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A Comparative Study of the Antioxidant and Antibacterial Activities of *Spermacocea pusilla* L and *Spermacocea ocymoides* L. Root

P. VIJAYALAKSHMI[1] and M. M. SUDHEER MOHAMMED*[2]

[1-2] Department of Botany, Government Arts College (Autonomous) (Affiliated to Bharathiyar University, Tamil Nadu), Coimbatore - 641 018, Tamil Nadu, India

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Abstract

Natural products have been a significant source of commercial medicines and bioactive compounds. The ethanolic crude root extracts of s*permacocea pusilla* and *Spermacocea ocymoides* were screened for secondary metabolites. It revealed the presence of Alkaloids, Flavonoids, Tannins, Triterpenoids, Saponins, and fixed oil. The ethanolic root extracts of *Spermacocea spp* correlation was found between DPPH, ABTS, and Phosphomolybdenum assay. The antioxidant capacity was evaluated using assays that detect different antioxidants: DPPH, ABTS (transition metal ion reduction), and Phosphomolybdenum assay (reducing power and nitric oxide reduction). The antibacterial activity of root extract against *Klebsiella pneumoniae, Streptococcus pneumonia, Streptococcus pyogenes*, *Staphylococcus aureus, Escherichia coli*, and *Enterococcus sp.,* was carried out using the agar well diffusion method. Minimum inhibitory concentration of *S. pusilla* root extract was found to be (15.06±0.66) against *Klebsiella pneumoniae* and *S. ocymoides* root was found to be (15.68±0.34) against *Klebsiella pneumonia. These* products have been a significant source of commercial medicines and bioactive compounds. The present study revealed the significance of the root as a drug.

***Key words*:** *Spermacocea pusilla* root, *Spermacocea ocymoides* root, Ethanol extract, Secondary metabolites, DPPH, ABTS, Antibacterial

Nature has a treasure of medicines to treat all kinds of ailments. Our prehistoric ancestors, roamed the earth in search of food, they perhaps earned better information about herbs. The importance of this information and experience was vital to the health of tribes, was delivered from generation to generation for thousands of years of human existence. Out of this fundamental knowledge came written and spoken knowledges on herbs, which was grown continuously to the present day [1]. Natural products from plants have played major, sustaining roles in the lives of humans, especially for food sources and medicinal products [2]. As of now majority of the drugs introduced to Western medicine are derived mostly from natural products and about 25% of commonly used prescription drugs are derived from traditionally used medicinal plants [3]. In addition, there are myriad plant extracts and plant materials that are employed commercially in various parts of the world. For approximately 85% of the world's population, these plant materials are the primary source of health care [4]. The Rubiaceae family comprises one of the largest angiosperm families, with 650 genera [5] and approximately 13,000 species, [6] distributed mainly in tropical and subtropical regions also reaching the temperate and cold regions of Europe and Northern Canada as well. The genera *Borreria* and *Spermacoce*, are the largest of the tribe *Spermacocea*, comprising about 280 species distributed in tropical and subtropical America, Africa, Asia, and Europe [8]. In Brazil, 36 *Borreria* species were recorded, of which 22 are endemics [9-10]. Both *Borreria* and *Spermacocea* species are used medicinally in various manners and are reputed in the traditional medicine of Latin America, Asia, Africa, and the West Indies. *B. pusilla* (Wall.) DC. [Syn.: *B. stricta* (Linn. f.) K. Schum., *S. pusilla* Wall.] is an annual erect herb native to tropical Africa and Asia. In India, the fresh buds associated with flowers are used for cuts and wounds [11] and crushed leaves are applied to the affected areas for bone fracture and scabies, and for snake and also for scorpion bites [12]. *B. ocymoides* (Burm. f.) DC. (Syn.: *S. ocymoides* Burm. f.) is common in all of America a, also occurs in eastern Africa and East India. In Nigeria, the juice of the leaves is applied for ringworm and eczema and the sap is squeezed onto the wound or lesion [13]. Rubiaceae is well known for its medicinal value, used in the treatment of malaria, diarrhea, digestive problems, skin diseases, fever, hemorrhage, urinary and respiratory infections, headache, and inflammation of eyes and gums [14]. Traditional medicinal systems like Ayurvedic, Chinese Medicine, and Unani developed from plant resources have been used to treat various diseases. The isolation of bioactive compounds such as tannins, terpenoids, alkaloids, flavonoids etc. for potential drug discovery has been extensively reported [16-17]. In biological system free radicals, together with secondarily formed radicals, are known to play an important role in the pathogenesis of many chronic conditions like atherosclerosis, arthritis, diabetes, ischemia, reperfusion injuries, central nervous system injury, and cancer [18-19]. The secondary metabolites such as alkaloids, glycosides, resins,

