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Phytochemical screening, antioxidant activity and antimicrobial activity of *Spathodeacampunalata* Stem extracts.

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Abstract

Spathodeacampunalata is African tulip tree, known for its medicinal properties, in the present study three extracts of stem Ethanolic, Methanolic and Aqueous extracts were evaluated for phenolic content, flavanoid content, total antioxidant capacity by Folin Ciocalteu method ,Colorimetric method, phosphomolybdenum assay respectively.Iinvitro antioxidant activity was evaluated by 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical method and by lipid peroxidation method. The antimicrobial activity was evaluated using agar well diffusion assay protocol against eight bacterial reference strains, Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Proteus vulgaris (ATCC 6380), Salmonella typhimurium (ATCC 25241), Salmonella paratyphi (ATCC 9150), Shigella sonnei (ATCC 25931) and Klebsiella pneumonia (ATCC 27736). Plant sources showing antioxidant activities which is safe has become growing interest across the world thus the above experiment was performed to screen the presence of phytochemicals, estimation of polyphenols, flavonoids, and their ability to show the effects of antimicrobial and antioxidant activities was investigated.

Results revealed the presence of phytoconstituents, with relevant quantity of polyphenol and flavonoid content of the extracts was found to decrease in the order of Methanolic extract > ethanolic extract>aqueous extract whereas total antioxidant activity evaluated by phosphomolybdenum assay showed decrease in the order of aqueous extract>methanolic extract>ethanolic extract.

The methanolic and ethanolic extracts exhibited better activity against eight bacterial strains used compared to the aqueous extract. Invitro antioxidant activity evaluated by DPPH assay of the extracts showed concentration dependent percentage inhibition of different radicals and raised gradually to its maximum level with higher concentrations, in the lipid peroxidation, aqueous extracts shows less percentage inhibition as compared to methanolic and ethanolic extracts. Thus extract possesses significant antioxidant activity.

Keywords: Spathodea campunalata, extracts, phytochemical screening, DPPH, lipid peroxidation, antibacterial activity.

Introduction

Plants are used for its medicinal or therapeutic purposes for various diseases for centuries throughout the world due to its biological active substances like secondary metabolites and antioxidants [1,2].

In recent years there has been corresponding rise in the universal demand for medicines obtained from the plants due to no side effects of plants and are more effective than the adverse effects of pharmaceutical drugs [3, 4]. Moreover, humans has showed resistance to pharmaceutical drugs, this has imposed the need for a permanent search and development of plants and given rise to herbal medicines that have been important part of primary health care on human. The search of effectiveness, affordability, low toxicity and acceptability for plants has gained increasingly [5, 6].

Reactive oxygen species production in excess is detrimental and can cause oxidative damage to lipids, proteins, DNA (single and double strand breaks, apurine/apyrimidine lesions [7] and many of which are toxic and mutagenic, increases the frequency of strand exchange intermediates during DNA replication leading to genomic instability). It also leads to ageing, atherosclerosis, cancer, neurodegenerative diseases. Microsatellite instability is caused by hydrogen peroxide induced oxidative DNA damage, this is associated with colorectal cancer. Oxidative damage to cellular components results in altered cellular function, compromised tissue and organ function, and ultimately death.

In recent years there has been increased interest in the therapeutic use of antioxidants for the treatment of diseases associated with oxidative stress [8-10].

Therefore protection of DNA damage by antioxidants is very important hence a balanced antioxidant status should be

maintained to reduce the levels of DNA damage it should be maintained a balanced antioxidant status by reducing the levels of DNA damage. Hence use of dietary antioxidants which play very important role in preventing the ROS induced DNA damage, endogenous antioxidants such as glutathione, -lipoic acid, coenzyme Q10, bilirubin and antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase) protects from oxidative stress. When they are insufficient, they are introduced as exogenous antioxidants, these compounds are primarily taken into the body by food and are predominantly of herbal origins like phenols, have redox properties which allow them to act as reducing agents, which are the hydrogen donors and singlet oxygen quenchers [11].

