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PRELIMINARY PHYTOCHEMICAL, ANTIOXIDANT AND ANTIBACTERIAL STUDIES ON THE BARK OF SPATHODEA CAMPANULATA P. BEAUV

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ABSTRACT
The African tulip tree, *Spathodea campanulata* was selected for performing the following study. Three extracts of the bark, namely hexane, ethyl acetate and ethanol extracts were taken and subjected to preliminary phytochemical analysis, performed by standard protocols. These revealed the presence of various biochemical compounds like phytosterols, triterpenes, glycosides, saponins, flavonoids, tannins, carbohydrates, alkaloids and oils. Antioxidant capacity of the three extracts was assessed using DPPH and hydroxyl radicals, with ascorbic acid as control. The ethanol and ethyl acetate extracts showed very high antioxidant activity. Finally, the three extracts were tested for antibacterial activity, with Rifampicin as the standard drug. The ethanol and ethyl acetate extracts showed fairly good antibacterial activity against the six test organisms (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aerogenosa, Bacillus pumulis & Bacillus subtilis*). Further research needs to be done to identify the specific drug candidate responsible for the observed antioxidant and antibacterial activities.

KEYWORDS: *Spathodea campanulata,* Phytochemical analysis, Antioxidant activity, Antibacterial activity.

1. INTRODUCTION

The world is endowed with a wide variety of plants, ranging from small herbs to medium sized shrubs, tall trees to creepers on the terrestrial environment and different algae in the aquatic environment. A large number of these plants have therapeutic property and are hence of great medicinal value. The presence of diverse secondary metabolites in plants is what makes them possess curative properties. Every country has its own traditional medicinal system, which employs plant extracts and preparations for the treatment of commonly occurring diseases in that area. In India, Ayurveda has been practiced for several centuries and is known to be highly effective. Many of these medicinal plants used traditionally have been evaluated scientifically and have shown remarkable curative or disease control capacity. Majority of the drugs available in the market today are plant based, i.e., they are synthetic or semi synthetic secondary metabolites of plants. The promising bioactive compounds are isolated, purified and characterized and are used directly or with slight modification to chemical structure as potent drugs. However, there are some diseases which still do not have a specific drug for complete cure, such as diabetes, cancer etc. There is also the sudden rise of a disease resulting in an epidemic such as the case with Ebola recently and Swine Flu, Bird Flu, Anthrax etc. in the past. The development of multi drug resistant strains of pathogens like *Mycobacterium tuberculosis, Klebsiella pneumonia* etc. has also been on the rise. All the above reasons make the discovery of bioactive compounds from new unexplored plant sources the need of the hour. Hence, there has been an accelerated global effort to identify plant species that display antioxidant and antimicrobial properties which can protect the human body [1].

Spathodea campanulata is a medium sized deciduous tree with dark foliage which is native to West Africa. It generally reaches a height of 10-35 m. The young bark is pale, grey-brown and smooth but turns grey-black, scaly and cracked vertically and horizontally with age [2]. The leaves are opposite, imparipinnate and exstipulate. Each leaf consists of 5-7 pairs of opposite leaflets and a terminal one. The leaflets are oblonge, elliptic, dark green on top, light green on the underside; there are granular swellings at the base of the lamina (usually a pair); the midrib and the nerves are yellow, raised and very slightly pubescent. The venation is reticulate; the petiole is short and thick, there are conspicuous lenticels on the rachis and rachis base is swollen. Flowers are large, red outside and orange inside, and are hermaphrodite. The calyx is green, split on the posterior