***Correspondence to:** M. M. Sudheer Mohammed, E-mail: mmsudheermohammed@gmail.com; Tel: +91 9443274469

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volatile oils, gums, and tannins of medicinal plants are the chemical compounds that are active pharmacologically and utilized to develop drugs [20-21]. Hence, the study of antioxidant status during a free radical challenge can be used as an index of protection against the development of these degenerative processes in experimental condition for therapeutic measures. Dietary intake of antioxidants can improve the protection against free radical damages. Antioxidants are substances that can prevent damage caused by reactive oxygen species [22]. Various studies revealed that the presence of phenolic compounds shows antioxidant activity. Phenolic compounds are found to have reducibility, metal chelating properties, and a hydrogen donor function [23]. Most medicinal plants have antimicrobial properties and their use is greatest in the tropics where the diversity and growth rates of microorganisms [15]. The review of the literature revealed that there is lack of scientific reports on pharmacognostic, pharmacological and phytochemical evaluation. So, the objective of the present study is to evaluate phytochemical, antioxidant and anti-microbial evaluation of *Spermacocea ocymoides* (Burm. F.) DC and *Spermacocea pusilla* root.

MATERIALS AND METHODS

The root of *Spermacocea pusilla* and *Spermacocea ocymoides* were collected from the local region of Karamadai, Coimbatore District, Tamil Nadu, India. The roots were removed directly from the plant to sterile polythene bags and transported to the laboratory. The roots were washed with tap water and followed a wash in distilled water and dried at room temperature.

Sample preparation

The dried root was grounded a fine powder using mortar and pistil followed by the preparation of different suspensions, viz., Petroleum ether, Ethyl Acetate, Ethanol and Aqueous were used to extract bioactive compounds from the sample. About 100 g of powdered root material from each plant was extracted by soxhlation using various solvents such as Petroleum ether, Ethyl Acetate, Ethanol and aqueous depending on this polarity. Extracts were then concentrated by evaporating in a room temperature and the concentrated residual extracts were stored in a dry airtight container until further use [38].

Secondary metabolites screening

Phytochemical analysis

The crude root extract was subjected to preliminary screening for bioactive compounds. Each root extract was tested individually with specific chemical reagents as per the methods described by [24-27]. Different chemical tests were carried out to detect Alkaloids, Flavonoids, Tannins, Steroids, Triterpenoids, saponins, Glycosides, Gum and mucilages, Fixed Oils and Anthraquinones. Visible color change or precipitate formation was taken into the presence (+) or absence (-) of particular bioactive constituents.

Detection of alkaloids

i) *Dragendorff's test* (Waldi 1965)

1 ml of Dragendorff's reagent is added to 1 ml of filtrate by the side of the test tube. A conspicuous orange or yellow precipitate indicates the presence of alkaloids.

ii) *Mayer's test* (Evans 1997)

Few drops of Mayer's reagent are added to few ml of filtrate by the side of the test tube. A white or creamy precipitate indicates the presence of alkaloids.

iii) *Hager's test* (Wagner *et al.* 1996)

1 or 2 ml of Hager's reagent (saturated aqueous solution of picric acid) is added to the plant extract by the side of the test tube. A prominent yellow precipitate indicates the test as positive.

Detection of flavonoids - 10% HCl and 5% NaOH test (Trease and Evans 2002)

1 ml of plant extract was treated with few drops of 5% NaOH solution, the filtrate turns yellow colour then it becomes colorless at the addition of few drops of 10% HCl solution, the colour transformation of yellow to colorless indicates the presence of flavonoids.

Detection of tannins - Ferric chloride test (Segelman *et al.* 1969)

1 ml of 0.1% $FeCl_3$ was added to the plant extract filtrate. Appearance of brownish green or blue black colour indicates the presence of tannins.

