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Total phenolic content, antioxidant and antibacterial activities of fruits of *Eucalyptus globulus* cultivated in Algeria

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ABSTRACT

Crude extract from fruit of *Eucalyptus globulus* (*E. globulus*) was screened for its *in vitro* antioxidant and antibacterial properties. Antioxidant activity was measured by two methods, namely the reducing power and lipid peroxidation inhibition. Antibacterial activity was determined by using disc diffusion method against three bacteria (*Staphylococcus aureus*: ATCC 6538, *Bacillus subtilis*: ATCC 6633 and *Klebsiella pneumoniae*: E 47). The extract exhibited moderate inhibition of lipid peroxidation of linoleic acid emulsion ($51.34 \pm 0.72\%$) and high reducing power ($IC_{50} = 39.52$ μg/mL). It also exhibited strong antibacterial activity against *B. subtilis* and *S. aureus* with minimum inhibitory concentration (MIC) values of 30 μg/mL and 80 μg/mL, respectively. These results suggest that fruits of *E. globulus* have interesting antibacterial and antioxidant activities.

1. Introduction

Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (antimicrobial activity) (Sengul et al., 2009). The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs. Since immemorial time, man has used various parts of plants in the treatment and prevention of various ailments. In recent years, secondary plant metabolites, previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (Parekh and Chanda, 2007). Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections. It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health-benefical effects (Göktürk Baydar et al., 2007; Doughari et al., 2008). They also serve in plant defence mechanisms to prevent damage by microorganisms, insects, and herbivores (Doughari et al., 2008).

In recent years, research has focused on medicinal plants to extract natural and low-cost antioxidant that can replace synthetic additives such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and terbutyl hydroquinone (TBHQ), that might have toxic, carcinogenic and abnormal effects on human (Göktürk Baydar et al., 2007). BHA and BHT have also 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.

E. globulus belong to the family of Myrtaceae. It is known as the blue gum and it is also called the fever tree. Because of its fast growth and increase in woody biomass, the genus *Eucalyptus* is extensively cultivated in the Mediterranean area. Its leaves have been used as traditional remedies for treatment of various diseases such as pulmonary tuberculosis (Sherryl and Warnke, 2004), influenza (Hou et al., 2000; Hasegawa et al., 2008), fungal infections (Takahashi et al., 2004) and diabetes (Alison and Peter, 1998; Gallagher et al., 2003; Jouad et al., 2003). Because of their antioxidant activity, leaf extracts of *E. globulus* have been used as food additives [Notification No. 120 (16 April 1996), Ministry of Health and Welfare, Japan] (Amakura et al., 2009).

Recently, we reported the characterization of several polyphenolic constituents in fruits of *E. globulus* including hydroxybenzoic acids, hydrolyzable tannins and flavonols (Boulekbache-Makhlouf et al., 2010). However, so far, no data have been published on the biological activities of the fruit extract of this plant. As a continuation of our studies, we have investigated the antioxidant and antibacterial capacities of the phenolic extract of *E. globulus* fruits.

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2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (represented by Algerian Chemical Society, Setif, Algeria). The antibacterial activity was screened against two Gram-positive bacteria, *S. aureus* (ATCC 6538) and *B. subtilis* (ATCC 6633), obtained from Saidal Antibiotical and one Gram-negative bacteria, *K. pneumoniae* (E 47), obtained from the Food Microbiology Laboratory of Biological Faculty of Bejaia University.

2.2. Sample preparation

Fruits of *E. globulus*, Myrtaceae family, were collected from plants grown in the arboretum of Derguinah (Bejaia, North East of Algeria). Samples were cleaned, dried in the drying oven at 40 °C during 5 days, and ground to granulometry lower than 250 μm prior to extraction.

2.3. Evaluation of moisture content of the sample

Thermal drying method was used in the determination of moisture content of the sample. 10 g of sample were placed in an oven at 105 °C for 3 h. The moisture content (MC) was calculated by expressing the weight loss upon drying as a fraction of the initial weight of sample used. MC $(\%) = W_0/W_i \times 100$, where W_0 correspond to the loss in weight (g) on drying and W_i correspond to the initial weight of sample (g).

