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RESEARCH ARTICLE

INVITRO STUDY ON ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF ETHYL ACETATE EXTRACT FROM THE *CASSIA ANGUSTIFOLIA*

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ABSTRACT

The objective of this research was to probe the *in vitro* antioxidant and antibacterial activities of ethyl acetate extract from the leaves of *Cassia angustifolia*. The total phenolic and flavonoid contents were estimated by Folin–Ciocalteu and Aluminium chloride method respectively. Antioxidant activities of *C. angustifolia* plants were tested on the basis of ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), radical scavenging assay, inhibition of lipid peroxidation, Super oxide radical scavenging activity, Nitric oxide radical scavenging activity and Metal chelating activity. Similarly, antibacterial activities were performed by disc diffusion method and MIC (Minimum Inhibitory Concentration) against *Xanthomonasoryzae pv. oryzae, Pseudomonas fuscovaginaeand Erwiniachrysanthemi.* The total phenolic and flavonoid content in ethyl acetate extract of *C. angustifolia*was 78.9±1.36 mg GAE/g and 53±2.45µg RE/g respectively. The ethyl acetate extract of *C. angustifolia* showed significant antioxidant activity. Similarly, the study on antibacterial activity of ethyl acetate extract of *C. angustifolia* revealed inhibitory activity. However ethyl acetate extract of *C. angustifolia* showed higher inhibitory zone against *Xanthomonas oryzae pv. oryzae, Pseudomonas fuscovaginae* and *Erwiniachrysanthemi* (16, 15.6 and 14.4 mm). This research work has made it clear that ethyl acetate extract of *Cassia angustifolia* possess excellent antioxidant and antibacterial activity and the extracts can be more widely used in developing countries for the prevention and treatment of ageing and infective related diseases and may be considered as good source for drug discovery.

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INTRODUCTION

Free radicals are extremely responsive molecules with an unpaired electron, twisted when the cells are exposed to stress. Further, starting chain reactions resulting in breakdown of cell membranes and cell compounds, together with lipids, proteins, and nucleic acids (Elzaawely, 2005). In pathogenesis, free radical oxidative stress had drawn a wide variety of disorders like cancer, autoimmune disease, diabetes, cardiovascular disease, multiple sclerosis and arthritis (Halliwell and Gutteridge, 1999). Generally, antioxidants slow down or prevent the oxidation of oxidizable substrate existing in foods or body at low concentrations. There are two classes of antioxidants, namely enzymatic antioxidants and non-enzymatic antioxidants. Examples of enzymatic antioxidants are superoxide dismutase, ascorbic peroxidase, polyphenoloxidase and catalase whereas non-enzymatic antioxidants examples are a-tocopherol (vitamin E), glutathione, ascorbic acid (vitamin C), carotenoids, and flavonoids (Boskou *et al.,* 2006). Thus, the natural plant antioxidants can be used as a preventive medicine. Many researchers have specified that two-thirds of the plant species in the world are recognized to possess medicinal properties and also, numerous medicinal plants with high antioxidant potential. The antioxidant resistance complements have the possibility to reduce oxidative tissue damage and prevent disease progression in human that are caused due to the imbalance between oxidative stress and antioxidative defense (Djeridane *et al.*, 2006). Moreover, increase in oxidative stress can result due to imbalance of pro-oxidants and can be controlled by a wide range of dietary augmented antioxidant enzymes, proteins and antioxidants. *Cassia angustifolia* Vahl. is a old-fashioned therapeutic plant belonging to the family Caesalpiniaceae. It is commonly known as Cassia senna. It is a rapid-growing shrub 3-5 m tall, extensively cultivated for its fruit and leaves in hot arid areas of India. The leaves and pods of *C. angustifolia* are used in the form of a decoction powder for intestinal worms as an anti-helmenthic.

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It is also widely used as an anti-pyretic in typhoid, splenic enlargements, cholera, laxative, anemia, toxicity and genotoxicity caused by *Escherichia coli*. This research study was conducted to investigate the antioxidant and antibacterial, possibilities of ethyl acetate extract of *C. angustifolia*.