Carotenoids in sufficiently high concentrations can protect lipids from peroxidation [12]. Ascorbic acid prevent free radical induced damage to DNA and acts as an antioxidant [13] and tocopherol-it is lipophilic, vitamin E is useful in protecting membranes from lipid peroxidation, flavonoids has chelating properties [14], also possess anti – carcinogenic, anti inflammatory and anti microbial activities. Saponins have been found to be potentially useful for the treatment of hypercholesterolemia which suggests that saponins might be acting by interfering with intestinal absorption of cholesterol [15]. Terpenoids avoid oxygen stress [16], tannins and terpenoids promotes wound healing. terpenoids also soothe irritated tissues and the mucous membranes that line the respiratory tract, which eases the respiratory ailments [17, 18].

Herbal antioxidants exhibits their activity through a wide variety of mechanisms, such as inhibition of oxidising enzymes, chelation of transition metals, transfer of hydrogen or single electron to radicals, singlet oxygen deactivation, or enzymatic detoxification of ROS. They can also stop lipid peroxidation process in either initiation or propagation step [19], thus herbal antioxidants have become leading interest and targets of numerous scientific research. Hence plants from various families is being investigated to explore as potential medical drugs or dietary supplements.

Spathodea campanulata is commonly known as African tulip tree belongs to the family bignoniaceae found in Africa.

It has many medicinal properties used as diuretic, urethra inflammation, used against fungal infection [20], diarrhea, stomachache, have molluscicidal activity, analgesic and anti-inflammatory, hypoglycemic, anti HIV and antimalarial activity. Spathodea campanulata showed presence of natural antioxidants such as plant – phenols, flavonoids and tannins which are potent antioxidants [21, 22]. Sterols like β-sitosterol have been reported for antioxidant activity [23]. Terpenoids are also reported to have antioxidant activity [24]. Phenols were used as antimicrobial and antioxidant compounds [25, 26]. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radicals' reactivity. Extracts are capable of performing scavenging activity, this plant also contains like spathodol, spathoside, Ajugol, Sitosterol, βsitosterol - 3-O-β- D-glucopyranoside, Oleanolic acid, pomolic acid, Ursolic acid tomentosolic acid, caffeic acid, p – hydroxybenzoic acid, Phenylethanol esters, phenolic acids and flavonoids [27-35].

These medicinal properties of this plant which is wide spread has stimulated for more pharmacological research on this plant. In the present study we investigated antibacterial and in-vitro antioxidant activity of Spathodea campanulata.

Methods

Chemicals

Sodium carbonate, Sodium nitrate, Aluminium chloride, Sodium hydroxide, Sulphuric acid, Sodium phosphate, Ammonium molybdate, Potassium chloride, Hydrochloric acid, trichloroacetic acid were purchased from Merck, India, Mueller Hinton broth (MHB) Mueller Hinton Agar (MHA) purchased from Hi-Media, India. Standard drugs 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteu, Gallic acid, Querecetin, Ascorbic acid, Fenton's reagent, thiobarbutaric acid, butylated hydroxytoluene (BHT), Chloramphenicol reagent were purchased from Sigma-Aldrich chemicals co. (St. Louis O., USA). Analytical grade solvents like Ethanol, Methanol were used in this study.

Plant material

Stem and Bark of Spathodea campanulata were collected from Central Research Institute of Unani Medicine, Hyderabad. The Stem and Bark were cleaned, dried under shade, ground to a coarse powder. Both the powders were mixed together and stored in an air-tight container at 25 C for further use.

Microbial cultures

Bacterial reference strains Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Proteus vulgaris ATCC 6380, Salmonella entericaparatyphi (Salmonella paratyphi) ATCC 9150 , Salmonella typhimurium ATCC 25241, Klebsiella pneumonia ATCC 27736 and Shigella sonnei ATCC 25931 were purchased from Hi-Media (Mumbai). The pure cultures were maintained on nutrient agar slants for the entire study. All the isolates were sub-cultured at regular time period and stored at 4 C as well as at -80 C by making their suspension in 10% glycerol.

Preparation of plant extracts

The Stem powder of Spathodeacampunalata were subjected to Soxhlet extraction apparatus for 16hrs with different solvents i.e., Methanol, Ethanol and Aqueous. Then the extracts were collected and concentrated in the Rotary evaporator at 50-60º C under reduced pressure leaving dark brown residue of stem.

