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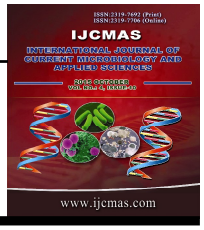

Original Research Article

In vitro Antioxidative Potential of Extracts from *Callistemon lanceolatus* Sweet. and *Eucalyptus lanceolata* Labill

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Keywords

Antioxidant activity, DPPH, plasmid nicking assay, Medicinal plants, *Callistemon lanceolatus* Sweet, *Eucalyptus lanceolata* Labill

A B S T R A C T

Present study focuses on the neutralization of free radical chain reaction using medicinal plants. Methanolic plant extracts (80 %) from *Callistemon lanceolatus* and *Eucalyptus lanceolata* were tested for their phenolic and flavonoid content and for antioxidative potential by DPPH and plasmid nicking method. Both the plants exhibit good phenolic and flavonoid content. *C. lanceolata* extracts showed good percent inhibition (64.60±0.40 at 200 µg/ml) with IC_{50} value 155 µg/ml whereas *E. lanceolata* showed (50.13±0.03 percent inhibition) IC_{50} value of 196 µg/ml. In plasmid nicking assay, *C. lanceolata* protected the native supercoiled form of DNA from hydroxyl radicals while *E. lanceolata* showed little effect. The results of the present *in vitro* study propose that both test plants possess promising antioxidant potential but *C. lanceolatus* exhibited better DPPH and pBR322 plasmid DNA protecting ability compared to *E. lanceolata* extract.

Introduction

In the last twenty years, the study of cellular injury caused by free radicals has become a significant thrust of cancer research. ROS are generated as byproducts of biological reactions or from numerous exogenous sources (Harman, 1994; Ames, 1998). Although, aerobes have evolved an antioxidant defence system to protect themselves against oxygen but even then 1-3% of the oxygen we consume is used to create reactive oxygen species (ROS) by mitochondria. Since, humans consume large quantities of oxygen, a simple calculation shows that over 2 kg of superoxide anion radicals ($O_2^{\cdot-}$) is made in the human body every year and people with chronic inflammation may produce even more (Fridovich, 1986). Additional radicals could be generated by exogenous sources, both natural e.g. radon gas, cosmic radiations,

and manmade sources. Low wavelength electromagnetic radiations (gamma rays) can split water in the body to generate ˙OH radicals (Von Sonntag, 1987). The ROS include three major radical species: superoxide anion ($O_2^{\cdot-}$), hydrogen Peroxide (H_2O_2) and extremely reactive hydroxyl radicals ($HO^{\cdot}$). In normal state, a balance is maintained between ROS and antioxidants, whenever there is a shift in this balance, the depletion of antioxidants occurs and causes damage in the body including atherosclerosis, arthritis, gastritis and injury to central nervous system (Dorge, 2002; Valko *et al.*, 2004, 2007; Bahorun *et al.*, 2006; Halliwell and Gutteridge, 2007; Nithiyantham *et al.*, 2012). At certain physiological level, ROS are crucial for proper regulation of cell functions like intracellular signalling, transcription activation, cell proliferation, inflammation and apoptosis (Alfadda and Sallam, 2012; Gomes *et al.*, 2012).

The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their harmful effects and to contribute to disease prevention. A direct relationship between oxidant activity and phenolic content of plant extracts has also been reported (Gollucke *et al.*, 2008; Conforti *et al.*, 2009; Du *et al.*, 2009; Shrififar *et al.*, 2009; Kalaivani and Mathew, 2010; Jaberian *et al.*, 2013; Li *et al.*, 2013) as the natural products are source of many modern medicines (Inayatullah *et al.*, 2012).