MATERIALS AND METHODS

Plant Materials: Leaves of *Cassia angustifolia* were collected from Government Siddha Medical College, Herbal Garden, Chennai, Tami Nadu, India. Plants were authenticated by Dr. S. Sankaranarayanan, Head, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamilnadu.

Extraction: The leaves of *Cassia angustifolia* were dried in hot air oven at (40 °C) for 1 hour, after which it was ground to uniform powder with house hold mixer grinder. The 80% aqueous methanol extracts were prepared by soaking 100 g of the dry powdered plant materials in 500 L of aqueous at 4 °C for 24 hour. The extracts were filtered first through a Whatmann filter paper No. 42 (125 mm) and then centrifuged at 5000 rpm for 10 min (Remi-R-8C, India). The clear solution was partitioned with petroleum ether for elimination of lipid and fatty acids compound and finally aqueous extracts were partitioned with ethyl acetate. It was concentrated using a rotary evaporator with the water bath set at 40 °C. The percentage yield of extracts ranged from 7–19% w/w.

Determination of total Phenolics: The concentration of total phenolics in the ethyl acetate extract of *C. angustifolia* were determined by using Folin-Ciocalteu reagent and calibrated externally with gallic acid. Briefly, about 0.2 ml of flavonoid extract and 0.2 ml of Folin-Ciocalteu reagent were added and mixed vigorously. After shaking for 4 min, 1 ml of 15% Na_2CO_3 was added, and finally the mixture was allowed to stand for 2 hours at room temperature. The absorbance was measured at 760 nm using Deep Vision 1371 spectrophotometer. The concentration of the total phenolic was estimated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The quantification of phenolic compounds in all the fractions was carried out in triplicate and the results were averaged (Singleton *et al*., 1999).

Determination of Flavonoid content: The amount of total flavonoids in the extracts was measured according to Quettier-Deleu *et al*. (2000). This method is based on the formation of a complex flavonoid-Aluminium, with the absorbance maximum at 430nm. Rutin was used to make a calibration curve. To 1ml of flavonoid rich fraction, added 1ml of 2% $AlCl_3$ and it was incubated at room temperature for 15 min. Then absorbance was measured at 430 nm using Deep Vision 1371 spectrophotometer.

Invitro antioxidants properties

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay: ABTS radical scavenging activity of ethyl acetate extract of *C. angustifolia* was determined according to Re *et al.,* (1999). ABTS radical was freshly prepared by adding 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to yield an absorbance of 0.70 at 734 nm and the same was used for the antioxidant assay.

The final reaction mixture of standard group was made up to 1 ml with 950µl of ABTS solution and 50µl of Ascorbic acid. Similarly, in the test group, 1 ml reaction mixture comprised 950µl of ABTS solution and 50 µl of different concentration of flavonoid rich fraction. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$

Where *A*0 is the absorbance of the control reaction and *A*1 is the absorbance of flavonoid rich fraction.

Inhibition of lipid peroxidation activity: Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk by the method of Bishayee and Balasubramaniyam1971, was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*.,(1979). The reaction mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH4)_2SO_4.7H_2O$ (0.06 mM); and various concentrations of ethyl acetate extract of *C. angustifolia*in a final volume of 0.5 ml. The reaction mixture was incubated at 37^{o}Cfor 1 hour. After the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance was measured at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the extract was calculated according to 1-(E/C) X 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample (Abs_{532}+TBA–Abs_{532}+TBA).

Superoxide radical scavenging assay: This assay was based on the capacity of the extract to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1979) in the presence of the riboflavin-light-NBT system, as described earlier Tripathi and Pandey (1999) and Tripathi and Sharma (1999). In brief, each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of test sample solution. It was kept in front of fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample solution.
% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$ Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Nitric oxide radical scavenging activity: Nitric oxide scavenging capacity of ethyl acetate extract of *C. angustifolia* was measured according to the method described by Olabinri *et al*.(2010). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of flavonoid rich fraction and incubated at room temperature for 150 min. After incubation period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the reaction mixture was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as the standard. The % inhibition was calculated by following equation.