2.4. Extraction of phenolic compounds

Extraction of phenolics was performed as described by Boulekbache-Makhlouf et al. (2010). 10 g of dried powder was extracted with 1000 mL of acetone–water (700:300, v/v) containing 0.5% acetic acid. The process of extraction continued for a week at room temperature, using magnetic blender. 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 × 3) to remove lipids, concentrated under reduced pressure, and lyophilized. The extraction yield was calculated as follows: Yield $(\%) = W_1/W_2 \times 100$, where W_1 was the weight of extract after lyophilization and W_2 was the weight of the dried powder of plant material.

2.5. Determination of phenolic composition

The amount of total phenolics in the extract was determined using the Folin–Ciocalteu reagent and gallic acid as standard as described by Singleton and Rossi (1965).

The total flavonoids content was determined by the methodology of Quettier-Deleu et al. (2000); that of flavonols was determined using the method of Abdel-Hameed El-Sayed (2009), and quercetin was used as standard.

Tannins were estimated spectrophotometrically according to the protocol developed by Hagerman and Butler (1978) and tannic acid was used as standard. All analyses were performed in triplicate and the mean value was calculated.

2.6. Antibacterial activity

The antibacterial activity of the extract was measured by a diffusion test using Mueller–Hinton agar previously inoculated with 1 mL of 18 h old of bacterial suspension (10^6 CFU/mL) (Changwei et al., 2008). Sterilized paper discs (6 mm) were impregnated with 20 μL of different concentrations of extract, (500, 1000, 1500, 2000 μg/mL) prepared in aqueous methanol (80%), and placed onto nutrient agar. The plates were incubated at 4 °C for 2 h to allow diffusion of the active compounds in the medium (Tagg and Mcgiven, 1971). Negative controls were prepared using the same solvent employed to dissolve the plant extract. Gallic and tannic acids are tested in the same conditions as positive controls. Incubation of plates was performed at 37 °C for 24 h. Inhibition zones in mm (without disc paper diameter) around discs were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extract against test microorganisms. The experiment was repeated in triplicate and the mean of diameter of the inhibition zones was calculated.

Minimum inhibition concentration (MIC) was determined as described by Zampini et al. (2005). Different concentrations (10–2000 μg/mL) of extract or standards (gallic and tannic acids) were tested. 1 mL of each solution was mixed with 9 mL of Muller Hinton medium and poured into sterilized Petri plates. Immediately after solidification, the plates were spot inoculated with 10 μL of suspension containing 10^6 CFU/mL of each bacterium. The inoculated plates were incubated at 37 °C for 24 h. The MIC values were determined as the lowest extract or standard concentration at which no growth was observed.

To determine the minimum bactericidal concentration (MBC) values, nutrient broth tubes were inoculated with a sample taken at the spot of the plates which did not show any growth. The mixture was incubated at 37 °C for 24 h. The lowest concentration of the extract or standards with no visible growth after incubation was taken as the minimum bactericidal concentration.

2.7. Antioxidant activity

The reducing power of the extract was evaluated according to the protocol of Hseu et al. (2008). 1 mL of different concentrations of the samples (25, 50, 75, 100 and 125 μg/mL) was mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1%). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (TCA) (1 mL, 10%) was added to the solution which was then centrifuged for 10 min at 3000 × *g*. The supernatant was gathered and mixed with distilled water (1.5 mL) and $FeCl_3$ (150 μL, 0.1%), and the absorbance was measured at 700 nm and compared to the standards (BHA and α-tocopherol), any increase in absorbance is synonymous of an increase in reducing power.