Stock solutions of 100 mg/ml of the extract were prepared and different concentrations were used in the experiments.

Preliminary Phytochemical Screening

Standard procedures consisting of Tannins, Terpenoids, Steroids, Alkaloids and Flavonoids were used for phytochemical studies [36, 37].

Determination of total phenolic content

Total phenolic content in both stem extracts was determined by modified Folin-Ciocalteau method [38]. 5µl of extract was added to 495µl of distilled water which was then mixed with 100µl of Folin-Ciocalteau reagent. Tubes were vortexed for 15s and incubated for 10 min. Then, 300µl of 20% Na2CO3 solution was added and tubes were vortexed again. The volume of the mixture was adjusted to 1 ml using distilled water. The mixture was incubated in dark for 2 h and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against blank sample. The total phenolic content was measured as Gallic acid equivalents (mg GAE)/gram of dry weight (dw).The experiment was repeated thrice.

Determination of total flavonoids content

Estimation of Total flavonoid content was done by a colorimetric method 5µl of extract was mixed with 495µl distilled water and 30µl of 5% NaNO2 solution. The tubes were incubated for 10 min at room temperature. Then, 60µl of 10% AlCl3 solution was added. Subsequently, 350µl of 1 M NaOH and 60µl of distilled water were added to make the final volume 1ml. Tubes were again incubated for 15 min at room temperature. The absorbance was measured at 510 nm. The values were expressed as means of triplicate analysis. The total flavonoid content was measured as Quercetin equivalents (mg QE)/gram of dry weight (dw).The experiment was repeated thrice [39].

Determination of total antioxidant activity (TAA)

The total antioxidant activity of both stem and bark extracts of Spathodea campunalata were evaluated by phosphomolybdenum method. 2.5µl of extract was dissolved in 97.5µl of distilled water and 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). Tubes were vortexed-mixed and incubated in a thermal block at 95 C for 90min. Then the tubes were cooled at room temperature. Absorbance was measured at 695nm against blank samples. Ascorbic acid (AA) was used as standard and the total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (mg AAE)/ gram of dry weight (dw) [40].

DPPH radical scavenging activity

The stable free radical antioxidant activity of the extracts was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH).

DPPH solution (0.004% w/v) was prepared in 95% methanol. Various concentrations of extracts were mixed with 900µL of DPPH solution, the reaction mixtures were incubated in dark for 30 min and then absorbance was measured at 517 nm. Methanolic (95%) DPPH solution was used as blank and ascorbic acid (AA) was used as standard. Antiradical activity (%) of the samples was calculated according to the formula [41]:

Antiradical activity (%) = 100 X (Absorbance of control-Absorbance of sample/ Absorbance of control)

Determination of inhibition of Lipid peroxidation

Lipid peroxidation inhibitory activity of Spathodea campunalata extracts and the standard (ascorbic acid) was carried out according to the standard protocol.

The goat liver was purchased from local slaughter house; its lobes were dried between blotting paper (to remove excess blood) and were cut into small pieces with a heavy duty blade. They were then homogenised in a glass Teflon homogenising tube with 0.15 M KCl. The homogenate was centrifuged at 800 g for 15 min at 4 C and the supernatant was used for the thiobarbutaric acid assay [7].

The extracts of Spathodeacampunalata at different concentrations were mixed with the liver microsome preparation, the mixtures were incubated in the presence and absence of Fenton's reagent (50µl of 10mM FeCl3; 10µl of 2.5mM H2O2) in phosphate buffer (0.2M, pH 7.4) and the final volume was made to 1ml. The reaction mixtures were incubated at 37 C for 30 min. After incubation, 2ml of ice-cold HCl (0.25N) containing 15% trichloroacetic acid, 0.5% thiobarbutaric acid and 0.5% butylatedhydroxytoluene (BHT) was added to the reaction mixture, followed by heating at 100 C for 60min. The reaction mixture was put in an ice bath for 10 min. The mixture was centrifuged at 1000 g for 10 min and the extent of lipid peroxidation was subsequently monitored by the formation of thiobarbutaric acid reactive substances (TBARS) as pink chromogen in the presence or absence of extracts and standard (ascorbic acid). The absorbance of the supernatant was measured spectrophotometrically at 532nm. The decline in the formation of pink chromogen in the pre-treated reactions was considered as inhibition of lipid peroxidation.