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Metal chelating activity: Metal chelating capacity of ethyl acetate extract of *C. angustifolia* was measured according to the method described by Iihami *et al*.,(2003). 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance of the solution was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as the standard. The % inhibition of ferrozoine-Fe2+complex was calculated by following equation.

% Inhibition of ferrozoine-Fe^{2+}complex = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Antibacterial Properties

Bacterial strains: Bacteria used for the determination of antibacterial activities were *Xanthomonas oryzae pv. Oryzae* MTCC 29213 and *Erwinia chrysanthemi* MTCC 1771, *Pseudomonas fuscovaginae* MTCC 2488. The bacterial strains were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology Sector 39-A, Chandigarh-160036, India. All bacterial strains were sub cultured on nutrient agar medium, incubated at 37 °C for 24 h and stored at 4 °C in refrigerator to maintain stock culture.

Antibacterial assay: Antibacterial activity was carried out using disc diffusion method (Velickovic and Smelcerovic, 2003). Petriplates were prepared with 20 ml of sterile nutrient agar (HIMEDIA). The tested cultures were swabbed on top of the solidified media and allowed to dry for 10 min. The test was conducted in four different concentrations of the flavonoid rich fraction (5, 10, 15 & 20 µl/ml) and impregnated discs with extracts (Whatman No.1 filter paper was used to prepare discs) were prepared and air dried well. The loaded discs were placed on the surface of the medium and incubated at room temperature for 24 h. The relative susceptibility of the organisms to the flavonoid rich fraction was indicated by the clear zone of inhibition around the discs. It was then observed, measured and recorded in millimeters with three replicates.

Determination of minimum inhibitory concentration: Microdilution broth susceptibility assay for bacteria was used, as recommended by Eloff, (1998). All tests were performed in Mueller Hinton broth (MHB) supplemented with Tween 80detergent (final concentration of 0.5%, v/v) to enhance the oil solubility. Bacterial strains were cultured overnight at 37 °C in MHA and the yeasts were cultured overnight at 30 °C in SDB. Test strains were suspended in MHB to give a final density of $5X10^5$ CFU/mL and these were confirmed by viable counts. Geometric dilutions ranging from 5 µmg/mL to 20 µg/mL of the ethyl acetate extract of *C. angustifolia* were prepared in a96-well microtiter plate, including one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + extract). Plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. The bacterial growth was indicated by the presence of a white ‘‘pellet” on the well bottom.

Statistical analysis: The influence of the ethyl acetate extract of *C. angustifolia* on its antioxidant activity was measured by the ABTS assay, lipid peroxidation, superoxide scavenging, metal chelating and nitric oxide radical were ascertained using one-way analysis of variance (ANOVA). Furthermore, Duncan’s post hoc test was applied, so as to determine the statistically significant different values. All statistical handling was performed using SPSS software, v. 14.0 (SPSS, Chicago, Ill., U. S. A.).