Myrtaceae family comprises of 140 genera distributed in 3800 species and the plants are mostly found in tropical and subtropical regions of the world (Ali *et al.*, 2011). Many studies have reported antibacterial and antifungal properties from *Eucalyptus* (Bachir and Benali, 2008; Vázquez *et al.*, 2008; Sen-Sung *et al.*, 2009) and *Callistemon* (Ali *et al.*, 2011; Shinde *et al.*, 2012). Keeping this background in view, the present study was designed to study the total phenolic (TPC) and flavonoid content (TFC) and antioxidant potential from methanolic extracts of two plants from *C. lanceolatus* and *E. lanceolata* using DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical scavenging method and inhibition of strand breakage of deoxyribonucleic acid in the plasmid nicking assay.

Materials and Methods

Chemicals

Supercoiled plasmid pBR322 and agarose was obtained from Genei, Bangalore. DPPH and ethidium bromide were obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Sodium carbonate (Na_2CO_3), bromophenol blue, Folin–Ciocalteu reagent, ferric chloride ($FeCl_3$), L-ascorbic acid, tris (hydroxymethyl) aminomethane, sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), ethylene diamine tetraacetic acid (EDTA), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), gallic acid, rutin and quercetin were of analytical grade.

Collection of plant material and preparation of methanolic extract

Leaves of test plants were collected from trees growing in botanical garden of Guru Nanak Dev University, Amritsar. Fresh leaves were washed with tap water twice and then air dried at room temperature. Dried leaves were finally powdered and three successive extractions with 80% methanol were carried out at room temperature for 24 hrs. The extracts were filtered using Whatman No. 1 sheet. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40˚C and the concentrated solution was then lyophilized to get the dry powder.

Phytochemical analysis

Determination of total phenolic content

Phenolic content of the extracts was determined according to method given by Yu *et al.* (2002). In this procedure 100 µl of extract (100 µg/ml) and 900 µl of double distilled water was added to make final volume 1000 µl. To this solution, 1.5 ml of 20% sodium carbonate solution and 0.5 ml of 1:1 Folin-Ciocalteu reagent was added. The volume was raised to 5 ml and the mixture was incubated for 2 hrs at room temperature.

Absorbance of the mixture was measured at 765 nm using UV-VIS spectrophotometer. Quantification was carried out on the basis of standard curve of gallic acid and described by equation y = 0.000x + 0.039 (R^2 = 0.987) where y = absorbance and x = concentration.

Determination of total flavonoid contents

Flavonoid content in methanolic extracts was assessed by the ammonium chloride procedure given by Kim *et al.* (2003). In this method, 4ml of ddH_2O was added to 1 ml extract (100 µg/ml concentration) and then 300 µl of $NaNO_3$ and 300 µl of $AlCl_3$ were added. The mixture was then incubated at room temperature for 5 minutes. Following which, 2 ml of sodium hydroxide (1M) was added and the final volume was raised to 10 ml using distilled water. The absorbance of sample and blank were taken at 510 nm by UV-VIS spectrophotometer. The total flavonoid content was then expressed as rutin equivalents (RE) in mg/g of dry sample. For rutin, the curve absorbance *versus* concentration is described by the equation, y = 0.0011x + 0.0409 (R^2 = 0.9892) where y = concentration; x = absorbance.

Antioxidant assay

The scavenging activity of plant extracts was measured spectrophotometrically by using method given by Blois (1958) with slight modifications. In this method, 200µl of extract solution (concentrations ranging from 20 to 200µg/ml) was used and to this solution freshly prepared 3ml of 0.1 mM DPPH was added. The absorbance of the reaction mixture was taken at 517 nm. The decrease in absorption was correlated with the scavenging action of the test compound. Gallic acid being a phenolic compound was used as a positive control. The radical scavenging activities were expressed as percent inhibition and calculated according to the equation;

$$\% \text{ DPPH Inhibition} = [(1-A_s/A_c) \times 100]$$

Where Ac = absorbance of control and As= absorbance of sample.