Detection of steroids - Libermann- Burchard's test (Finar 1986)

1 ml of extract treated with 1 ml of acetic anhydride followed by 1 ml of concentrated H_2SO_4 by the side of test tube, brown ring is formed at the junction two layers. The upper layer turned to green colour indicates the presence of steroids.

Detection of triterpenoids

i) *Libermann- Burchard's test* (Finar 1986)

1 ml of filtrate treated with 1 ml of acetic anhydride followed by 1 ml of concentrated H_2SO_4 by the side of test tube, brown ring is formed at the junction two layers. Formation of deep red colour in lower layer indicates the presence of triterpenoids.

ii) *Salkowski's test* (Roopashree *et al.* 2008)

1 ml of extract treated with 1 ml of chloroform and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenoids.

Detection of saponins - Foam test (Kokate 1999)

Few ml of filtrate is diluted with the same amount of distilled water. The suspension is shacked for 15 to 30 seconds and allowed to stand for minimum 10 seconds. Formation of one or two centimeter layer of foam at the end indicates the presence of saponins.

Detection of glycosides - Keller – Kiliani test (Camporese *et al.* 2003)

One ml glacial acetic acid, three drops of Iron (III) chloride and few drops concentrated sulphuric acid added with two ml of filtrate. Appearance of Green-blue colour indicated the presence of cardiac glycosides.

Detection of gum and mucilages - Whistler and BeMiller test (Whistler and BeMiller 1993)

The extract (100 mg) is dissolved in 10 ml of distilled water and to this; 25 ml of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of gums and mucilages.

Detection of fixed oils and fats - Spot test (Kokate 1999)

A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil.

Detection of anthraquinones - Sanker - Nahar test (Sanker and Nahar 2007)

One ml of filtrate was treated with the same volume of aqueous base NaOH or NH_4OH solution by the side of test tube. A pink or violet colour in the base layer indicates the presence of anthraquinones.

Antioxidant activity

DPPH free radical- scavenging activity

The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH, according to the method of Braca *et al*. [28]. Sample extracts at various concentrations were taken and the volume was adjusted to 100 µL with methanol. About 3 mL of a 0.004% methanolic solution of DPPH was added to the aliquots of samples and standards (BHT and Rutin) and shaken vigorously. Negative control was prepared by adding100 µL of methanol in 3 mL of methanolic DPPH solution. The tubes were allowed to stand for 30 minutes at 27 °C. The absorbance of the samples and control were measured at 517 nm against the methanol blank. The test was triplicated. Radical scavenging activity of these samples was expressed as IC 50 which is the concentration of the sample required to inhibit 50% of $DPPH^{\cdot}$ concentration.

ABTS radical scavenging activity

The ABTS radical action decolonization assay was used to determine plants crude extracts free radical scavenging activity [34]. The reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1) produced the ABTS cation radical. Before use, the mixture was kept in the dark at room temperature for 12-16 hours. After that, the ABTS solution was diluted with methanol to achieve a 734 nm absorbance of 0.700. At 30 minutes after the initial mixing, the absorbance of 3.900 ml of diluted ABTS solution was measured following the addition of 100 µl of plant extracts with a different concentration in mg/mL (100, 50, 25, 12.5, 6.25) of *Spermacocea ocymoides* and *Spermacocea pusilla*. In each assay, a suitable solvent blank was used. At least three O.D. measurements were taken on each one. Trolox was used as a standard reference drug for comparison.

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{Ab control} - \text{Ab sample})}{(\text{Ab control})} \times 100$$

Where Ab control is the absorbance of ABTS radical + methanol
Ab sample is the absorbance of ABTS radical + crude extracts / standard

Phosphomolybdenum assay

The total antioxidant capacity of the extracts. An aliquot of 0.5 mL of plant root crude extracts was added with 4.5 mL of the reaction mixture (28 mm sodium phosphate, 0.6 M sulfuric acid, and 4 mM ammonium molybdate). For blank, 0.5 mL of 45% ethanol was used instead of plant extracts. After that, 90 minutes, the tubes were placed in a 95 °C boiling water bath. The UV-2450 spectrophotometer (Shimadzu, Japan) was used to measure the absorbance of the plant extracts in comparison to the blank at 695 nm after the samples had been cooled to room temperature. The absorbance of the sample at 695 nm was used to represent the total antioxidant activity. Higher antioxidant activity was indicated by the higher absorbance value. The standard reference drug was ascorbic acid. The following equation was used to estimate antioxidant capacity:

$$\text{Antioxidant activity (\%)} = \frac{(\text{Absorbance control} - \text{Absorbance extract})}{(\text{Absorbance control})} \times 100$$

Statistical analysis

The mean ± standard deviation (SD) of the three replicates represented the experimental outcomes. The regression plots provided the Effective Concentration (EC_{50}) values, or the concentration at which 50% inhibition was achieved.