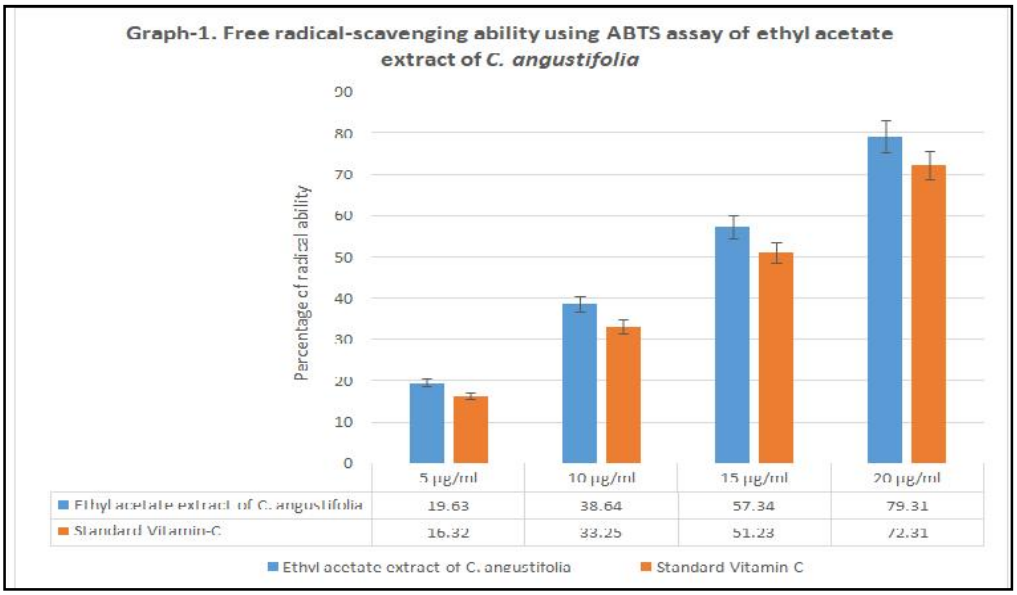
RESULTS AND DISCUSSION

Total phenolic and flavonoid content: The total phenolic and flavonoid contents of ethyl acetate extract from the leaves of *C. angustifolia* showed the highest amount of phenolic compounds78.9±1.36 mg GAE/g and 53±2.45µg RE/g.

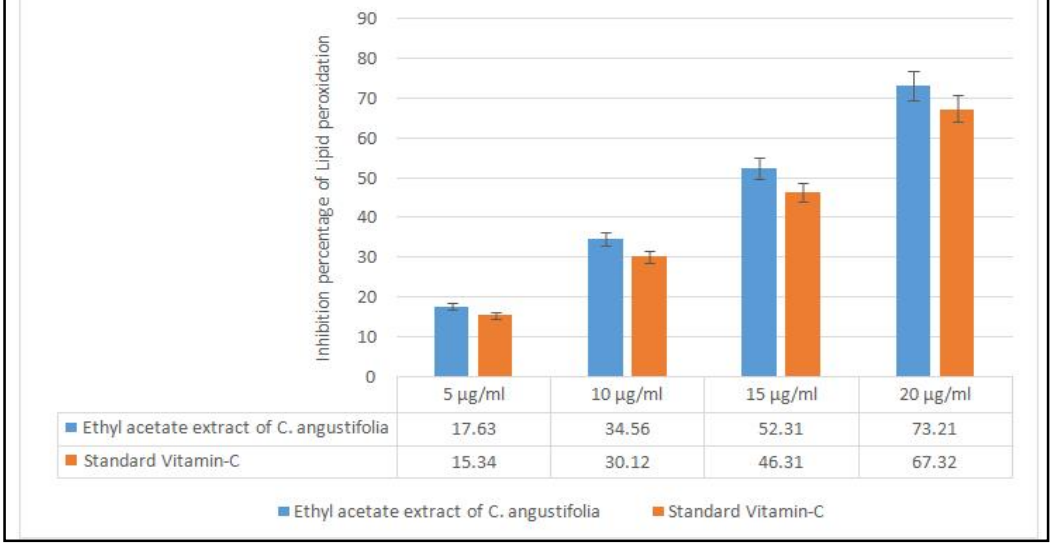
Free radical-scavenging ability using ABTS assay: The radical scavenging capability was dignified by ABTS assay as given in table 2. The Percentage embarrassment of the ABTS radical activity was assayed on average, and higher free radical-scavenging values were found in ethyl acetate extract of *C. angustifolia*. In ABTS assay, the scavenging ability of the ethyl acetate extract of *C. angustifolia* (79.31%) and lower ability scavenging properties of standard Vitamin-C (72.31%) (Graph-1). However, compounds other than phenolics in the ethyl acetate fraction may also involve in the antioxidant properties of *C. angustifolia*. These data were in agreement with earlier research (Gorinstein *et al*., 2003; Maisuthisakul *et al*, 2007), which have shown that great total polyphenols content intensifications antioxidant activity and there was a linear correlation between phenolic content and antioxidant activity.