A percent inhibition *versus* concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

Plasmid nicking assay

Plasmid protecting efficiency of the extracts was analyzed using method given by Lee *et al.* (2002) with slight modifications. The reaction was initiated by mixing 0.5 µg of plasmid DNA (pBR322) in a micro centrifuge tube with 10 µl of Fenton's reagent (30 mM H_2O_2 + 50 µM ascorbic acid and 80 µM $FeCl_3$). To this mixture, plant extract (200 µg/ml) was added and final volume of the mixture was brought up to 20 µl by using ddH_2O. The mixture was then incubated for 30 minutes at 37˚C followed by the addition of 2–5 µl of loading buffer (0.25% bromophenol blue, 50% glycerol). Ellagic acid (100 µg/ml); a positive

scavenger of hydroxyl radical, was used as a control. DNA was analysed using Gel Doc XR system (Bio-Rad, USA) after agarose gel electrophoresis, using 1% agarose gel, in TBE buffer, at 50 V (1.5–2 V/cm) for 4 hrs.

Statistical analysis

Experiment was performed in triplicates and the results were expressed as mean ± SE. One way analysis of variance (ANOVA) and Tukey's HSD post hoc test were carried out to determine significant differences between the mean at $p \leq 0.05$.

Results and Discussion

The antioxidative effect of phenolic and flavonoid compounds may depend on the inhibition or termination of free radical chain reaction (Dawidowicz *et al.,* 2006; Abdullah *et al.,* 2012; Agbafor and Nwachukwu, 2012; Shahwar and Raza, 2012) and findings from many studies have suggested that phenolics and flavonoids are responsible for antioxidant activities in plants (Alothman *et al.,* 2009; Isabelle *et al.,* 2010, Hossain *et al.,* 2011). Total phenolic and Flavonoid content of plants is expressed as gallic acid (GAE) and rutin equivalents (RE) per gram dry weight of extracts respectively. *E. lanceolata* showed highest phenolic content of 409.66 ± 2.02 mg GAE whereas *C. lanceolatus* shows 261 ± 0.88 mg GAE per gram dry weight of extract (Table 1). On the other hand, in case of flavonoids *C. lanceolatus* (282.06 mg) showed higher content than in *E. lanceolata* (126.23 mg) RE/g dry wt. The results infer that no correlation is there between the flavonoid and phenolic content of the given plants.

In vitro radical scavenging ability was measured by DPPH assay - a popular test to assess antioxidative activity (hydrogen donating) in plant systems. Table 2 represents concentration dependent percent inhibition in two plants. *C. lanceolatus* exhibited higher DPPH radical scavenging activity of 64.60 ± 0.40% with IC_{50} value of 155 µg/ml whereas *E. lanceolata* showed 50.13 ± 0.03% inhibition with IC_{50} value of 196 µg/ml. Gallic acid used as a standard in DPPH test showed IC_{50} value of 24.01 µg/ml (Figure 1). High percentage inhibition in *C. lanceolatus* may be because of high flavonoid content (Abdelhady *et al.,* 2011; Salem *et al.,* 2013; Kumar *et al.,* 2011). However, the antioxidant activity in plants does not depend only on these compounds but also depends on some individual active phenolic compounds (Djeridane *et al.,* 2006; Surveswaran *et al.,* 2007).

Table.1 Total phenolic and flavonoid content in the leaves of two plants used in the study

Plant	Total Phenolic content (mg GAE/g dry wt)	Total Flavonoid content (mg RE/g dry wt)
Callistemon lanceolatus	261.00±0.88	282.06±0.84
Eucalyptus lanceolata	409.66±2.02	126.23±1.53

*The results are expressed as mean± SE (n=3) and significance of results was tested at $p \leq 0.05$

Table.2 Concentration dependent free radical scavenging ability of methanolic leaf extracts of *C. lanceolatus* and *E. lanceolata* using 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH)