Lipid peroxidation inhibition (LPI) activity was determined using the β-carotene bleaching assay, as described by Chan et al. (2009). 0.5 mL of extract (4 mg/mL) was added to 2 mL of β-carotene/linoleic acid solution. The mixture was incubated at 50 °C for 60 min along with two controls, one containing the antioxidant BHA (positive control) and the other one without BHA or extract (negative control). The absorbance was measured at 470 nm. LPI activity expressed as AOA (%) was calculated as follows:

$$\text{Bleaching rate of β-carotene } (\text{BR}) = \frac{\ln[(A_{\text{initial}})/A_{\text{sample}}]}{60}$$

$$\text{AOA } (\%) = \left(\frac{1 - \text{BR}_{\text{sample}}}{\text{BR}_{\text{control}}}\right) \times 100$$

where A_{initial} and A_{sample} are absorbance of the emulsion before and 1 h after incubation, and $\text{BR}_{\text{sample}}$ and $\text{BR}_{\text{control}}$ are bleaching rates of the sample and negative control, respectively.

2.8. Statistical analysis

All experiments were conducted in triplicate and results are expressed as mean ± standard deviation (SD). Analysis of variance was performed by ANOVA procedure with one factor for the

Table 1
Antibacterial activity of fruits extract of *E. globulus*.

Microorganisms	Inhibition zone (mm)		
	Extract	Gallic acid	Tannic acid
S. aureus	8.67 ± 0.58	13.67 ± 0.58	10.00 ± 0.00
B. subtilis	5.5 ± 0.5	1.00 ± 0.00	5.33 ± 1.15
K. pneumoniae	ND	ND	ND

ND: not determined.

determination of moisture and phenolic contents. Statistical analysis of the antioxidant and antibacterial activities was performed by analysis of variance with two factors in the software STATISTICA 5.5 Fr. IC_{50} value were determined by regression analysis. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Moisture content of sample, extraction yield and phenolic contents

Moisture content of sample is 57.14 ± 0.59% and the extraction yield is about 30.83%. Fruits extract of *E. globulus* was rich in total phenolics and tannins (464.71 ± 1.52 mg GAE/g CE, 332.05 ± 9.31 mg GAE/g CE, respectively), but poor in flavonoids (2.99 ± 0.01 mg QE/g CE), and flavonols (2.3 ± 0.22 mg QE/g CE).

3.2. Antibacterial activity

Table 1 presents diameters of inhibition zones exerted by the extract and the two standards towards tested microorganisms. *E. globulus* fruits extract was effective against the two Gram-positive strains (*S. aureus*, *B. subtilis*) but no activity was observed against the Gram-negative strain (*K. pneumoniae*). Higher inhibition was detected against *S. aureus*, which is one of the most common of the Gram-positive bacteria causing food poisoning. The activity of eucalypt fruit extract is lower than that of gallic and tannic acids. In the case of *B. subtilis*, tannic acid gave comparable inhibition zone (5.33 ± 1.15 mm) compared to that of extract (5.5 ± 0.5 mm) while gallic acid exhibited only weak activity (1.00 ± 0.00 mm) against this strain.

The sensitivity of *S. aureus* to fruit extract of *E. globulus* is consistent with published data about eucalypt species, but the results are difficult to compare because literature assays were carried out at different conditions. Takahashi et al. (2004) found a remarkable antibacterial effect of extracts of leaves of *E. maculata* and *E. viminalis* against *S. aureus*. Khan et al. (2009) have reported the antistaphylococcol activity of leaf extract of *E. globulus* (diameter of inhibition zones ranged from 16 to 25 mm). Egwaikhide et al. (2008) have reported an inhibitory effect of methanol, hexane and ethyl acetate extracts of *E. globulus* against *S. aureus*. Other studies have revealed the sensitivity of this strain to several genera of Myrtaceae family. The extracts of leaves of guava (*Psidium guajava*) and cloves (*Syzgium aromatica*) showed inhibitory effects on growth of *S. aureus*, with inhibition zones ranging from 10 to 20 mm and from 21 to 30 mm, respectively (Ahmad and Beg, 2001).

