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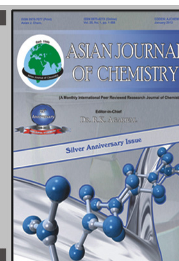

Antioxidant Potential Profile of Extracts from Different Parts of Stem of *Eucalyptus camaldulensis*

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Eucalyptus camaldulensis is a powerful antiseptic and is used for relieving coughs, cold, sore throats and other infections. It is conventionally used against deteriorating diseases so it is an important task to gauge their antioxidant activity. Antioxidant potential and radical scavenging activity of the five fractions were examined by using different antioxidant assays such as ferric reducing antioxidant power, ABTS {2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)}, total phenolic contents and DPPH (diphenyl-1-picrylhydrazyl radical) radical scavenging assay. Trolox equivalent antioxidant activity (TEAC) of the aqueous and organic fraction of these herbs was determined by calculating the per cent inhibition of the coloured radical solution after reaction with sample and standard antioxidants by comparing with the standard curve formed by Trolox as standard antioxidant. Total phenolic contents and ferric reducing antioxidant power decolourization assays of bark, sapwood heartwood of *Eucalyptus camaldulensis* showed a broad range of antioxidant activity. The results of such anti disease activities of the herb can be attributed to antioxidant compounds, which may be present in the plant under study.

Key Words: 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulpohonic acid), *Eucalyptus camaldulensis*, Trolox equivalent antioxidant capacity, Radical cation, Ferric reducing antioxidant power.

INTRODUCTION

Eucalyptus is an evergreen tall tree; native to Australia, widely cultivated in many countries including India[1]. Eucalyptus is a large genus of the myrtaceae family that includes around 900 species and subspecies[2]. Eucalyptus is used as antiinflammatory, analgesic and antipyretic therapies for the symptoms of lung infections such as cold, flu and sinus blocking[3]. Eucalyptus extracts show various biological effects, such as antibacterial, antifungal, anti hyperglycemic and antioxidant activities[4] with essential oils playing a main role in these biological functions. The essential oils produced by *E. citriodora* are used for medicinal and pharmaceutical purposes[5,6]. Studies have confirmed the antimicrobial properties of Eucalyptus essential oils against a broad range of microorganisms. *E. citriodora* oil has shown to have a wide range of antifungal activity. In addition, *Eucalyptus camaldulensis* and *Eucalyptus urophylla* are also well branded for their antibacterial[7] and antifungal[8] activities. In current years, naturally occurring antioxidants have come to be ideal for both consumers and food makers, mainly because of increasing uncertainties about the safety of man-made antioxidants such as butylated hydroxyl toluene and butylated hydroxyl anisole, which are doubted to act as promoters of carcinogenesis[9]. Antioxidants function by decreasing the rate of initiation in the free radical chain reactions and are efficient at very low concentrations of 0.01 % or less. Several extracts of herbs and spices have been evaluated as potent antioxidants in lipid systems[10-15]. However no comprehensive study of the parts of stem of *Eucalyptus camaldulensis* has been undertaken as yet. The aim of the present study is to estimate radical scavenging and antioxidant potential of different organic extracts of bark, sapwood and heartwood.

EXPERIMENTAL

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2,4,6-tripyridal-*s*-triazine (TPTZ), 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6- sulfonic acid (ABTS) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (USA). All other chemicals and reagents of analytical grade were purchased from Merck (Germany).

Plant was collected from BRB canal, Lahore. Height of plant was 12.2 m and diameter of stem was 14.4 cm and the age of plant was about 8 to 9 year. Voucher specimen for plant was deposited in the Sultan Herbarium at Department of Botany, Government College University, Lahore.

The plant material *i.e.* bark (250 g), sapwood (250 g) and heartwood (250 g) was shade dried, powdered, and extracted

by percolation method for 7 days at room temperature with 100 % methanol. After complete extraction, methanol was evaporated under reduced pressure to obtain a crude residue. The aqueous solution was partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (4 × 25 mL) for each extraction.

