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BIOACTIVITY OF METHANOL EXTRACTS OF *EUCALYPTUS SARGENTII* MAIDEN CULTIVATED IN IRAN

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This study was designed to examine the antioxidant activities of methanol sub-fractions (polar and non-polar) of *Eucalyptus sargentii* Maiden cultivated in Iran. The Samples were subjected to screening for their possible antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assay methods. The polar sub-fraction of methanol extract showed good antioxidant activity (IC_{50} = 17.27 ± 0.84 μg/ml), comparable to that of synthetic antioxidant standard butylated hydroxyl toluene (BHT, IC_{50} = 19.8 ± 0.5 μg/ml) in DPPH assay. In β-carotene/linoleic acid assay, the best inhibition was belonged to non-polar sub-fraction (86.07%). The amount of total phenolic compounds for polar and non-polar sub-fractions was 189.03 ± 1.89 μg/mg and 24.11 ± 0.17 μg/mg, respectively. A positive correlation which was observed between the total phenolic content and antioxidant activity of the extracts in DPPH assay, suggesting the main attribution of phenolic compounds of the plant in its antioxidant potential.

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1. Introduction

The active oxygen and nitrogen species may induce some damage to the human body. Over production of various forms of activated oxygen species, such as oxygen radicals and non-free radical species are considered to be the main contributor to oxidative stress [1-3]. As a consequence of oxidative stress, many diseases, e.g. cancer, arteriosclerosis and other cardiovascular problems and diabetes and even ageing are promoted [4]. The antioxidant serves as a defensive factor against free radicals in the body. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as potential inhibitors of lipid peroxidation and thereby stabilizing fat-containing food-stuffs. But, according to toxicologists and nutritionists, the side effects of these synthetic antioxidants have already been documented. For example, these substances can show carcinogenic effects in living organisms [5, 6]. Recently, plant products such as essential oil and various extracts [7-9] have been of great interest for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of the food from toxic effects of the oxidants.

The genus *Eucalyptus* from Myrtaceae family consists of about 700 species of evergreen tree and shrubs, natives of Australia and Tasmania and naturalized in various tropical and subtropical countries [10-13]. Traditionally, Native Australians (Aborigines) have used *1*been approved as food additives [14], and are also currently used in cosmetic formulations. *Eucalyptus*

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extracts exhibited biological effects, such as antibacterial [15], antihyperglycemic [16] and antioxidant [17, 18] activities.

According to our literature survey, the antioxidant of methanolic sub-fractions from the leaves of *Eucalyptus sargentii* Maiden have not reported previously. Thus, this study is the first report on these aspects of the plant.

2. Experimental

Plant material

The aerial parts of *E. sargentii* were collected from cultivated sample in Kashan Botanical Garden (Kashan, Isfahan Province, Iran) at an altitude of ca. 1000 m in November 2009. The leaves were separated from the stem and dried in shade. A voucher specimen of the plant has been placed in the herbarium of Research Kashan Botanical Garden, Kashan, Iran.

Preparation of extracts

Ground, dried leaves of *E. sargentii* (20 g) were Soxhlet-extracted, with 300 ml of methanol for 8 h at a temperature not exceeding the boiling point of the solvent [19]. The extract was concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) at a maximum temperature of 45 ºC. The yield of dried extract was 9.27 g (46.3% W/W).

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.

Antioxidant activity

DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay usually involves hydrogen atom transfer reaction, but, based on kinetic data, an electron transfer mechanism has also been suggested for this assay [20, 21]. Radical scavenging activity (RSA) of *E. sargentii* extracts was determined using a published DPPH radical scavenging activity assay method [22] with minor modifications.

It is a widely used reaction based on the ability of antioxidant molecule to donate hydrogen to $DPPH^{\cdot}$ which consequently turns into an inactive form. Briefly, stock solutions (10 mg/ml each) of the extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions were made to obtain concentrations ranging from 1 to 5×10^{-10} mg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of a freshly prepared 80 µg/ml $DPPH^{\cdot}$ methanol solution and allowed to stand for 30 min in dark and room temperature for any reaction to take place. Ultraviolet (UV) absorbencies of these solutions were recorded using a spectrometer (Cintra 6, GBC, Dandenong, Australia) at 517 nm and using a blank containing the same concentration of extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I %) was calculated in following way:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC50) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC50 values were reported as means ± SD of triplicates.

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 [23] 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

Many natural molecules, especially those produced in the plant kingdom, have at least one benzene ring with a hydroxyl functional group in their skeleton. Such compounds are collectively known as phenolic compounds and, due to their hydrogen or single electron donating potentials, usually play important rules in the antioxidant activity of the plant extracts. Total phenolic constituents of the polar and nonpolar subfractions of methanol extract of *E. sargentii* were determined by literature methods involving Folin-Ciocalteu reagent and gallic acid standard [24]. Solutions of the extracts (0.1 ml each) containing 1000 µg of the extracts were taken individually in volumetric flasks, 46 ml of distilled water and 1 ml Folin-Ciocalteu reagent were added, and the flasks were thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixtures were allowed to stand for 2 h with intermittent shaking. Absorbencies were measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0–1000 mg/0.1 ml) and a standard curve obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid } (\mu g) + 0.0033$$

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

Antioxidant activity of the polar and non-polar sub-fractions of methanol extract of the plant was evaluated using two complementary (i.e. DPPH free radical scavenging and β-Carotene/linoleic acid) assay systems. The IC_{50} of the polar sub-fraction of methanol extract was 17.27 ± 0.84 µg/ml, comparable to that of synthetic standard antioxidant BHT (19.8 ± 0.5 µg/ml).

The non-polar sub-fraction (IC_{50} = 254.69 ± 2.12 µg/ml) did not show an appreciable antioxidant activity in this test. While non-polar methanol extract of *E. sargentii* established 86.07% inhibition in this test, polar sub-fraction were much weaker inhibitors with 61.53% inhibitions. Thus, for an extract or fraction to be considered as a good antioxidant, it is not necessary to obtain good results in both tests.

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 189.03 ± 1.89 µg/mg and 24.11 ± 0.17 µg/mg for polar and non-polar sub-fractions, respectively.

It was found that the total phenolic content of polar sub-fraction of *E. sargentii* was superior to ethanol extract of fruit and both fresh bark and volatile oil of *Eucalyptus camaldulensis* var. *brevirostris* [25]. 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.

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