Concentration (µg/ml)	*C. lanceolatus* (Inhibition %)	*E. lanceolata* (Inhibition %)	Gallic acid (Inhibition %)
20	10.08± 0.27	6.50± 0.27	38.09± 0.38
40	11.62± 0.13	10.60± 0.46	62.99± 0.49
60	18.72± 0.45	16.86± 0.30	86.49± 0.27
80	25.28± 0.26	20.70± 0.40	87.68± 0.19
100	32.75± 0.44	24.34± 0.20	89.59± 0.11
120	38.4± 0.50	31.64± 0.66	90.16± 0.03
140	43.93± 0.32	37.13± 0.17	90.06± 0.11
160	51.40± 0.43	41.74± 0.43	90.03± 0.05
180	59.11± 0.16	45.44± 0.29	90.03± 0.09
200	64.60± 0.40	50.13± 0.03	90.03± 0.05
IC_{50} (µg/ml)	155	196	24.01

*The results are expressed as Mean Percent Inhibition ± SE (n=3) and significance of results was tested at $p \leq 0.05$

Figure.1 Antioxidant activity in terms of percent inhibition of *C. lanceolatus* and *E. lanceolata* methanolic extracts in comparison to natural antioxidant (gallic acid)

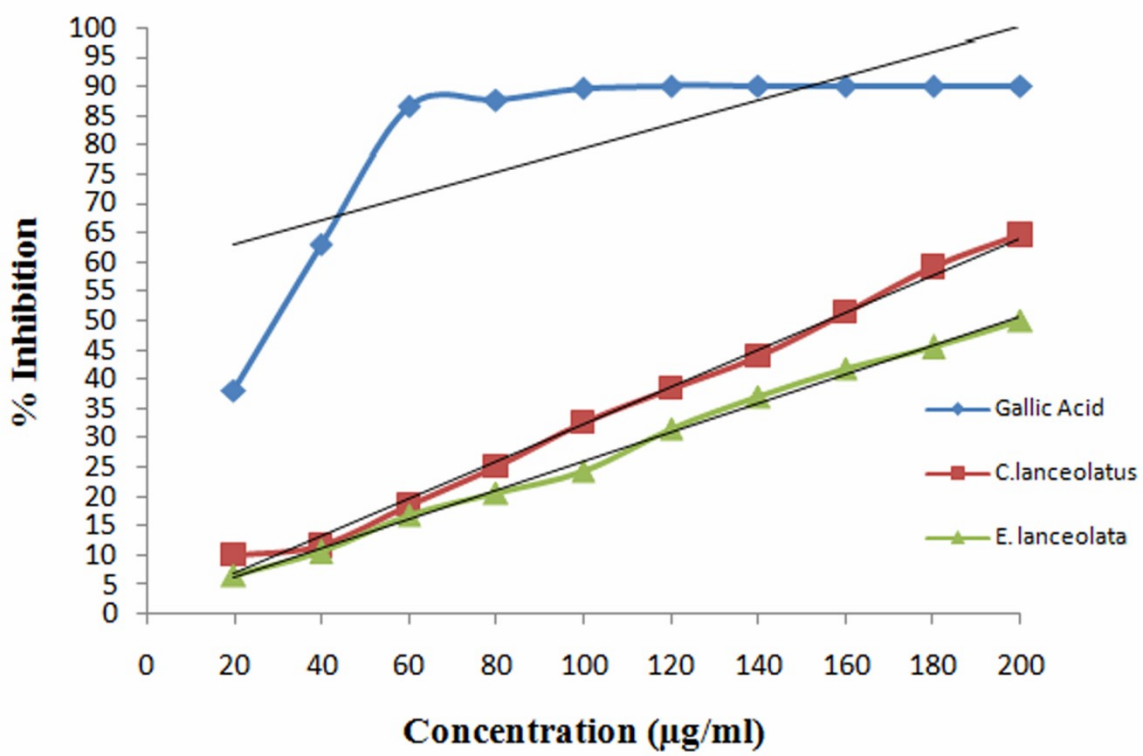
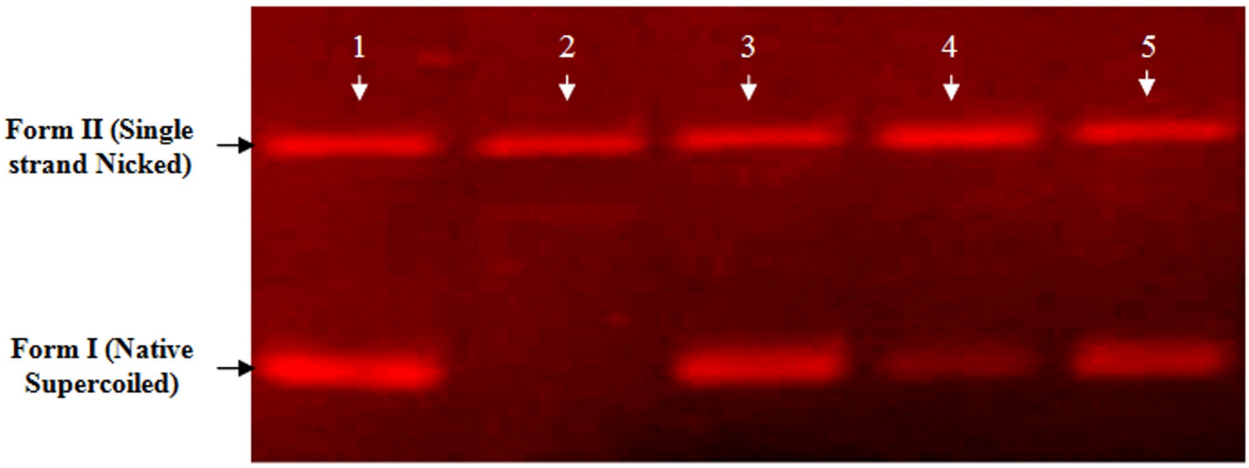


