Digest Journal of Nanomaterials and Biostructures Vol. 7, No 2, April - June 2012, p. 657 - 660

BIOACTIVITY OF *EUCALYPTUS OLEOSA* VAR. OBTUSE LEAVES AND FLOWERS

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This study was designed to evaluate antioxidant activities of the sub-fractions of methanol extract from the leaves and flowers of *Eucalyptus oleosa* var.obtusa which were cultivated in Kashan area. The samples were subjected to a screening for their possible antioxidant activities by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and b-carotene-linoleic acid assays. Among the extracts, the strongest activity was exhibited by the polar sub-fraction of the methanol extract from the flowers with an IC_{50} value of 18.2 ± 0.42 µg/ml. The amount of total phenolics was highest in the polar sub-fractions. Particularly, a positive correlation was observed between the total phenolic content and the antioxidant activity of the extracts. As estimated from the results, amounts of phenolic compounds were less in hexane and dichloromethane extracts than in the others. In conclusion, antioxidant potentials of polar methanol sub-fractions could be attributed to their high phenolic contents. Antioxidant capacity of BHT was also determined in parallel experiment.

(Received September 2, 2011; Accepted May 12, 2012)

Keywords: Eucalyptus oleosa, methanol extract, DPPH, Total phenolics, Antioxidant activity

1. Introduction

The pathology of numerous chronic diseases, including cancer and heart disease, involves antioxidative damage to cellular components [1].Today, we well known that radicals cause molecular transformations and gene mutations in many type of organism. Oxidative stress is well-known to cause many diseases [2]. Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases [3]. The phenolic compounds extracted by solvents showed antioxidant activity [4]. A direct relationship has been found between the phenolic content and antioxidant capacity of plants [5]. The phenolic content and composition of plants and the products produced from them depend on genetic and environmental factors, as well as post-harvest processing conditions [6].

Eucalyptus contains many chemical compounds that play several roles in the plant [7-9]. The best known compounds are the terpenoids. However, *Eucalyptus* is also a rich source of phenolic constituents such as tannins and simpler phenolics [10, 11]. Some of these compounds have formed the basis of industries in the past [12]. The components of "eucalyptus leaf extract", such as hydrolyzable tannins have antioxidant activity [13]. Antioxidant activity of the tannins and acylated flavonol glycosides, all with galloyl groups, was much higher than that of a synthetic antioxidant [14].

Our literature surveys show that there are no reports on the antioxidant activity of the extracts and the amount of total phenolics of *E. oleosa*. The aims of this work are to evaluate the in vitro antioxidant properties of sub-fractions of methanol extracts obtained by using Soxhlet extraction and to determine the amount of total phenolics of the extracts. In vitro antioxidant

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activities were determined by using two complementary assays, namely inhibition of DPPH radical and β-carotene-linoleic acid systems.

2. Experimental

Plant material

The leaves and flowers of *Eucalyptus oleosa* were collected on November 2010 from Kashan in Isfahan. The voucher specimen has been deposited at the Herbarium of the voucher specimens of the plant were deposited in the herbarium of research institute of Forests and Rangelands, Kashan, Iran. The aerial parts of plant were separated from stems, dried in shade at room temperature, and ground in a grinder.

Preparation of extracts

The plant material (20 g of flowers and leaves) was successively extracted with 200 cc of methanol by using a soxhlet extraction for 8h. The methanol extracts were filtered and then the extracts were concentrated in a rotary evaporator (Buchi, Flawil, Switzerland) at 50 ºC to get crude extracts. Crude methanol leaves extract of the plant (6 g) was fractionated to polar and non-polar sub-fractions using distilled water and chloroform as fractionation solvents. The extract was dispersed in distilled water and extracted with chloroform (4×100 ml). Each fraction was concentrated using rotary evaporator and dried in vacuum oven. Polar and non-polar fractions were 4.5 g (34.7%, W/W) and 1.5 g (11.6%, W/W) respectively. All extracts obtained were dried at 50–60 ºC temperature in a drying oven and kept in the dark at +4 ºC prior to use.

Antioxidant activity

DPPH assay

The free radical scavenging activity of each extract (polar and non polar sub-fractions) from flowers and leaves of *E.oleosa* was examined by comparing to those of known antioxidant such as BHT by 1,1-diphenyl-2-picrylhydrazyl (DPPH) using a published DPPH radical scavenging activity assay method [15] with minor modifications [16].

Briefly, 2 ml of various concentrations of polar sub-fraction of methanolic extract in methanol were added to 2.0 ml of methanolic solution of DPPH (10 mg/ml).