side and is ribbed. It has 5 petals and 4 stamens with orange filaments, the style extruding with a 2-lipped stigma. The flower buds are curved and contain a red sap. Fruit is upstanding, dark brown, cigar-shaped with a woody pod, split on the ground into 2 boat shaped valves, releasing many flat-winged seeds; 1-4 pods usually develop from 1 flower cluster. The seeds are thin, flat and surrounded by a filmy wing[3]. *Spathodea campanulata* has many medicinal uses, both where it is native and introduced. It is used in traditional herbal medicine [4]. It is used for the treatment of ulcers, filaria, gonorrhea, diarrhea and fever [5][6][7]. It is commonly employed to control epilepsy [8]. Its leaf is used in the treatment of painful inflammation [9],[10], constipation and dysentery[11]. The plant is also reported to exhibit anti plasmodial activity [12] and anti-inflammation activity [13]. In the rural communities of Iboland, Nigeria, its leaf decoction has been widely used for the control of epilepsy and convulsions. It may be effective as a malaria prophylactic [14]. Bark extracts of *Spathodea canpanulata* showed hypoglycemic, anti-complement and anti-HIV activities according to G. Niyonzima *et al* [15]. According to Patil *et al*., 2009[16], the flower extract of *Spathodea campanulata* showed the ability to absorb UV radiation and hence proved its UV protection ability. Prophylactic and curative effects of an aqueous extract of *Spathodea campanulata* on liver injury induced by carbon tetrachloride in rats was reported by Charles *et al*., 2013[17]. Pone *et al*., 2014[18] found that the methanol extract of shade dried leaves, its various fractions and the isolated pure compounds possess anti-inflammatory activity. The curative activity of the stem bark isolate on rat's model of lung cancer was observed by Masruri *et al*., 2014[19].

The aim of the present study is to investigate the antioxidant and antibacterial properties of *Spathodea campanulata*.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals and reagents used for the following studies were analytical grade. 1, 1- diphenyl-2-picrylhydrazyl was purchased from Sigma Chemical Company, St. Louis, USA, Ascorbic acid from Loba Chemicals Pvt. Ltd, Mumbai. Other chemicals Rifampicin, DMSO and individual components of agar media were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai. The other reagents were purchased from Desai Chemicals, Visakhapatnam, India.

2.2 Test Organisms

Six bacterial species were used to test the antibacterial activity of the hexane, ethyl acetate and ethanol extracts of *Spathodea campanulata*. The bacterial species were purchased from National Collection of Industrial Micro-organisms (NCIM), Pune, India. Each of the test bacterial species was maintained in nutrient broth medium on shaker in separate culture tubes. Out of the six bacteria, three were Gram positive (*Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus pumilis*), and three were Gram negative (*Pseudomonas aerogenosa*, *Escherichia coli* and *Klebsiella pneumonia*).

2.3 Culture Medium

For assessing the antibacterial activity of the three different extracts of *Spathodea campanulata*, Nutrient Agar media was used. Nutrient broth was used for maintaining the bacterial species.

2.4 Plant Material

The plant material selected for the following study is the bark of *Spathodea campanulata*. It was collected from Andhra University campus, Visakhapatnam, Andhra Pradesh, India, during the month of December, 2012. The authentication of the above plant was done by Prof. M. Venkaiah, Department of Botany, Andhra University, Visakhapatnam.

2.5 Preparation of Extracts

The bark of *Spathodea campanulata* was collected and cut into smaller pieces. These were then dried in the shade for a week after which they were ground to yield a coarse powder. The shade dried bark powder of *S. campanulata* was separately extracted by maceration successively with hexane, ethyl acetate and ethanol. The liquid fractions were collected and were concentrated to dryness under vacuum according to Harborne, 1973[20] at a temperature of 54°C using a rotary evaporator (Buchi R-210). The soft mass so obtained was dried completely in a desiccator so as to remove all traces of solvent. Then, the weight was taken. The weight of the powdered plant materials taken for extraction and the weight of the crude fractions obtained are given in Table I.

2.6 Phytochemical Analysis

Preliminary phytochemical analysis was carried out on the hexane, ethyl acetate and ethanol fractions of *Spathodea campanulata* to detect the presence of alkaloids, carbohydrates, flavonoids, saponins, steroids, tannins and triterpenes by the procedures according to Faraz *et al*., 2003[21]; Harborne 1998[22]; Edeoga *et al*., 2005[23].