The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the following formula i.e. Eq. (1) [42]:

Inhibition (%) = (Control Absorbance-Test Absorbance) X 100/ Control Absorbance

Evaluation of anti-bacterial activity

Anti-Bacterial activity of the extract was determined by Agar diffusion assay. Bacterial strains were first grown in Mueller Hinton broth (MHB) under shaking condition for 24 h at 37 C and after the incubation period, 0.1ml of the test inoculums was spread evenly with a sterile glass spreader on Mueller Hinton Agar (MHA) plates. The seeded plates were allowed to dry in the incubator at 37 C. Wells were made using sterile 6mm cork borer in the inoculated MHA plate. The wells were filled with 150µl of the extracts (re-suspended in respective solvents). The concentration of stock extracts was 100 mg/ml. The inoculated plates were incubated at 37 C for 24 h. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone

around the wells. The size of the zones of inhibition was measured and the antibacterial activity was expressed in terms of average diameter of the zone of inhibition in millimetres. The results were compared with the standard antibiotic Chloramphenicol (25mcg). The photograph was taken in U.V-Visible documentation system [43].

Results

Phytochemical screening

Phytochemical screening was performed using standard protocols which showed the presence of tannins, terpenoids, steroids, alkaloids and flavonoids.

The total phenolic content

The total phenolic content of S.campunalata is shown in Table 1. polyphenol content was found in the decreasing order of methanolic extract(4.05 mg GAE/g)> ethanolic extract(3.494 mg GAE/g)> aqueous extract (3.09mg GAE/g).

Phenolic components are responsible for the antimicrobial, antioxidant and antiradical activities. This can be concluded that phenolic content is higher in methanolic extract and thus showed better antibacterial activity than the ethanol and aqueous extract. This conclusion can be made by the obtained result that shows that methanolic extract which contain higher phenolic content showed better antibacterial activity than ethanol and aqueous extract. Phenolic compounds significantly contribute to the antioxidant activity; antioxidant activity appears to be a result of combined activity of phenolics, peptides, organic acids and enzymes.

Total flavonoid content

Total flavonoid content was found in the decreasing order of, methanol (8.897 mg QE/g dw)>ethanol (6.329mg QE/g dw) and aqueous extracts(5.95 mg QE/g dw) (Table 1

Table 1: Total Antioxidant Activity, Flavonoid Content and Polyphenols Content of S. Campunalata.

	Total Antioxidant Activity[a] (mean±SD)	Flavanoids[b] (mean±SD)	Polyphenols[a] (mean±SD)
S. Campunalata Aqueous	5342.66 ± 207.96	5950.66 ± 760.014	3122.66 ± 458.95
S. Campunalata Ethanolic	3212.66 ± 78.799	6329 ± 49.56	3494.33 ± 151.44
S. Campunalata Methanolic	4338 ± 967.9	8897.66 ± 602.72	4064 ± 127.16

a: gallic acid; b: quercetin; c: ascrobic acid equivalents µg/ml dw plant material respectively; Results represented in means ± standard deviation.

Total antioxidant activity (TAC)

The total antioxidant capacity was based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acidic pH. It evaluates both water – soluble and fat- soluble antioxidants (total antioxidant capacity). The results indicate higher TAC (expressed as ascorbic acid equivalent) of the aqueous extract. This suggest the potential comparable antioxidant constituents of the all the three extracts because antioxidant capacity of ascorbic acid has been used as a reference standard with which plant extracts with potential antioxidants are compared [44].

The results of the antioxidant activity measurements are summarized in Table 1. The antioxidant activities were found in decreasing order of aqueous (5.342mg AAE/g dw) >methanol (4.33mg AAE/g dw) >ethanol (3.21mg AAE/g dw).

DPPH radical scavenging activity

The free radical scavenging capacity of the extracts against common free radical (DPPH) shows the dose-response curve of DPPH scavenging activities of the extracts. The results (Table 2) revealed that all the three extracts, ethanolic, methanolic and aqueous extract had DPPH radical scavenging ability was raised gradually to its maximum level with higher concentrations.