$ABTS^+$ radical cation decolourization assay: (In buffer media) ABTS radical scavenging assay depends upon the scavenging activity of ABTS radical cation produced from a reaction between ABTS and 3.68 mL of (10 mM) potassium persulfate and making the total volume to 13.88 mL by adding 0.820 mL of deionized water in it. The antioxidant components reduce bluish green ABTS radical to colourless native ABTS depending upon their nature and amount on a time-dependant scale. Trolox is generally used as a standard antioxidant for comparison purposes. For the determination of trolox equivalent antioxidant capacity of the extracts, $ABTS^+$ decolourization assay was followed[16]. ABTS radical cation was produced by a reaction between ABTS and potassium persulfate (7 mM and 2.45 mM final concentrations, respectively) and permitting the mixture to stand in the shaded room temperature for 14-16 h before use and strong bluish green coloured stable radical cation ($ABTS^+$) was produced. To study the antioxidant activity of standard antioxidant and native medicinal herb, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.71 ± 0.01 at 734 nm and equilibrated at 30 ºC.

Then 2.90 mL of diluted $ABTS^+$ solution (A 734 nm = 0.71 + 0.010) was shifted into the cuvette and noted down the absorbance as A_o. Then added 10 µL of sample solution, the absorbance reading was taken as 25 ºC exactly in 1 min after initial mixing and upto 6 min. Appropriate blank were run in each case. All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards. The percentage inhibition of absorbance was calculated by the following formula.

$$\text{Inhibition (at 734 nm) (\%)} = (1 - I_f/I_o) \times 100$$

where I_o is the absorbance of radical cation solution before addition of sample/ standard antioxidants and if is the absorbance after addition of the sample/standard antioxidants. Total equivalent antioxidant capacity values of ABTS are more prominent in Buffer medium.

Total phenolic contents assay: Total phenolic contents of the extracts were determined by an reported method[17]. Stock solution of gallic acid was made by dissolving 0.495 g gallic acid in 12 mL of C_2H_5OH in a 100 mL conical flask and diluted it to volume with double distilled water. Anhydrous sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 850 mL of distilled water. After boiling and successive cooling of the solution, a small number of Na_2CO_3 crystals were added. The solution was stand for 26 h, filtered and volume was raised to 1000 mL with distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of stock solution of phenol were added into 100 mL conical flask separately and then diluted to volume with distilled water. The final solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid. From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of distilled water was added. Folin-Ciocalteu's reagent (200 µL) was added and mixed well. After 10 min, 600 µL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 20 ºC for 2.5 h and absorbance of each solution was noted at 765 nm against the blank. A concentration *versus* absorbance linear plot was thus obtained. Alternately, they can be left at 40 ºC for 40 min before reading the absorbance and noted the absorbance at 765 nm. Create a calibration curve with standard and determine the level in sample.

Ferric ion reducing antioxidant power assay (FRAP): The ferric ion reducing capacity of plant extract was measured according to the reported method[18]. Freshly prepared FRAP solution contained 24 mL of 300 mM acetate buffer (pH 3.6), 2.7 mL of 10 mM 2,4,6-tripyridyl-s-triazine solution in 40 mM HCl solution and 2.4 mL of 20 mM ferric chloride ($FeCl_3$) solution. The mixture was incubated at 37 ºC throughout the reaction period. 2.99 mL of FRAP reagent was mixed with 100 µL of sample and 250 µL of distilled water. Absorbance readings were taken at 593 nm after every minute for 8 min. Results were compared with standard curve of ferrous sulphate.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging potential was found by using a previously reported method[19]. 2,2-Diphenyl-1-picrylhydrazyl is one of a little stable and commercially available organic nitrogen radicals and has a UV-VIS absorption maximum at 515 nm. Upon reaction, solution colour fades and the reaction progress is monitored by spectrophotometer.