2.7 In vitro Anti-oxidant Activity

All the fractions (hexane, ethyl acetate and ethanol) of *S. campanulata* were screened for free radical scavenging activity against DPPH and hydroxyl radicals at different concentrations. The percentage inhibition and 50% inhibition concentrations (IC_{50}) were calculated. All experiments were performed thrice and the results were averaged.

2.8. DPPH Radical

DPPH is an abbreviation for the organic compound, 2, 2-diphenyl-1-picrylhydrazyl. This compound is a dark colored crystalline powder composed of stable free radical molecules. The DPPH radical is well known and widely used to investigate the scavenging activity of natural compounds. The DPPH radical has a deep violet

color in solution because of a strong absorption band centered at about 520 nm and it becomes colorless or pale yellow when neutralized. DPPH solution loses the characteristic deep purple (λ_{max} 515–517 nm) color on accepting hydrogen from a corresponding donor. This property allows visual monitoring of the reaction. The rate of reaction of various antioxidants with DPPH differs according to Janaszewska & Bartosz, 2002[24].

The scavenging activity of the three extracts for DPPH free radicals was measured according to the procedure described by Braca *et al*., 2003[25]. The percentage inhibition activity and the 50% inhibition concentration of extract/ ascorbic acid was then determined.

2.9 Hydroxyl Radical

The neutral form of the hydroxide ion (HO–) is hydroxly radical (•HO). They are short-lived owing to their property of high reactivity. Most notably hydroxyl radicals are produced from the decomposition of hydroperoxides (ROHO). The hydroxyl radical has a very short in-vivo half-life of approximately 10^{-9} seconds and a high reactivity. This makes it a very dangerous compound to the organism [26]. The destructive action of hydroxyl radicals has been implicated in several neurological autoimmune diseases. The hydroxyl radical can damage virtually all types of macromolecules; nucleic acids (mutations), lipids (lipid peroxidation) and amino acids. The hydroxyl radical cannot be eliminated by an enzymatic reaction. This damage causes ageing, cancer and several diseases [27]. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases.

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fenton reaction which results in the formation of thiobarbituric acid reacting substances [28]. The percentage inhibition activity and the 50% inhibition concentration of extract/ ascorbic acid was then determined.

2.10 Calculation of Percentage Inhibition

The percentage inhibition of the extract was calculated using the formula:

$$\text{Inhibitory ratio} = \frac{(A_0 - A_1)\times 100}{A_0}$$

Where, A_0 is the absorbance of control;

A_1 is the absorbance with addition of plant extract/ ascorbic acid.

2.11 Calculation of 50% Inhibition

The optical density obtained with each concentration of the extract/ ascorbic acid was plotted taking concentration on X-axis and percentage inhibition on Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ ascorbic acid.

2.12 In-vitro Antibacterial Activity

In vitro antibacterial activity is determined by well diffusion method [29].This method is based on measuring the diameter of zone of inhibition of microbial growth surrounding the wells containing various dilutions of extracts/standard drug. Nutrient agar was prepared by weighing each of the components separately and dissolving them in 400ml of double distilled water. The final volume was made up to 500ml. The pH was adjusted to 6.8 using a pH meter. Similarly, the Nutrient broth was prepared by dissolving each of the components (without agar) in 500ml distilled water.

All the media were autoclaved for 20min at 15lbs pressure at 121ºC. Immediately after autoclaving, they were allowed to cool till it reaches a temperature of 30 to 35º C. The Nutrient agar media was inoculated (1ml culture) with the test organism at a temperature of 35ºC and was poured into sterile Petri plates by pour plate method. After solidification, a sterile metal borer was used to prepare the wells of 4mm diameter in the agar plates. Then the test samples (100µl) and the standards (50µl) were added into the wells using a micropipette. All the Nutrient agar plates were incubated at 28ºC for 48-72hr. The presence of definite zone of inhibition of any size around the well indicated antimicrobial activity. The diameter of the zones were measured and recorded.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Analysis

The percentage of the fractions obtained from *Spathodea campanulata* are as follows:

Ethanol > Ethyl acetate > Hexane.