Antibacterial activity

Preparation of test solution

The test solution was prepared with a known weight of extracts dissolved in 5% Dimethyl sulphoxide (DMSO). The solution was freshly prepared before the antibacterial experiments.

Agar well diffusion method

The test bacterial cultures were swabbed over the solid Muller Hinton Agar plates. A well of 6mm diameter was punched into the agar medium and filled with 100 microlitres of root extract of *Spermacocea pusilla* and *Spermacocea ocymoides* (25 µl, 50 µl, and 75 µl in 10% DMSO of different concentrations were used. Chloramphenicol (5 µl) was used as the positive control and 10% dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated in an upright position at 37°C for overnight in an incubator. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms [39].

RESULTS AND DISCUSSION

Phytochemical constituents

Plants continue to be crucial to medicine production and play a significant role in healthcare. According to estimates, many people in many developing countries rely heavily on traditional medicines and healers to take care of their primary medical needs. Phytochemical screening of *Spermacocea ocymoides* root and *Spermacocea pusilla* root ethanolic crude extract shows the presence of various bioactive compounds such as alkaloids, flavonoids, saponins and triterpenoids.

Table 1 Presence of secondary compounds

S. No.	Chemical constituent	Tests	*Spermacocea ocymoides*				*Spermacocea pusilla*			
			Organic solvents			W	Organic solvents			W
			PE	EA	E		PE	EA	E	
1	Alkaloids	a. Dragendorff's test	-	-	+	-	-	-	-	-
		b. Mayer's test	-	-	-	+	-	-	-	-
		c. Wagners test	-	-	-	-	-	-	+	-
		d. Hagers test	-	-	+	+	-	-	-	-
2	Flavonoids	10% Hcl and 5% Naoh test	-	-	-	-	-	-	-	-
3	Tannins	5% $Fecl_3$ test	-	-	+	-	-	-	-	++
4	Steroids	Liebermann - Burchard's test	-	+	++	-	-	-	++	-

5	Triterpenoids	a. Liebermann - Burchard's test	-	-	+++	++	+	-	++	++
		b. Salkowskis test	-	-	++	+	-	-	-	-
6	Saponins	Foam test	-	-	-	+	-	-	-	-
7	Glycosides	Keller - Kiliani test	-	-	-	-	-	-	-	-
8	Gum and Mucilages	Whistler and Bemiller test	+	-	-	-	-	-	-	-
9	Fixed Oils	Spot test	-	+	-	-	-	-	+	-
10	Anthraquinones	Sanker and Nahar test	-	-	-	-	-	-	-	-

PE- Petroleum Ether; **EA-** Ethyl Acetate; **E-** Ethanol; **W-** Water

+ Indicates the presence of compounds, - Indicates the absence of compounds

In present study the ethanolic extract of *Spermacocea pusilla* root and *Spermacocea ocymoides* root extracts showing the occurrence of various secondary metabolites.

In vitro antioxidant activities

DPPH radical scavenging activity

The ability of the ethanol extract of root samples to scavenge free radicals was assessed using DPPH radical as substrate. The DPPH radical scavenging activity of the *S. pusilla root* IC_{50} was 27.11 and *S. ocymoides root* IC_{50} was 30.62 at 120 µg/mL concentration**.** Thus, the *S. pusilla* root extract showed good radical scavenging activity then *S. ocymoides* and it was compared with the standard rutin (IC_{50} = 7.73 µg/mL concentration) and BHT (IC_{50} = 6.87 µg/mL concentration).