Table 2: DPPH Radical Scavenging Activity of Spathodaea campanulata

DPPH			
Concentration	Methanol (mean±SD)	Ethanol (mean±SD)	Aqueous (mean±SD)
1	12.49± 0.4689	22.29 ± 0.3214	21.24 ± 0.1913
2	27.63± 0.1664	49.29 ± 0.217	38.21 ± 0.1907
3	42.48± 0.5342	74.67 ± 0.4903	55.46 ± 0.1858
4	59.36± 0.3534	85.31 ± 0.5755	69.14 ± 0.02
5	65.39± 0.3987	86.29 ± 0.14	73.72 ± 0.1833
6	81.59± 0.3536	89.24% ± 0.205	82.75 ± 0.2706
7	86.42± 0.0907	89.59% ± 0.2484	85.3 ± 0.4176
8	90.03± 0.1006	90.37% ± 0.1571	89.14 ± 0.0264
9	91.3± 0.0435	90.50% ± 0.036	93.5 ± 0.2402
10	92.45± 0.5839	90.70% ± 0.2066	94.62 ± 0.204

Inhibition of lipid peroxidation

The results of inhibition of lipid peroxidation by S.campunalata extracts are presented in Figure (3). It showed that both methanolic and ethanolic extracts showed dependent percentage inhibition of scavenging activity which was raised gradually to its maximum level with higher concentrations whereas aqueous extracts shows low level percentage inhibition with same concentrations as Compared to methanolic and ethanolic extracts.

Figure 1: Total Antioxidant Activity, Flavonoid Content and Polyphenols Content of S. Campunalata.