The preliminary phytochemical analysis revealed the presence of steroids, saponins, alkaloids, carbohydrates in all the three extracts (hexane, ethyl acetate and ethanol). Triterpenes were absent in the hexane and ethyl acetate extract, flavonoids, tannins and glycosides were absent in the hexane extract. The findings are represented in Table II.

This is in accordance to the findings of Brindha *et al.*, 2012[30] who reported the presence of alkaloids (1.92mg/kg), flavonoids (2.50mg/kg), tannins (0.35mg/kg), lignin (0.29mg/kg), glycosides (0.05mg/kg), serpentines (0.05mg/kg), terpenoids (0.03mg/kg), saponin (0.01mg/kg) and phenols (0.12 mg/kg) in the bark of *S. campanulata*. Moreover they also reported the presence of important nutraceuticals such as carbohydrates (1.36mg/kg), protein (0.32mg/kg), fats (0.03 mg/kg) and energy (5.69Kcal/gm). Zahid *et al.*, 2011[31] also reported the presence of various phytochemicals in the different extracts; chloroform, petroleum ether and methanol from the bark and flowers of *S. campanulata*. The presence of various phytochemicals was also reported in the leaves of *S. campanulata* by Sowjanya *et al.*, 2013[32].

Table I: Percentage of the fractions obtained from *Spathodea campanulata*

S.No	Name of the solvent	Weight of the powdered material (g)	% of soluble extractives (Wt/Wt)
1	Hexane	590	0.27
2	Ethyl acetate	590	1.11
3	Ethanol	590	6.90

Table II: Preliminary phytochemical analysis

Phytochemicals	*Spathodea campanulata*		
	Hexane Extract	Ethyl acetate Extract	Ethanol Extract
Phytosterols	+	+	+
Triterpenes	-	-	+
Glycosides	-	+	+
Saponins	+	+	+
Flavonoids	-	+	+
Tannins	-	+++	+
Carbohydrates	+		++
Alkaloids	+	+	+
Oils	+	+	+

– Absent, + Present
++Present in good amount resulting in dark color
+++Present in high amount resulting in very dark color

3.2. In vitro Antioxidant Activity

DPPH Radical Scavenging Activity: The hexane, ethyl acetate and ethanol extracts of *Spathodea campanulata* were found to possess concentration dependent scavenging activity on DPPH radicals and the results are given Table III and Fig. I. The tests showed that the percentage inhibition was dependent on the concentration of the plant extract used; the maximum activity was obtained at a concentration of 1 mg (1000 µg). Among the three extracts of *Spathodea campanulata*, the ethanol extract showed better activity compared to other extracts, but slightly less than the standard drug, ascorbic acid. The order of reactivity obtained is as follows:

Ascorbic acid > Ethanol extract > Ethyl acetate extract> Hexane extract.

Table III: Concentration dependent percent inhibition of DPPH radical by different extracts of *Spathodea campanulata* and ascorbic acid in *in-vitro* studies

Name of the extract of *Spathodea campanulata*	Percentage inhibition of DPPH radical					
	Quantity of extracts/ ascorbic acid in micrograms (µg)					
	100	200	400	600	800	1000
Ethanol	53.8±2.72	65.23±0.4	72.69±0.3	84.12±1.7	86.18±0.8	87.9±0.3
Ethyl Acetate	46.13±1.2	52.34±3.5	65.23±0.5	79.41±0.5	81.50±1.6	82.49±0.2
Hexane	-	-	-	3.61±0.48	8.46±0.2	20.45±0.6
Ascorbic acid	48.0±0.5	88.08±1.0	90.68±0.3	93.63±0.5	94.21±0.3	94.74±1.1