Inhibition of lipid peroxidation: Ethyl acetate extract of *C. angustifolia* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum lipid peroxidation inhibition was apparent in ethyl acetate extract of *C. angustifolia* with percentage value 73.21 and standard vitamin-C67.32 µg/ml. As it is known that lipid peroxidation is the net result of any free radical attack on membrane and other lipid constituents present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). Here we have used egg yolk as a substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method of ethyl acetate extract of *C. angustifolia* significantly, inhibited the degree of lipid peroxidation than positive control (Graph-2).

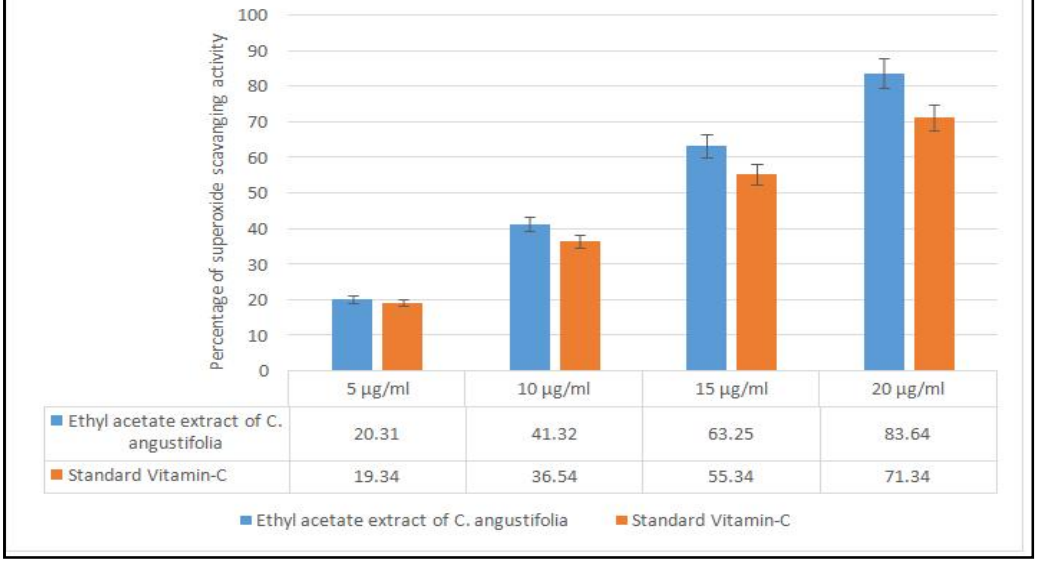
Graph-1. Free radical-scavenging ability using ABTS assay of ethyl acetate extract of *C. angustifolia*