After 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated in following was :

$$I\% = (Ablank\text{-}A_{sample} / A_{blank)} \times 100,$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition ($IC_{50)}$ was calculated from the graph plotting inhibition percentage against extract concentration. Test was carried out in triplicate and butylated hydroxytoluene (BHT), was used as a positive control.

β-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari and Shahidi [17] was used with slight modifications. A stock solution of *β*-carotene and linoleic acid was prepared with 0.5 mg of *β*-carotene in 1 ml chloroform, 25 µl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of oxygenated distilled water were then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 µl of each sample solution were added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one contained BHT as a positive control and the other contained the same volume of DMSO instead of the samples. The test tube with BHT maintained its yellow colour during the incubation period. The absorbencies were measured at 470 nm on an ultraviolet spectrometer (Cintra 6, GBC, Dandenong,

Australia). Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A\beta\text{-carotene after 2 h assay}/A\text{initial }\beta\text{-carotene}) \times 100$$

Where Aβ-carotene after 2 h assay is the absorbance of β-carotene after 2 h assay remaining in the samples and Ainitial β-carotene is the absorbance of β–carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

Assay for total phenolics

The concentrations of phenolic compounds in the polar sub-fraction extracts from leaves and flowers of *E.oleosa* var.obtusa were determined by literature methods involving Folin-Ciocalteu reagent and gallic acid as standard [18]. Extract solution (0.2ml) containing 10 mg of extract was taken in a volumetric flask; 46 ml of distilled water and 1 ml Folin-Ciocalteu reagent were added and the flask was thoroughly shaken. After 3 min, 3 ml of a solution of 2% Na_2CO_3 were added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0-11.0 mg 0.1 ml^{-1}) and a standard curve was obtained with the equation given below:

Absorbance = 0:0012 × Gallic acid (μg) + 0.0033

Total phenols of the extract, as gallic acid equivalents, was determined by using the absorbance of the extract measured at 760 nm as input to the standard curve and the equation. All tests were carried out in triplicate andphenolic contents as gallic acid equivalents were reported as means ± SD of triplicate determinations.

3. Results and discussion

Antioxidant activity

The free radical-scavenging activity was determined by the DPPH test. This test aims to measure the capacity of the extracts to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) formed in solution by donation of hydrogen atom or an electron [19].

In our study, we have investigated the free radical scavenging activities of both sub-fractions (polar and non polar) of the methanol extracts from the leaves and flowers of *E.oleosa*. Free radical scavenging activities of the extracts were measured in DPPH assay. The results from the radical-scavenger assays are presented in Table. Free radical scavenging activity of the extracts is concentration dependent and lower IC_{50} value reflects better protective action. The free radical scavenging activity of polar sub-fraction of the methanol extract from the flowers of *E.oleosa* var .obtusa was superior to other extracts. The concentration of the positive control (BHT) required to scavenge 50% of the free radical (IC_{50}) was 18.2 ± 0.42 μg/ml.

Table . Free radical scavenging activities of the methanolic extracts of E.oleos.

Sample	DPPH,IC_{50}(μg/ml)
Polar extract from leaves of *E.oleosa*	39.82 ± 1.55
Non polar extract from leaves of E.oleosa	217.77 ± 1.33
Polar extract from flowers of E.oleosa	18.2 ± 0.42
Non polar extract from flowers of E.oleosa	264.23 ± 2.63

A great number of simple phenolic compounds as well as flavonoids can act as antioxidants, however, their antioxidant power depends on some important structural prerequisites, particularly on the number and the arrangement of hydroxyl groups, the extent of structural conjugation and the presence of electron-donating and electron-accepting substituents on the ring structure [20-22].

Considerable antioxidant capacity of the polar and non-polar sub-fractions of the extract respectively in DPPH and β-Carotene/linoleic acid assays landmarks the plant as a good candidate for its application in food, hygiene and pharmaceutical products.

Amount of total phenolics

Total phenolic content of the plant extracts were determined using a colorimetric assay method based on Folin-Ciocalteu reagent reduction. Results, expressed as gallic acid equivalents were 175.03 ± 1.9 µg/mg and 25.10 ± 0.15 µg/mg for polar and non-polar sub-fractions, respectively.

High gallic acid equivalent of the polar sub-fraction, which is in good agreement with its antiradical DPPH antioxidant capacity, suggests a possible contribution of water soluble phenolic compounds of the plant in its electron transfer based antioxidant activity.

Acknowledgments

The authors gratefully acknowledge the financial support of this work by the Research Affairs Office of the Islamic Azad University, Qom Branch, Qom, I. R. Iran.

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