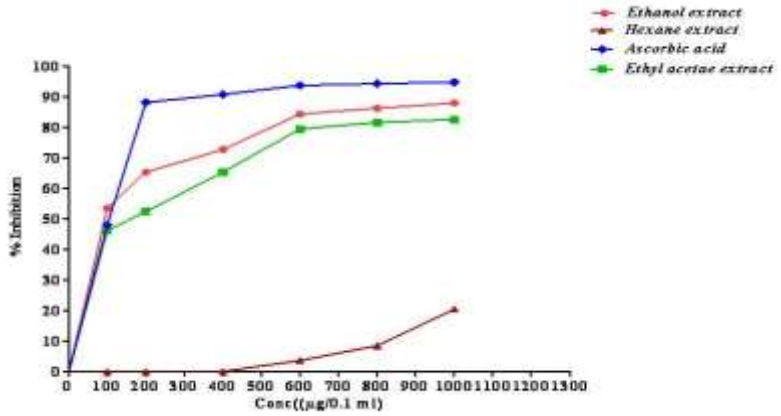

Fig I: Concentration dependent percent inhibition of DPPH radical by different extracts of *Spathodea campanulata* and Ascorbic acid in *In-vitro* studies

3.3 Hydroxyl Radical Scavenging Activity

The hexane, ethyl acetate and ethanol extracts of *Spathodea campanulata* were found to possess concentration dependent scavenging activity on hydroxyl radicals and the results are given in Table IV and Fig II. The percentage inhibition was dependent on the concentration of the plant extract used; the maximum inhibition was obtained at a concentration of 1 mg (1000 µg). As in the case with DPPH radical, the ethanol extract showed highest activity among the other plant extracts, but slightly less than that of the standard drug, ascorbic acid. The order of reactivity obtained is as follows:

Ascorbic acid > Ethanol extract > Ethyl acetate extract> Hexane extract.

Table IV: Concentration dependent percent inhibition of hydroxyl radical by different extracts of *Spathodea campanulata* and ascorbic acid in *in-vitro* studies

Name of the extract of *Spathodea campanulata*	Percentage inhibition of Hydroxyl radical					
	Quantity of extracts/ ascorbic acid in micrograms (µg)					
	100	200	400	600	800	1000
Ethanol	16.22±0.6	24.32±0.4	38.34±0.5	49.54±0.4	62.61±0.3	76.15±0.4
Ethyl Acetate	17.99±0.4	26.32±0.8	34.71±0.8	47.33±0.4	59.79±0.3	69.61±0.4
Hexane	7.84±0.7	10.41±0.4	14.51±0.4	19.72±0.5	28.22±0.7	38.07±0.2
Ascorbic acid	24.32±0.4	34.12±0.6	54.61±1.0	64.31±0.6	76.25±0.4	82.11±1.0

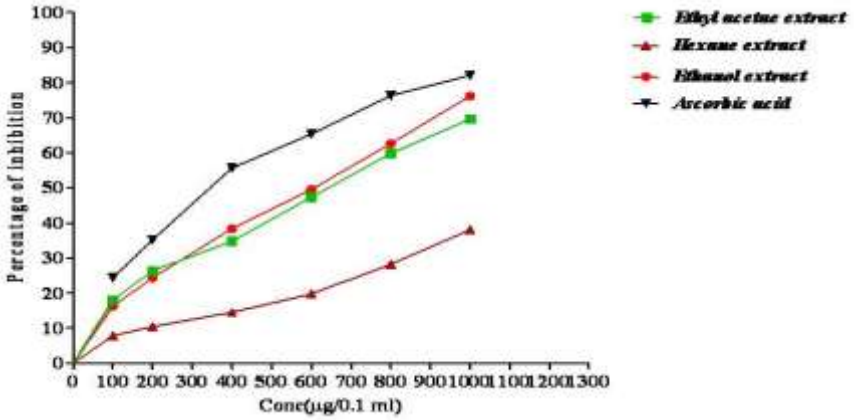

Fig II: Concentration dependent percent inhibition of Hydroxyl radical by different extracts of *Spathodea campanulata* and Ascorbic acid in *In-vitro* studies

I_{50} Values

The mean IC_{50} values for DPPH radical of ethanol and ethyl acetate extracts *Spathodea campanulata* were found to be 93.6µg, 166.0µg respectively. The mean IC_{50} value of ascorbic acid was found to be 107.0µg.