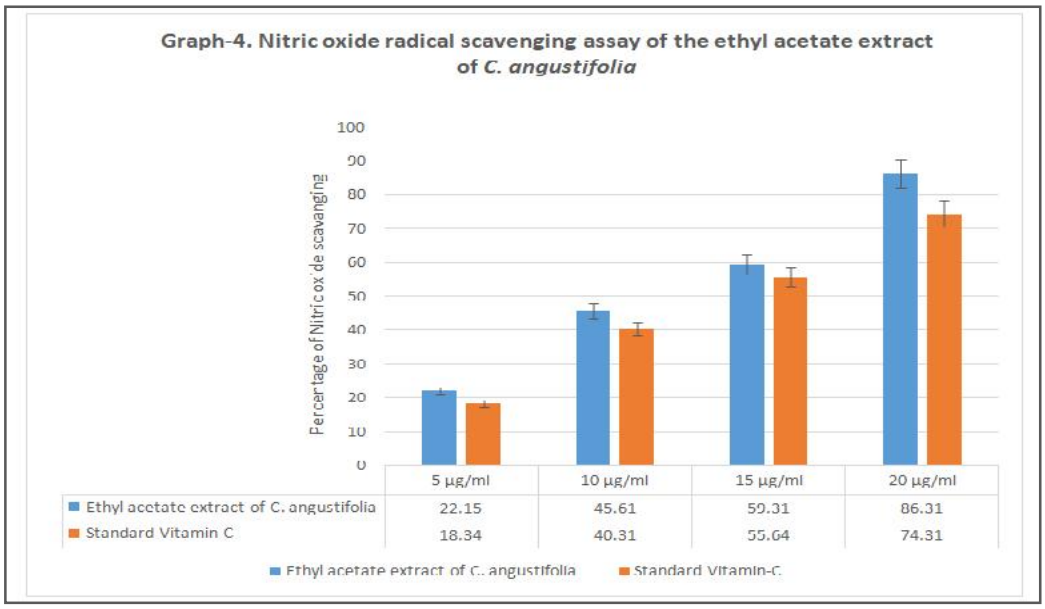


Graph-2. Inhibition of lipid peroxidation by Ethyl acetate extract of *C. angustifolia*



Graph-3. Superoxide scavenging activity of ethyl acetate extract from the leaves of *C. angustifolia*