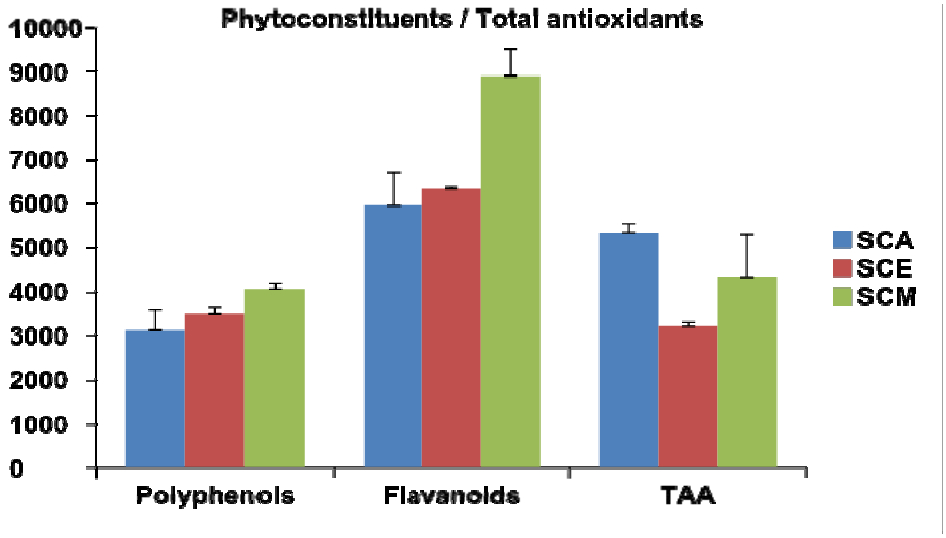


Figure 2: DPPH Radical Scavenging Activity of Spathodaea campanulata

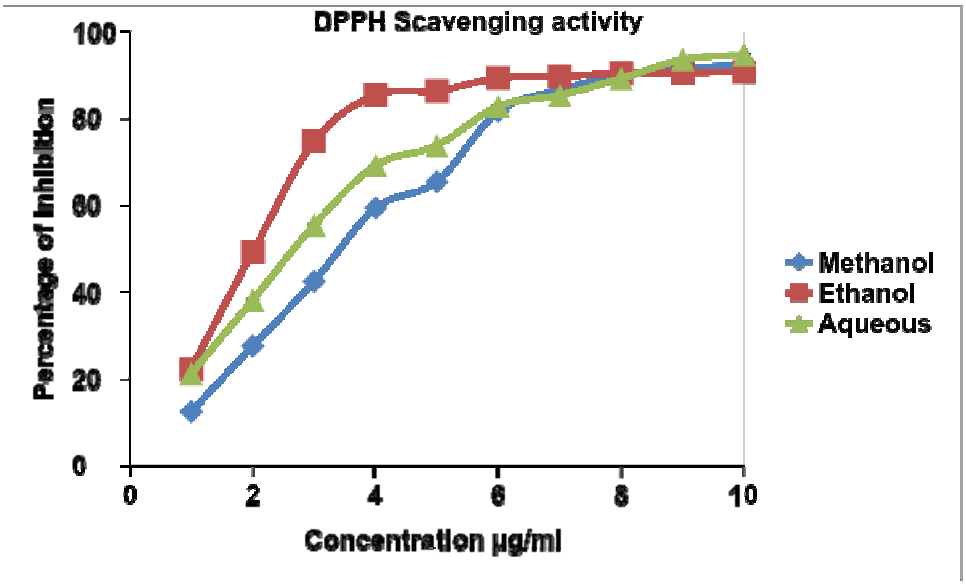
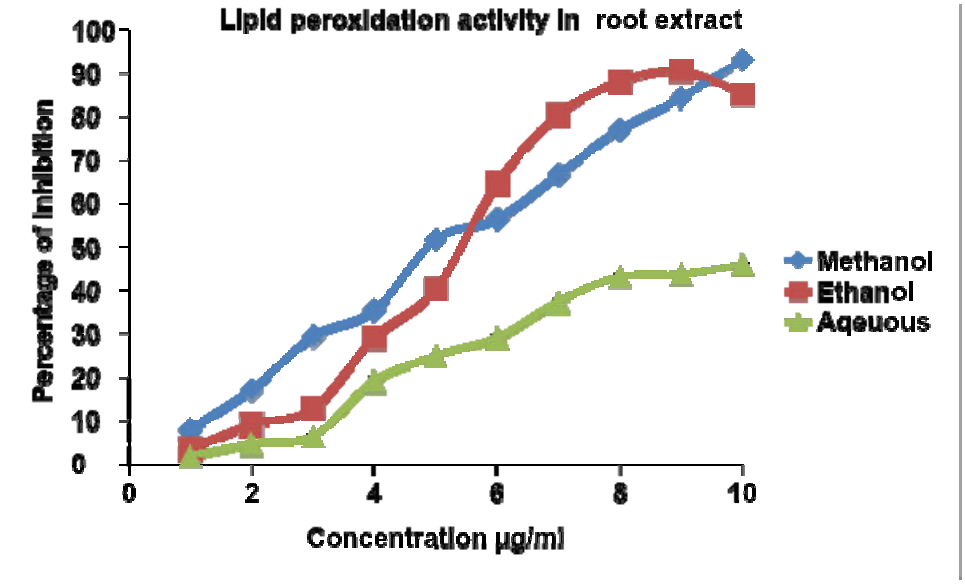


Figure 3:



Antimicrobial activity

The methanolic and ethanolic extract showed better antimicrobial activity compared to aqueous extract. The aqueous extract possess considerable antimicrobial potential against four organisms out of eight organisms; it is found to be affective on E.coli with a zone of inhibition of 25mm , Proteus vulgaris with a zone of inhibition of 9mm, Klebshiella with a zone of inhibition of 10mm and Shigella with a zone of inhibition of 6mm. (table 3); the highest inhibition zone of 17 mm was observed in Pseudomonas aeruginosa. The results were compared with standard antibiotic chloramphenicol (25mcg).