The mean IC_{50} values for hydroxyl radical of ethanol and ethyl acetate extracts of *Spathodea campanulata* were found to be 606.0µg, and 650.0µg, respectively. The mean IC_{50} value of ascorbic acid was found to be 353.0µg. The results are given in Table V.

Table V: *In-vitro* 50% Inhibition Concentration (IC_{50}) of different extracts of *Spathodea campanulata* on DPPH and hydroxyl free radical.

S. No.	Name of the extract of *Spathodea campanulata*	IC_{50} value (µg)	
		DPPH radical	Hydroxyl radical
01.	Ethanol extract	93.6	606
02.	Ethyl Acetate extract	166.0	650
03.	Hexane extract	-	-
04.	Ascorbic acid	107.0	353.0

In-vitro antioxidant activity of the bark of *S. campanulata* was assessed by Shanmukha *et al.*, 2010[33] using reducing power method and nitric oxide radical scavenging activity method, while the reducing power activity increased in a dose dependent manner, the scavenging activity of nitric oxide was highest for the standard i.e. ascorbic acid followed by the different concentrations of plant extracts. Similar dose-dependent free radical scavenging activity of *S. campanulata* leaves was reported by Ravi Kumar J., and Ganga Rao 2013[34]. Kowti *et al.*, 2011[35] reported the protective effect of *S. campanulata* flowers against pre-oxidant induced DNA

damage. Significant antioxidant capacity of flowers of *S. campanulata* was observed by Heim *et al.*, 2012[36], however the bark showed five times more antioxidant capacity than the flowers.

3.4 In vitro Antibacterial Activity

All the fractions (hexane, ethyl acetate and ethanol fractions) showed significant activity at a concentration of 4mg/ml. Among all the fractions, the ethanol fraction showed more activity towards *E.coli*, *Pseudomonas aeruginosa* and *Bacillus pumilis.* Results of average in triplicates are given in Tables VI to VIII.

Antibacterial activity of the stem bark of *S. campanulata* was also reported by Ofori-Kwakye *et al.*, 2009[37]. They also formulated four topical products by incorporating the methanol extract of *S. campanulata* (20% w/w) into aqueous cream, soft paraffin, emulsifying ointment and simple ointment bases. Similar antibacterial activity of the leaves and flowers of *S. campanulata* was reported by Akharaiyi *et al.*, 2012[38]. Rajesh *et al.*, 2010[39] reported that the ethanol extract of the flower showed more potent antibacterial activity than that of the leaf.

Table VI: Table showing the antibacterial activity of hexane extract of *Spathodea campanulata*

Concentrations (per 100 µl)	*Zone of Inhibition (mm)*					
	Ec	***Sa***	***Kp***	***Pa***	***Bp***	***Bs***
0.5mg	-	9	-	10	-	14
1.0mg	-	11	-	10	-	14
2.0mg	-	12	10	11	-	14
4.0mg	14	13	11	12	-	15
Rifampicin	30	30	32	30	30	32
DMSO(control)	0	0	0	0	0	0

Table VII: Table showing the antibacterial activity of ethyl acetate extract of *Spathodea campanulata*

Concentrations (per 100 µl)	*Zone of Inhibition (mm)*					
	Ec	***Sa***	***Kp***	***Pa***	***Bp***	***Bs***
0.5mg	12	8	8	10	-	11
1.0mg	12	10	10	11	-	14
2.0mg	14	11	13	11	-	14
4.0mg	15	12	14	12	-	14
Rifampicin	30	30	32	30	30	32
DMSO(control)	0	0	0	0	0	0

Note: *Ec-Escherichia coli, Sa-Staphylococcus aureus, Kp- Klebsiella pneumonia, Pa-Pseudomonas aerogenosa, Bp-Bacillus pumulis, Bs-Bacillus subtilis*

Table VIII: Table showing the antibacterial activity of ethanol extract of *Spathodea campanulata*

