyphenols especially hydrolyzable tannins which have been previously reported in *E. globulus* (Cadahia et al., 1997; Conde et al., 1997; Hou et al., 2000; Amakura et al., 2009). Eyles et al. (2004) reported that antioxidative activity of wounded wood of *E. globulus* extracts is due to hydrolyzable tannins including pedunculagin, di-, tri- and tetragalloylglucose. In addition, it has been reported that the antioxidant activity of gallotannins increased with increasing number of galloyl groups (Tian et al., 2009). Thus, antioxidant activity of fraction c (eluted by acetone/water 60%) was probably due to the presence of gallotannins with *O*-dihydroxyl groups such as tetra and pentagalloylglucose, which are mainly responsible for their hydrogen donating abilities.

In an attempt to establish a potential relationship between phenolic contents and antioxidant activities of extracts, we have evaluated the correlation coefficient between these two parameters. Results are shown in Figure 5. The correlation values of the phenolic contents and antioxidant activities of extracts are moderate and

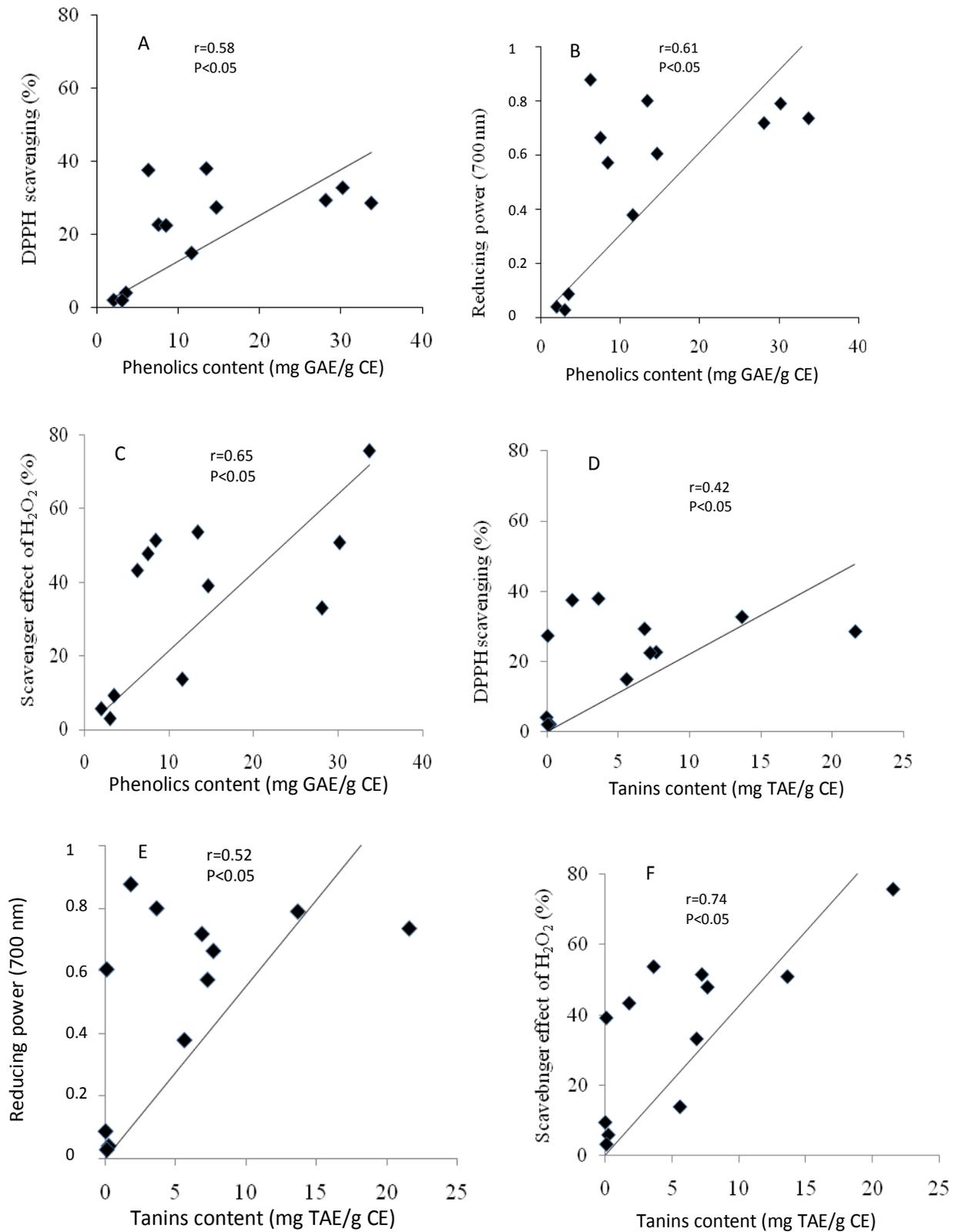


Figure 5. Correlation between total phenolic content and antioxidant activities of *E. globulus* extracts (A) Correlation between total phenolic content DPPH activities. (B) Correlation between total phenolic content and reducing power activity. (C) Correlation between total phenolic content and scavenging activity of H_2O_2 . (D) Correlation between tannins content DPPH activities. (E) Correlation between tannins content and reducing power activity. (F) Correlation between tannins content and scavenging activity of H_2O_2 .

positive for DPPH ($r=0.58$, $p < 0.05$), reducing power ($r=0.61$, $p < 0.05$) and hydrogen peroxide ($r=0.65$, $p < 0.05$) assays. These values are also positive between tannins content and antioxidant activities of extracts; they were moderate in case of reducing power ($r=0.52$, $p < 0.05$) and hydrogen peroxide ($r=0.74$, $p < 0.05$) assays, but lower in case of DPPH ($r=0.42$, $p < 0.05$) assay. These results are consistent with those reported by Vazquez et al. (2008).

Conclusion

The fractionation of the crude extract by Sephadex LH-20 allowed separation of three fractions with different phenolic contents and antioxidant activity. These characteristics are attributed to varying hydrogen and electron-donating capacities of the phenolic compounds present in the crude extract and its fractions. Crude extract showed the highest antioxidant activity. This approach leads to the use of crude extract as antioxidant, instead of pure compounds or purified fractions. Moreover, this alternative is more favourable from an economic point of view. However, the components responsible for its activity are currently unclear. Therefore, it is suggested that further works be performed on the isolation and identification of the antioxidant components.

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