Briefly, DPPH solution (3.5 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions (0.10 mL). The reaction progress of the mixture was monitored at 517 nm over a time period of 0.5 h until the absorbance becomes stable. Upon appropriate reduction, the purple colour of the solution changed to yellow diphenylpicrylhydrazine. The percentage of the DPPH remaining ($DPPH_{rem}$ %) was calculated as:

$$(\%)\ DPPH_{rem} = 100 \times [DPPH]_{t=t}/[DPPH]_{t=0}$$

where, $[DPPH]_{t=0}$ is the concentrate of ion of DPPH radical before reaction with antioxidant samples and while $DPPH_{rem}$ is proportional to the antioxidant concentration and $[DPPH]_{t=t}$ is the concentration of DPPH radical after reaction with antioxidant sample at time t. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min) was plotted for each fraction of the samples. EC_{50} value, which is the concentration of a substance that reduces the amount of DPPH radical to half of the original concentration under experimental conditions, was also determined for each fraction.

RESULTS AND DISCUSSION

ABTS assay: The ABTS decolourization assay was applied to estimate *in vitro* radical scavenging potential of several fractions of bark, sapwood and heartwood of *Eucalyptus camaldulensis*. The reduction potential of the ABTS radical cation is equivalent to that of hydroxyl radical formed during metabolic reactions *in vivo*. Trolox equivalent antioxidant

capacity values were obtained by comparing the % inhibition values of fractions samples with the standard trolox curve. trolox equivalent antioxidant capacity values ranged from 0.996-4.365 µM of trolox equivalents.

Bark: In different fractions, *n*-butanol, methanol and ethyl acetate fractions of the bark showed higher TEAC values than those of *n*-hexane and chloroform fractions. A general trend of increase in the TEAC value with increasing polarity of extractive solvent may be seen in Fig. 1. The fractions with less polar extractive solvent showed small TEAC values showing low solubility of phenolic and other antioxidant components in non-polar medium. Order of radical scavenging activity of different fractions in buffer medium as *n*-butanol > methanol > ethylacetate > chloroform > *n*-hexane.

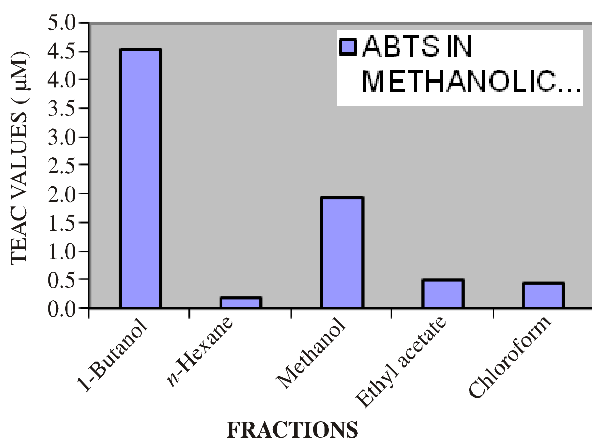

Fig. 1. ABTS assay of extracts of bark of *Eucalyptus camaldulensis*

Heartwood: Amongst different fractions ethyl acetate, *n*-butanol and methanol fractions showed higher TEAC values than those *n*-hexane and chloroform. The overall trend of increase in the TEAC value with increasing polarity of extractive solvent may be seen in Fig. 2. The fractions with less polar extractive solvent indicated small TEAC values showing low solubility of phenolic and other antioxidant components in non-polar medium. Sequence of radical scavenging activity of different fractions in buffer medium as *n*-butanol > methanol > ethyl acetate > chloroform > *n*-hexane.