Concentrations (per 100 µl)	*Zone of Inhibition (mm)*					
	Ec	***Sa***	***Kp***	***Pa***	***Bp***	***Bs***
0.5mg	11	-	-	11	-	10
1.0mg	15	8	9	12	-	13
2.0mg	17	10	11	13	-	14
4.0mg	18	11	14	13	12	14
Rifampicin	30	30	32	30	30	32
DMSO(control)	0	0	0	0	0	0

Note: *Ec-Escherichia coli, Sa-Staphylococcus aureus, Kp- Klebsiella pneumonia, Pa-Pseudomonas aerogenosa, Bp-Bacillus pumulis, Bs-Bacillus subtilis*

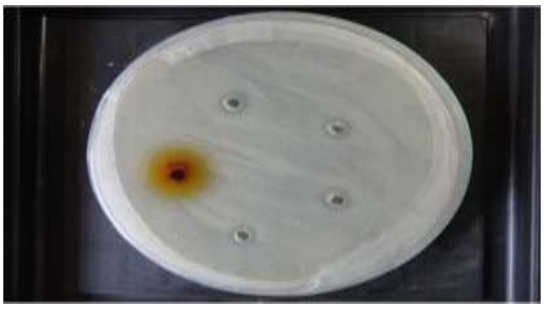

Fig. III: Antibacterial activity of Hexane extract of *S. campanulata* against *B. subtilis*

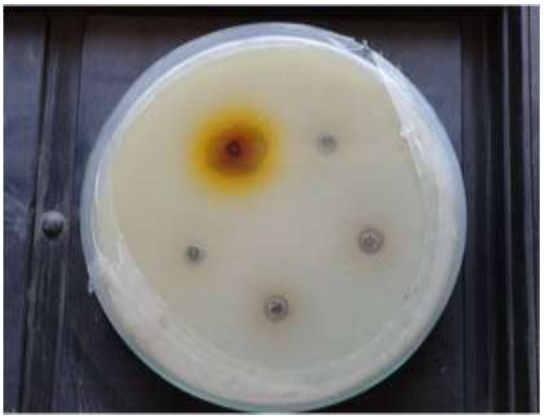

Fig. IV: Antibacterial activity of Ethyl acetate extract of *S. campanulata* against *E. coli*

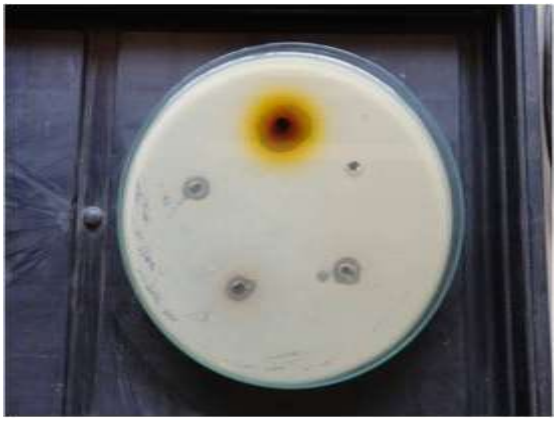

Fig. V: Antibacterial activity of Ethanol extract of *S. campanulata* against *E. coli*

CONCLUSION

Phytochemical analysis of the test extracts of *S. campanulata* was determined by standard protocols. The hexane, ethyl acetate and ethanol fractions showed dose dependence radical scavenging activity for DPPH and Hydroxyl radicals, producing a maximum activity at a dose of 1mg. The standard drug Ascorbic acid also showed similar dose dependent activity and produced maximum scavenging activity at a dose of 1mg.

All the fractions (hexane, ethyl acetate and ethanol extracts) showed significant antibacterial activity at a concentration of 4mg. Among all the fractions, the ethanol fraction showed significant zone of inhibition towards gram negative and gram positive bacteria (*E. coli, B. subtilis, K. pneumonia* and *P. aeruginosa*).

Overall, it can be concluded that the study revealed that the bark extracts of *Spathodea campanulata* showed potent antioxidant and antibacterial activity .However, further research has to be done to identify the specific drug candidate responsible for the observed antioxidant and antibacterial activities.

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