Quantitative evaluation of the antibacterial activity of the extract of *E. globulus* fruit and of the standards was carried out against selected microorganisms; the MICs of the tested samples are presented in Table 2.

MIC values of the eucalypt extract and tannic acid were lower for *B. subtilis* while gallic acid was a more potent inhibitor of *S. aureus* and much less efficient against *B. subtilis*. Indeed, extracts of leaves and bark of *E. globulus* have been reported to be effective against *B. subtilis* (MIC = 100; 6250 μg/mL, respectively) (Cruz et al., 2005; Khan et al., 2009). Another study, has reported the effective antibacterial capacity of extract of leaves of this plant against the same strain (MIC = 500 ppm) (Hou et al., 2000).

Aligiannis et al. (2001) have proposed a classification of plant extracts on the basis of their MIC values: strong inhibition: MIC < 500 μg/mL; moderate inhibition: 600 μg/mL < MIC < 1500 μg/mL and low inhibition: MIC > 1600 μg/mL. On the basis of this classification, the fruit extract and the tested standards exert a strong inhibitory activity on *S. aureus* (MIC = 80 μg/mL). The extract and tannic acid also have a strong inhibitory activity on *B. subtilis* (MIC = 30; 50 μg/mL, respectively), whereas gallic acid exerts just moderate inhibitory activity on this strain (MIC = 600 μg/mL).

The MBC values of fruits extract against *S. aureus* is less than that of *B. subtilis* (Table 2). The comparison of MICs and MBCs values allows a better evaluation of antibacterial effect of bioactive compounds. According to Biyiti et al. (2004), a substance is bactericidal when the ratio MBC/MIC ≤ 2, and bacteriostatic if the ratio MBC/MIC > 2. Based on these data, the extract of eucalypt fruit and gallic acid exert bactericidal effects against S. aureus (MBC/MIC = 1 and 1.4, respectively), while tannic acid exerts a bacteriostatic effect on the same bacterium (MBC/MIC ≈ 3.33). The fruit extract and tannic acid show a bacteriostatic effect against B. subtilis (MBC/MIC ≈ 13.33 and 40, respectively), whereas gallic acid exhibits a bactericidal effect against this bacteria (MBC/MIC ≈ 1.33).

Results from this study suggest that phenolic compounds are responsible of the antibacterial activity of extract of E. globulus fruits. Numerous works have reported the antibacterial effects of these metabolites against a wide range of bacteria (Ahmad and Beg, 2001; Rodríguez et al., 2009). It has been reported that hydrolyzable tannins have potent antibacterial effects on various bacteria including *B. subtilis* and *S. aureus* (Taguri et al., 2006; Rodríguez et al., 2009). Hou and colleagues have reported the antibacterial activity of eucaglobulin and tellimagrandin isolated from *E. globulus* leaves, against *S. aureus* (MIC values are, 1000 and 31 ppm, respectively), indicating that the observed activity of our extract could be due to its richness in hydrolyzable tannins (Boulekbache-Makhlouf et al., 2010).

Phenolic compounds can act at two different levels: the cell membrane and cell wall of the microorganisms (Taguri et al., 2006). They can interact with the membrane proteins of bacteria by means of hydrogen bonding through their hydroxyl groups which can result in changes in membrane permeability and cause cell destruction. They can also penetrate into bacterial cells and coagulate cell content (Tian et al., 2009). Phenolic compounds are known to be synthesized by plants in response to microbial infection (Doughari et al., 2008; Sengul et al., 2009); it is therefore logical that they have been found *in vitro* to be effective antimicrobial substances against a wide array of micro-organisms (Cowan, 1999).

This study shows that *E. globulus* fruits extract was effective against the two Gram-positive strains (*S. aureus*, *B. subtilis*) but no activity was observed against the Gram-negative bacteria (*K. pneumoniae*). This is consistent with previous studies reporting that Gram-negative bacteria are more resistant to antimicrobials than Gram-positive microorganisms due to their outer lipopolysaccharide membrane (Khan et al., 2009; Al-Zoreky, 2009).