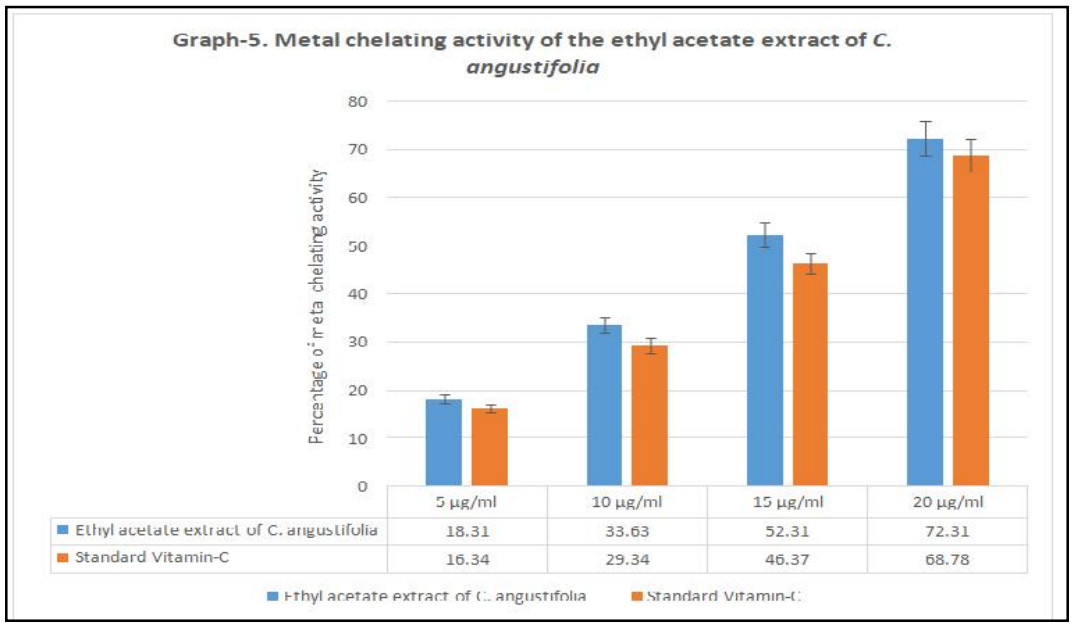


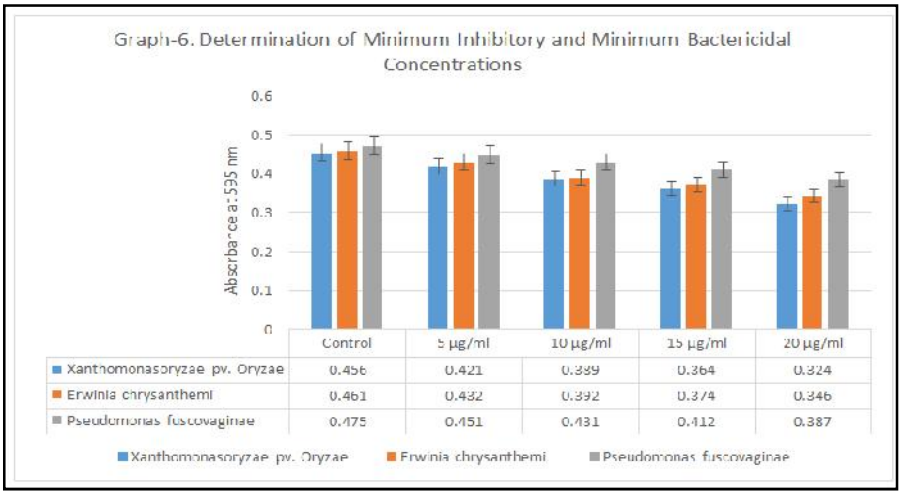
Table-1. Anti-bacterial activity of ethyl acetate extract of *C. angustifolia*tested against plant pathogenic bacteria

Ethyl acetate extract of *C. angustifolia*	Different concentrations of extract µl/ml							
	[a]Inhibition zone of the Xanthomonasoryzae pv. Oryzae measured (mm)				[a]Inhibition zone of the *Pseudomonas fuscovaginae* measured (mm)			
	5 µl/ml	10 µl/ml	15 µl/ml	20 µl/ml	5 µl/ml	10 µl/ml	15 µl/ml	20 µl/ml
	8.63±1.2	11.34±.23	13.2±1.6	16.0±0.8	7.8±0.4	10.2±1.3	12.8±1.4	15.6±0.7
	[a]Inhibition zone of the *Erwinia chrysanthemi* measured (mm)							
	7.1±0.6	9.1±0.54	11.6±2.3	14.4±1.3				

[a] Mean diameter of the zone of inhibition is in millimetres. Figures in parentheses are inhibition percentages compared to streptomycin.

Generally, the mechanisms of phenolic compounds for antioxidant activity are neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.In the present study, ethyl acetate extracts significantly inhibited lipid peroxidation in a dose-dependent manner. Similar results were obtained in a study by Tatiya *et al*. (2010) which evaluated anti-lipid peroxidation activities of *C. adscendens*.

Superoxide scavenging activity: Ethyl acetate extract of *C. angustifolia* exhibited potent scavenging activity for superoxide radicals in a concentration dependent manner than positive control. The percentage of Superoxide scavenging belongings in ethyl acetate extract of *C. angustifolia* had 83.64% and the positive control least potent with 71.34% (Graph-3). Superoxide radicals generated by photochemical reduction of nitro blue tetrazolium (NBT) in the



presence of a riboflavin-light-NBT system, is one of the standard methods. These superoxide radicals are highly toxic and may be generated either through xanthine activity or through mitochondrial reaction. Superoxide anion is also very harmful to cellular components. Robak and Glyglewski (1988) reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions.

Nitric oxide radical scavenging assay: In the present study, the nitric oxide radical scavenging activity of the ethyl acetate extract of *C. angustifolia* was detected and compared with the standard ascorbic acid. The ethyl acetate extract of *C. angustifolia* exhibited the maximum inhibition of 86.31% at a concentration of 20 µg/ml, in a concentration-dependent manner when compared to ascorbic acid that showed lowest activity against nitric oxide 74.31% (Graph-4). In the present study, the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by flavonoid rich fraction. Significant scavenging activity was observed for ethyl acetate extract of *C. angustifolia*. This species antioxidant activity is mostly due to the presence of the nonvolatile components, mainly polyphenolic compounds such as caffeic acid, gallic acid, hyperin, quercetin, 3',4'dimethoxyflavone and kaempferol previously identified in this species water, methanol, and ethanol extracts (Di *et al*., 2016).