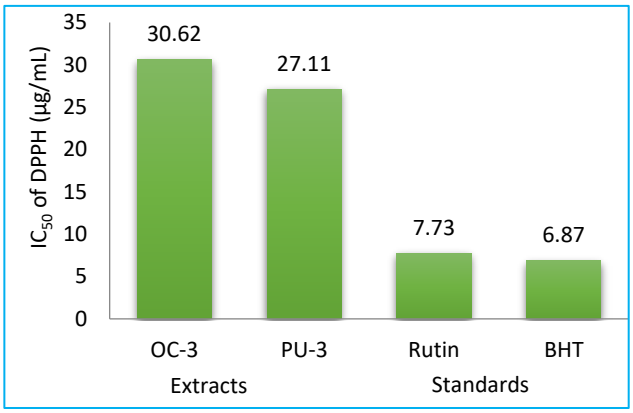


Fig 1 DPPH assay of *ocymoides root* and *pusilla root*

ABTS radical scavenging activity

The ABTS radical was quickly and effectively eliminated by the extracts. The standard drug Trolox has been compared to its antioxidant capacity to scavenge the radical ABTS.

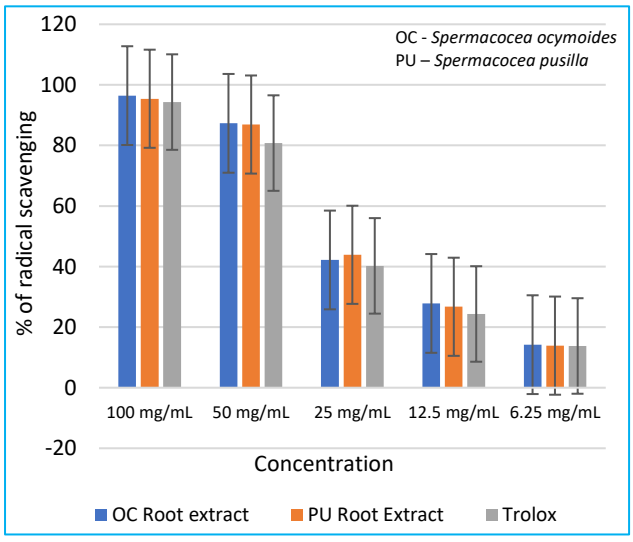


Fig 2 *ABTS radical scavenging activity*

Utilizing potassium persulfate, the stable form of the ABTS radical cation was produced. Subsequent to getting the steady absorbance, the plant extracts are added to the reaction mixture and the anti-oxidant activity was estimated by concentrating on decolourization ability. An important property of antioxidants is their ability to scavenge proton radicals. Protonated radical ABTS has characteristic absorbance maxima at 734 nm that decrease with proton radical scavenging. The root crude extracts from *Spermacocea ocymoides* and *Spermacocea pusilla* were fast and effective scavengers of ABTS radical shown in.

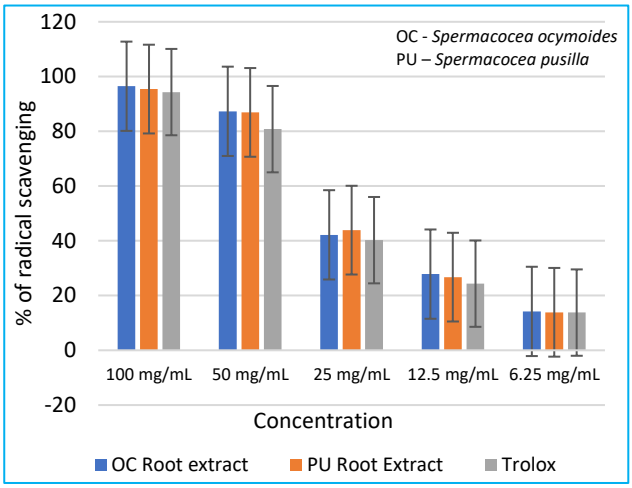


Fig 3 Root crude extracts from *Spermacocea ocymoides* and *Spermacocea pusilla*

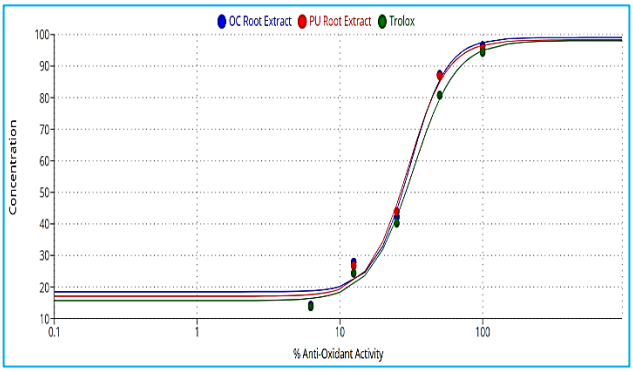


Fig 3 EC50 of plant extracts and trolox standard in ABTS radical scavenging assays