Figure.2 The inhibitory effects of two plant extracts on DNA nicking caused by hydroxyl radical. Here, **Lane 1:** pBR322 DNA + H_2O; **Lane 2:** pBR322 DNA + Fenton reagent + H_2O; **Lane 3:** pBR322 DNA + Fenton reagent + Quercetin; **Lane 4:** pBR322 DNA + Fenton reagent + *Eucalyptus lanceolata* extract; **Lane 5:** pBR322 DNA + Fenton reagent + *Callistemon lanceolatus* extract



In addition to the above, the protective effect of the methanolic extracts (200µg/ml) of *C. lanceolatus* and *E. lanceolata* was tested using pBR322 plasmid nicking assay. Hydroxyl radicals produced by Fenton's reagent has the capacity to cause strand break in DNA (Walia *et al.,* 2012; Guleria *et al.,* 2011). Phenolics and flavonoids from the plant extracts exhibit hydroxyl radical scavenging avtivity against oxidation byproducts (Halliwell and Gutteridge, 1990; Zafar *et al.,* 2011; Albishi *et al.,* 2013). The addition of plant extracts to the reaction mixture considerably reduces the DNA strand breakage and preserves the native supercoiled form, thus effectively protects DNA. Extracts from *C. lanceolatus* showed significant reduction in the formation of nicked DNA and helped in retaining the supercoiled form of DNA. On the other hand, ˙OH radical scavenging ability of *E. lanceolata* extract at highest test concentration showed weak effect, therefore the supercoiled DNA form was not found to be protected and was converted to single stranded DNA form (Figure 2).

In conclusion, despite the major research in this area, extensive knowledge has not been gained with regard to the antioxidants derived from plants. The results of the present *in vitro* study suggest that both test plants possess promising antioxidant potential with high percent inhibition. *C. lanceolatus* exhibited better DPPH and pBR322 plasmid DNA protecting ability whereas the activities were found to be comparatively less in the *E. lanceolata* extract.

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