Metal chelating activity: The metal chelating activity of the ethyl acetate extract of *C. angustifolia*. The ethyl acetate extract of *C. angustifolia* was assessed for their ability to compete with ferrozine for ferrous iron in the solution. In this assay, ethyl acetate extract of *C. angustifolia* interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are capable of capturing ferrous iron before ferrozine. The ethyl acetate extract of *C. angustifolia*reduced the red color complex immediately and showed the highest chelating activity (EC_{50} 7.59 µg/ml) than positive control (EC_{50} 27.11 µg/ml) (Graph-5). This would mean that the flavonoid compounds in extract should be responsible for antioxidant activities of scavenging free radicals and reducing ferric ions might not be directly involved in ferrous ion chelation. The screening and characterization of antioxidants derived from natural sources has gained much attention and efforts have been put into identifying compounds as suitable antioxidants to replace synthetic ones (Wong and Leong, 2006).

Anti-bacterial activity of ethyl acetate extract of *C. angustifolia* tested against plant pathogenic bacteria: Antibacterial activities of ethyl acetate extract of *C. angustifolia* against the tested organisms are shown in Table-1. The plants differ in their activities against the micro-organisms tested. Ethyl acetate extract of *C. angustifolia* showed maximum antibacterial activity against *Xanthomonasoryzae pv. Oryzae*, *Erwinia chrysanthemi* and *Pseudomonas fuscovaginae.* Highest antibacterial activity was observed with ethyl acetate extract of *C. angustifolia* against *Xanthomonasoryzae pv. Oryzae* and *Pseudomonas fuscovaginae* (16and 15.6 mm) respectively while lowest activity was observed against *Erwinia chrysanthemi* with the inhibition zone of 14.4 mm.

Results obtained in the current investigation revealed that the ethyl extract possess potential antibacterial activity against entire tested organisms.These findings are quite similar with the results of Chao *et al*. (2000) reporting that Cassia sp fully inhibited the growth of some Gram positive and Gram negative bacteria, fungi and yeasts. As the main component, cinnamaldehyde, has proven to be particularly effective against some species of Gram positive and Gram negative bacteria including Clostridium, Pseudomonas and yeasts, Candida strains (Shan *et al*. 2007).Bameri *et al*., (2010) recorded that methanol extract of *C. angustifolia* displayed the highest activity and a broad spectrum of activity against pathogenic bacterial strains. It was reported that the bactericidal activity was due to the presence of flavonoids found in the methanol extract. Bameri *et al*., (2013) have been studied that methanol and ethanol extracts of *C. angustifolia* possess significant antibacterial activity against *E. coli*, *Klebsiella pneumoniae* and *Shigella shinga*.

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

Ethyl acetate extract of *C. angustifolia* have been shown to possess antimicrobial, properties. The MIC obtained from ethyl acetate extract of *C. angustifolia* was shown in Graph-6. The MIC of the ethyl acetate extract of *C. angustifolia* ranged from 5 to 20 µg/ml. The MIC was defined as the lowest concentration of ethyl acetate extract that was able to inhibit bacterial growth, i e., as lower the concentration is

obtained, less product is needed to inhibit the bacterial growth. All plant species with MIC values of up to 8 mg/ml are measured to possess at least some degree of inhibitory effect, and any concentration more than this should not be considered effective, conferring to Fabry *et al*. (1998).
Sensible microbial inhibitors are described by Aligiannis *et al*. (2001) as those plant extracts with MIC values ranging between 0.60 mg/ml and 1.50 mg/ml.

Conclusion

In conclusion this is the first study concerning the antioxidant capacity, antibacterial activity and total phenolic content ethyl acetate extract of *C. angustifolia*. Since this plant is dispersedin a large quantity in many tropical regions of theIndia and its leaf extract showed a significant antioxidantand antibacterial activities, our work suggest that the leafof *C. angustifolia* may be utilized as effective and safe antioxidant and antibacterial source. However, searching for further bioactive compounds which are responsible for the biological activities of *C. angustifolia* is needed.

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