3.3. Antioxidant activity

The reducing power of different concentrations of extract (25, 50, 75, 100, 125 μg/mL) was compared with that of BHA and α-tocopherol (Fig. 1). The reducing capacity of all samples was dose dependent. The fruit extract and BHA showed significantly higher activity than α-tocopherol ($p < 0.05$). IC_{50} value of extract was calculated, it was about 39.52 μg/mL, significantly lower ($p < 0.05$) than that of α-tocopherol (117.76 μg/mL).

Table 2
MIC and MBC for fruits extract of *E. globulus* and standards.

Microorganisms	Extract	Gallic acid	Tannic acid	Extract	Gallic acid	Tannic acid
	MIC (μg/mL)			MBC (μg/mL)		
S. aureus	80	50	90	80	70	300
B. subtilis	30	600	50	400	800	>2000

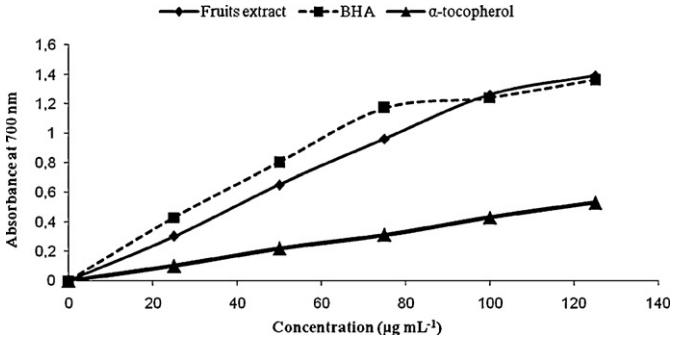

Fig. 1. Reducing power of fruits extract of *E. globulus*, α-tocopherol and BHA.

Concerning the lipid peroxidation activity, the addition of extract and BHA prevented the bleaching of β-carotene. The extract of *E. globulus* fruit showed moderate antioxidant activity (inhibition of 51.34 ± 0.72%) compared to that of BHA, the difference being statistically significant ($p < 0.05$). β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. In this study, the extract of fruit of *E. globulus* was found to limit β-carotene bleaching by neutralizing the linoleate-free radical.

The antioxidant capacity observed with the extract of *E. globulus* fruits is probably due to its high content in phenolic compounds. Activity of putative antioxidants has been attributed to various mechanisms, binding of transition metal ion catalysts and reductive capacity (Hayouni et al., 2007). The activity of fruits extract may be related to the presence of compounds with high molecular weight, especially hydrolyzable tannins which were the main compounds detected in this extract (Boulekbache-Makhlouf et al., 2010). Indeed, this class of tannins has been reported to have potent antioxidative activities (Amakura et al., 2009). Eyles et al. (2004) 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 hydrolyzable tannins 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, antioxidant activity of our extract was due probably to presence of gallotannins with *O*-dihydroxyl groups such as pentagalloylglucose, which is mainly responsible for their hydrogen donating abilities. Antioxidant activity of this extract may also due to the presence of ellagic acid which was detected in our previous study in fruits extract (Boulekbache-Makhlouf et al., 2010). Cruz et al. (2005) have reported that a very remarkable antioxidant activity was determined for extract of wood of *E. globulus*, which had ellagic acid as its main phenolic component. This hydroxybenzoic acid has been reported to be very effective in protecting lipids and LDL from oxidation and an effective *in vivo* metal chelating agent (Cruz et al., 2005).

4. Conclusion

E. globulus has been used as traditional remedies for treatment of various diseases and its leaves have been used as food additive. In this study we report for the first time, the antioxidant and antibacterial activities of a fruit extract of this plant. The acetonic extract of *E. globulus* fruits, that was shown to contain phenolic compounds and especially hydrolyzable tannins, exhibited high reducing power and moderate lipid peroxidation inhibition activity. This extract exhibited also strong antibacterial activity against the two Gram-positive strains: *S. aureus* and *B. subtilis*.

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