This activity is comparable with that of trolox, the standard antioxidant used in this study. The percentage inhibition was 96.45% in ethanol extract of *Spermacocea ocymoides* and 95.42% in ethanol extract of *Spermacocea pusilla* and 94.32% inhibition exhibited by trolox respectively at 100 mg/mL, the highest concentrations tested. The plant roots extracts were more effective at neutralizing the system free radicals at higher concentrations (100 mg/mL). The strong correlation between the extracts ABTS radical scavenging capacity and standard trolox.

Table 2 EC50 values and ABTS radical scavenging activity

Ethanolic root extracts	EC50 values in (mg/mL concentration)
Spermacocea ocymoides	31.19
Spermacocea pusilla	30.06
Trolox standard	32.51

Phosphomolybdenum assay

The phosphomolybdate method has frequently been utilized to study plant extracts total antioxidant capacity. Mo (VI) is reduced to Mo (V) in the presence of plant extracts, resulting in a green-coloured Phosphomolybdenum V complex with a maximum absorbance at 695 nm. (Fig 3) shows the anti-oxidant activity of ethanolic root extracts of *Spermacocea ocymoides* and *Spermacocea pusilla.* The antioxidant activity of almost both the plant crude root extracts was not significantly different. *Spermacocea ocymoides* and *Spermacocea pusilla* ethanolic crude root extracts showed effective concentration of antioxidant capacity i.e. EC 50 value.

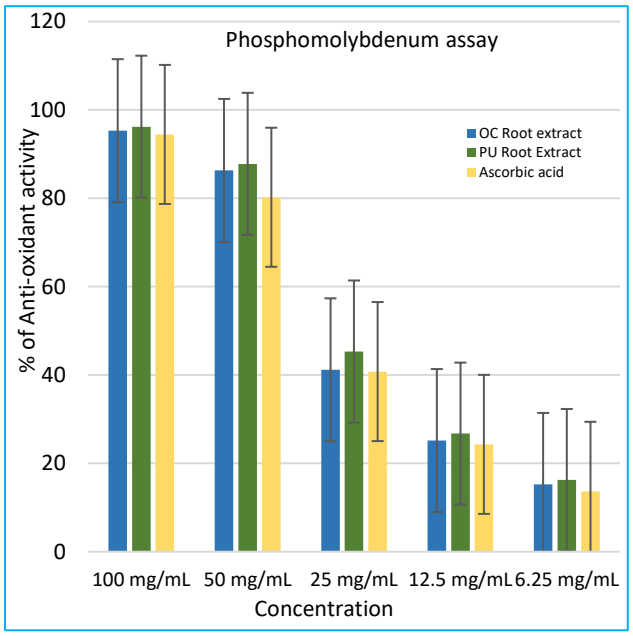


Fig 4 Phosphomolybdate method of the root extracts from *S. ocymoides* and *S. pusilla*

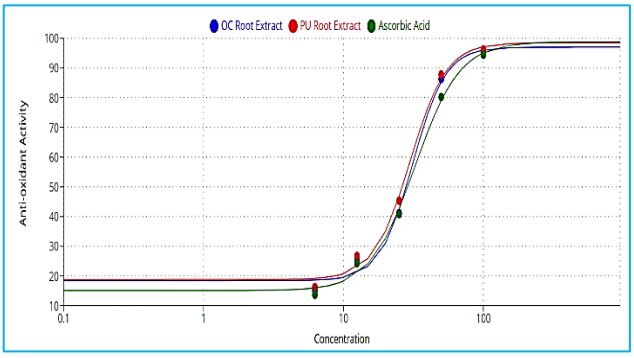


Fig 5 EC50 of plant extracts and Trolox standard anti-oxidant activity by phosphomolybdate method