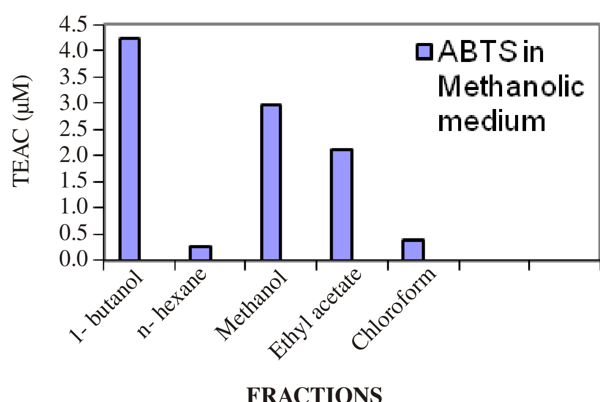

Fig. 2. ABTS assay of extracts of heartwood of *Eucalyptus camaldulensis*

Sapwood: Amongst different fractions *n*-butanol, ethyl acetate and chloroform showed higher TEAC values than those n-hexane and methanol. With few exceptions a general trend of increase in the TEAC value with increasing polarity of extractive solvent may be seen in Fig. 3. The fractions with less polar extractive solvent showed small TEAC values indicating low solubility of phenolic and other antioxidant components in non-polar medium. Order of radical scavenging activity of different fractions in buffer medium as *n*-butanol > ethyl acetate > chloroform > methanol > *n*-hexane.

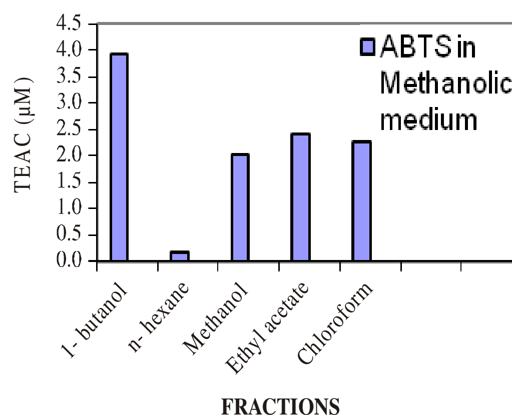

Fig. 3. ABTS assay of extracts of sapwood of *Eucalyptus camaldulensis*

Total phenolic contents: For the determination of phenolic components in plants extracts and other fractions Follin-Ciocalteu's reagent is usually used in the laboratories. Phenolic compounds react with Follin-Ciocalteu's reagent only under basic conditions at pH 9.90. Hydroxyl moieties of phenolic compounds have the ability to reduce yellow coloured Follin-Ciocalten's reagent to blue colour. Total phenolic content values ranged from 0.292 to 5.415 µM of GAE/100 g of dry weight. All the fractions of bark of *Eucalyptus camaldulensis* showed high values, fractions of sapwood showed above moderate values while all the fractions of heartwood showed sharp values of total phenolic contents as shown in Figs. 4-6.

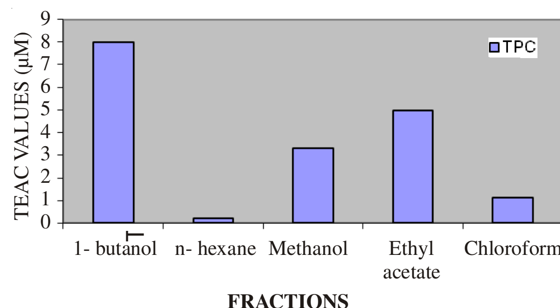

Fig. 4. Total antioxidant activity of bark extract of *Eucalyptus camaldulensis*

Antioxidant activities: The FRAP assay is used to evaluate antioxidant power by knowing the capability of the sample to reduce ferric to ferrous ion at low pH. The FRAP assay as established by Benzie and Strain[18] which involves a single electron reduction of the Fe(TPTZ)2(III) complex (pale yellow) to the Fe(TPTZ)2(II) complex (blue) by sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe(III)TPTZ salt (0.7 V) can reduce Fe^{3+}-

TPTZ to Fe^{2+}-TPTZ participating to Ferric reducing antioxidant power value[20]. This reduction is progressed spectrophotometrically at 593 nm. Appearance of intense blue colouration showed the presence of reducing components in the sample. The actual method of Benzie and Strain[18] used a 4 min interval but we saw that the colour of the reaction changed even after 4 min interval. Absorbance readings, therefore, were taken at a 6 min interval after adding sample to TPTZ reagent allowing the reaction to reach a steady state.