Table 3: Lipid Peroxidation Inhibition Activity of Spathodea campanulata

Concentration	Methanol (mean±SD)	Ethanol (mean±SD)	Aqueous (mean±SD)
1µl	8.04 ± 0.2571	3.556 ± 0.455	1.94 ± 0.3176
2 µl	17.23 ± 0.6671	9.166 ± 0.75	4.83 ± 0.1473
3 µl	29.46 ± 0.7182	12.8 ± 0.15	6.5 ± 0.4
4 µl	35.55 ± 0.0866	29.14 ± 0.166	19.18 ± 0.8607
5 µl	51.72 ± 0.4161	40.36 ± 0.892	24.95 ± 0.3214
6 µl	56.48 ± 0.5107	64.77% ± 0.946	29.05 ± 0.6392
7 µl	66.69 ± 0.52	80.48% ± 0.6255	37.5 ± 0.5169
8 µl	77.05 ± 0.7274	87.96% ± 0.7218	43.19 ± 0.6638
9 µl	84.42 ± 0.4531	90.55% ± 0.586	43.79 ± 0.2007
10 µl	93.04 ± 0.2247	85.13% ± 0.567	46 ± 0.3044

Discussion

Phytochemical screening of the extracts revealed the presence of tannins, terpenoids, steroids, alkaloids and flavonoids. Natural antioxidants like phenols, flavonoids and tannins possess potent antioxidant [21, 22]. Phenols present in the extracts have antioxidantive properties used in preparation of antimicrobial and antioxidant compounds [25, 26]. Sterols like β – sitosterol have also shown antioxidant activity [23]. Terpenoids are also potent antioxidant [24].Many studies suggested that flavonoids exhibit antioxidant, anti-inflammatory, antimicrobial, vascular activities and other medicinal properties [45]. Related studies of antimicrobialactivity indicates that crude extracts containingflavonoids, triterpenes and steroids have showedsignificantive activity against various strains of Staphylococcus aureus and Escherichia coli [46].

The DPPH free radical scavenging activity is due to the antioxidants and their ability of hydrogen donating [47]. Antioxidants reacts with DPPH, which is a stable free radical and converts it to 1,1-diphenyl-2- picryl hydrazyl. DPPH radical is scavenged by antioxidants through the donation of hydrogen forming the reduced DPPH-H. The colour changes from purple to yellow after reduction which can be quantified by its decrease of absorbance at wavelength 517nm. Hence, the ethanol extract of flowers of S. campanulata showed dose dependent DPPH radicals scavenging activity.Plants showing scavenging activity indicates that the extracts is capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radicals reactivity, or the extract were capable of scavenging superoxide and DPPH in a concentration manner.The ethanol and methanol extracts shows a significant protection against lipid peroxidation whereas aqueous extract suggest this activity with more concentration. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl- perferryl complex or through OH but this is less reactive than OH. The inhibition could be caused by the absence of ferryl – perferryl complex or by scavenging of OH radical or reducing the rate of conversion of ferrous to ferric ion or by chelating the iron itself [48-51]. Phenolic compounds usually work as inhibitors of lipid peroxidation chain reaction [52]. Flavonoids shown to inhibit peroxidation and protect tissue from the damage caused by oxygen reactive species [53, 54]. Inhibition of lipid peroxidation by the extracts were dose –dependent and it is possible that identified phenolic compounds in the extract from the table….. was responsible for this activity. Thus the chain break reaction has been postulated as the main mechanism by which phenolic compounds and plant extracts inhibit lipid peroxidation [52]

Table 4: Antibacterial activity of Spathodaea campanulata

	Diameter of Zone of Inhibition (in mm)			
Organism	Ethanol	Aqueous	Methanol	Chloramphenicol
S.TYPHI	12.12	Nil	12.5	09
E.coli	14	25	14.12	08
Pseudomonas	13.16	Nil	17	08
Shigella	10.33	6	13.87	08
Proteus	13.33	9	12.37	08
Klebsiella	11.33	10	13.37	19
S.paratyphi	12.5	Nil	13.62	09
Staph. aureus	11.75	Nil	12.37	21

Conclusion

On basis of the results obtained in the present study, it is concluded that ethanol, methanol and aqueous extract of stem of Spathodea campanulata has shown potent invitro - antioxidant and free radical scavenging activity. It possess significant activity in antimicrobial, DPPH and inhibition of lipid peroxidation. Thus, antioxidant potential of extracts may be attributed to the presence of secondary metabolites (tannins, terpenoids, steroids, alkaloids and flavonoids). Therefore, further investigation is needed to isolate and identify the active compounds responsible for different pharmacological activities present in the plant and its efficacy needs to be done.

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