Table 3 EC50 values phosphomolybdate method of *S. ocymoides* and *S. pusilla*

Ethanolic root extracts	EC50 values in (mg/mL concentration)
Spermacocea ocymoides	31.26
Spermacocea pusilla	29.99
Ascorbic acid standard	32.51

Most medicinal plants have a number of different pharmacologically active compounds that can work together, individually, or in combination to improve health. More than 100 human diseases are caused by free radicals in the body, including atherosclerosis, arthritis, ischemia and reperfusion injury to many tissues, injury to the central nervous system, gastritis, cancer, and AIDS. The therapeutic potentials of medicinal plants as antioxidants in reducing tissue damage caused by free radicals have recently received increased attention. In addition, in the search for novel antioxidant from plant species have been investigated. There is still a need to learn more about the antioxidant capabilities of plant species.

Antibacterial activity

Spermacocea pusilla root

The ethanol crude extract of *Spermacocea pusilla* root was tested for antibacterial activity against the chosen bacterial pathogens on MHA plates. The antibacterial activity of ethanol crude extract of *Spermacoce pusilla* root showed effective growth inhibitory activity against all bacterial pathogens tested.

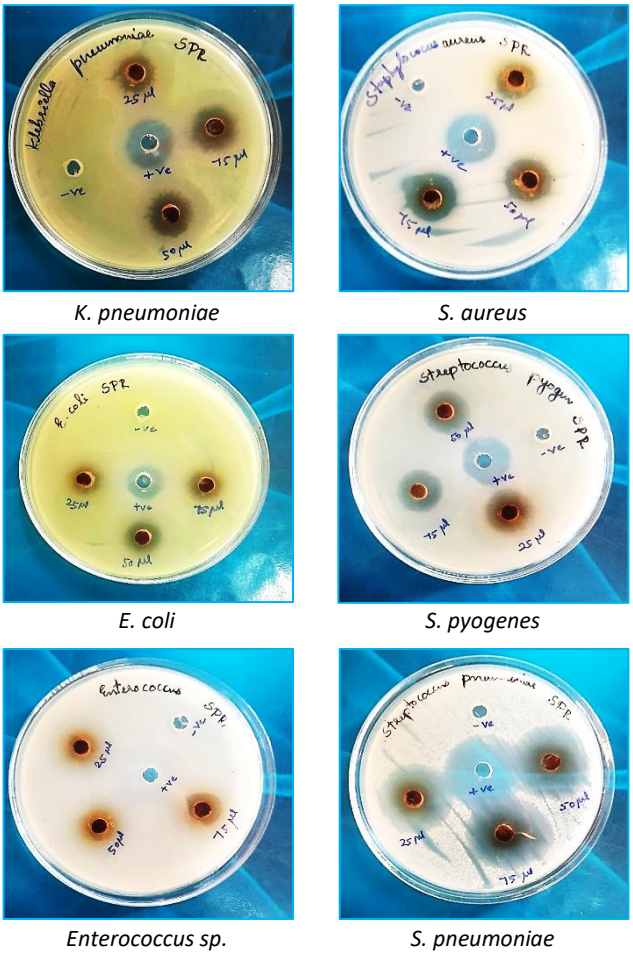
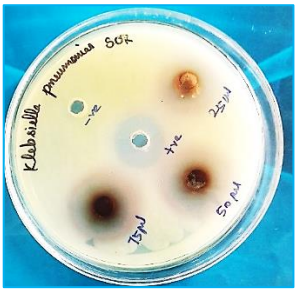


Fig 6 Antibacterial activity of *S. pusilla* root

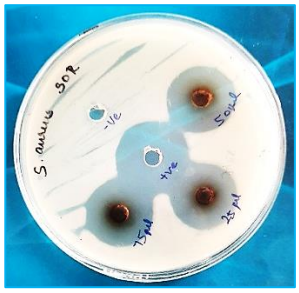
The zone of inhibition varied greatly depending on the concentration utilized. The ethanol root extract effectively inhibited the *Streptococcus pneumoniae* (15.06±0.66) with the zone of inhibition followed by *Streptococcus pyogenes* (11.05±0.24) and *Klebsiella pneumoniae* (11.25±0.44). Compared to other bacterial pathogens, the ethanol crude extract of *Spermacocea pusilla* root showed less inhibitory efficacy against *Staphylococcus aureus* (9.15±0.29), *Escherichia coli* (9.05±0.64) and *Enterococcus sp.*, (9.25±0.24). The standard antibiotic Chloramphenicol recorded zone of inhibition ranged from 21 to 25 mm.