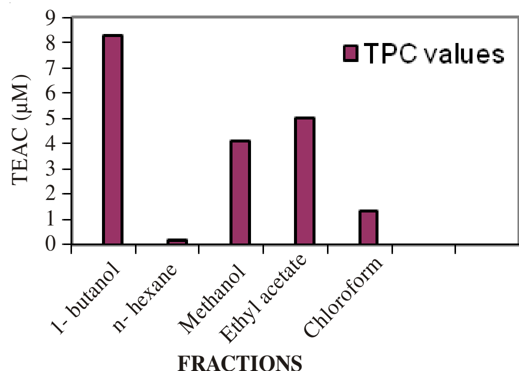

Fig. 5. Total antioxidant activity of sapwood extract of *Eucalyptus camaldulensis*

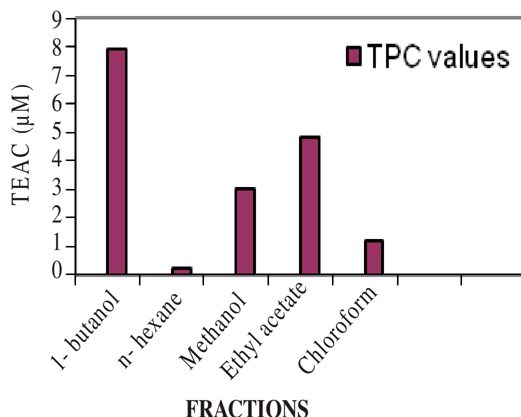

Fig. 6. Total antioxidant activity of heartwood extract of *Eucalyptus camaldulensis*

Ferric reducing antioxidant power values for different fraction of bark ranged from 1.83 to 7.11 µM. Higher TAC values were observed for *n*-butanol, methanol and ethyl acetate, these values were ranged from 0.913 to 5.89 µM for sapwood and 2.60 to 7.48 µM for heartwood as shown in Figs. 7-9.

DPPH is a preformed stable radical applied to measure radical scavenging activity of antioxidant samples and has a UV. Upon reaction the solution colour diminishes. The progress of the reaction is checked by a spectrophotometer. The assay was performed by taking parameters, the absorbance of antioxidant and the time for completion of the reaction of antioxidants, into consideration.

Kinetic curves obtained by plotting absorbance *versus* time showed that all the fractions of bark, sapwood and heartwood of Eucalyptus camaldulensis comprized high levels of DPPH radical scavenging agents. For bark extract, the curves obtained from *n*-butanol is much steeper in the first 05 min, showing fast reaction of antioxidant components with DPPH radical and curve obtained from ethyl acetate just steeper in the first 10 min, showing moderate reaction of antioxidant component with DPPH radical and all other fractions show the curve almost parallel at its origin, indicates the completion of the antioxidants (Figs. 10-12).

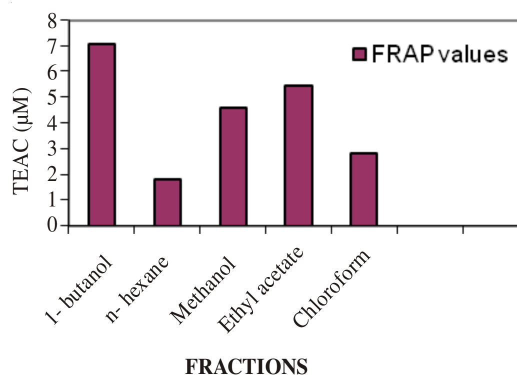

Fig. 7. FRAP assay of bark extracts of *Eucalyptus camaldulensis*

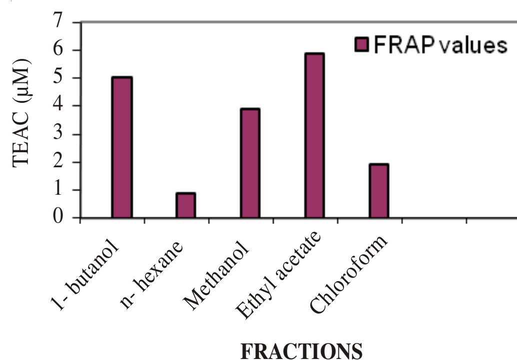

Fig. 8. FRAP assay of sapwood extracts of *Eucalyptus camaldulensis*

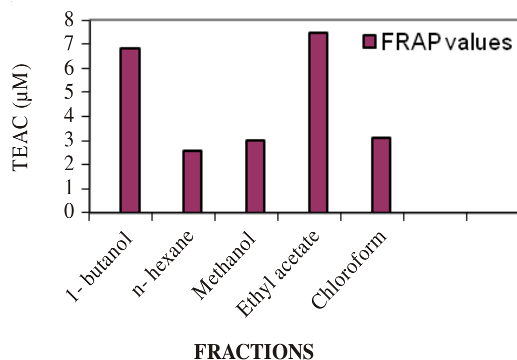

Fig. 9. FRAP assay of heartwood extracts of *Eucalyptus camaldulensis*

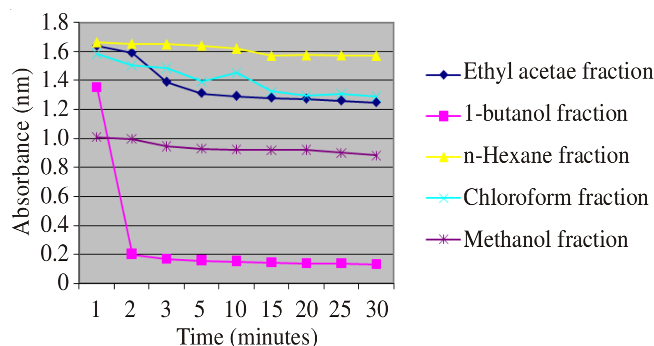

Fig. 10. DPPH scavenging activity of bark extracts of *Eucalyptus camaldulensis*

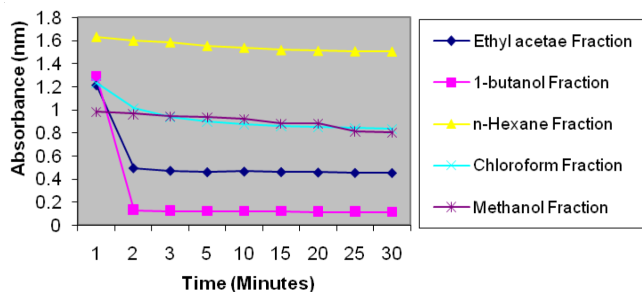

Fig. 11. DPPH scavenging activity of sapwood extracts of *Eucalyptus camaldulensis*

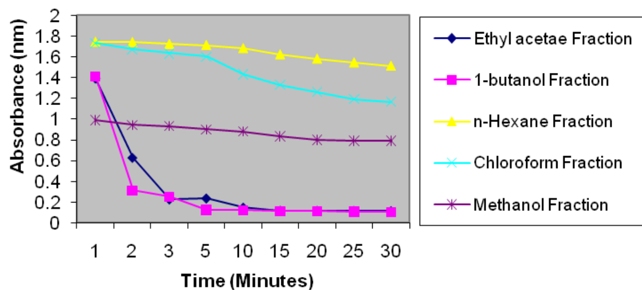

Fig. 12. DPPH scavenging activity of heartwood extracts of *Eucalyptus camaldulensis*

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