Table 4 Antibacterial activity of *Spermacocea pusilla* root

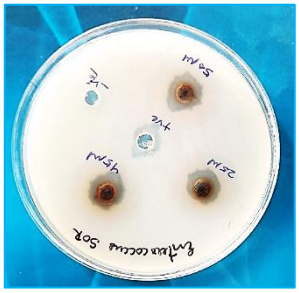
Test bacterial pathogens	Zone of inhibition (mm)			
	25 µl	50 µl	75 µl	Chloramphenicol (5 µl)
K. pneumoniae	9.34±0.48	11.36±0.76	11.25±0.44	25.08±0.71
S. aureus	7.12±0.48	8.54±0.04	9.15±0.29	20.55±0.04
E. coli	7.24±0.20	8.58±0.09	9.05±0.64	22.95±0.51
S. pyogenes	9.26±0.32	10.25±0.76	11.05±0.24	23.04±0.64
Enterococcus sp.	7.25±0.64	8.36±0.55	9.25±0.24	21.43±0.33
S. pneumoniae	9.25±0.34	10.25±0.54	15.06±0.66	24.47±0.62



K. pneumoniae



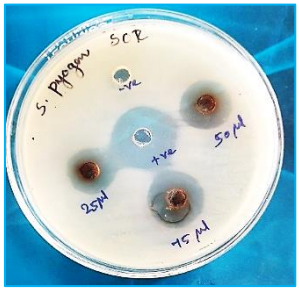
S. aureus



Enterococcus sp.

S. pneumoniae

E. coli



S. pyogenes

Fig 7 Antibacterial activity of *Spermacocea ocymoides root*

Spermacoce ocymoides root

The ethanol crude extract of *Spermacocea ocymoides* root was tested for antibacterial activity against the hosen bacterial pathogens on MHA plates. The antibacterial activity of ethanol crude extract of *Spermacocea ocymoides* root showed effective growth inhibitory activity against all bacterial pathogens tested. The zone of inhibition varied greatly depending on the concentration utilized.

Table 5 Antibacterial activity of *Spermacocea ocymoides* root

Test bacterial pathogens	Zone of inhibition (mm)			
	25 µl	50 µl	75 µl	Chloramphenicol (5 µl)
K. pneumoniae	13.82±0.04	14.08±0.78	11.26±0.34	25.08±0.71
S. aureus	10.17±0.18	13.34±0.25	15.16±0.06	20.55±0.04
E. coli	8.66±0.09	10.05±0.04	13.05±0.04	22.95±0.51
S. pyogenes	8.12±0.18	11.24±0.17	12.09±0.09	23.04±0.64
Enterococcus sp	7.10±0.08	9.00±0.14	10.36±0.26	21.43±0.33
S. pneumoniae	9.02±0.04	10.17±0.78	15.68±0.34	24.47±0.62

The ethanol root extract effectively inhibited the *Streptococcus pneumoniae* (15.68±0.34) with the zone of inhibition followed by *Staphylococcus aureus* (15.16±0.06) and *Escherichia coli* (13.05±0.04). Compared to other bacterial pathogens, the ethanol root extract of *Spermacocea ocymoides* root showed less inhibitory efficacy against *Klebsiella pneumoniae* (11.26±0.34), *Streptococcus pyogenes* (12.09±0.09) and *Enterococcus sp.*, (10.36±0.26). The standard antibiotic Chloramphenicol recorded zone of inhibition ranged from 21 to 25 mm.

CONCLUSION

The result demonstrates the highest zone of inhibition suggesting high antibacterial activity of ethanol extract of *Spermacocea pusilla* root and *Spermacocea ocymoides* root against *Streptococcus pneumoniae* found under noticing active compounds, which are responsible for antibacterial activity are abundant in ethanol extract. These findings implies that the ethanol root extract have effectively inhibits pathogenic bacterial growth. Also *S. aureus*, the most common pathogen to cause skin infections was inhibited in low dose. The results suggest that usage of larger doses can overcome this problem. Further the study observed that the dose employed and the types of bacterial strains used can be factors which influence the inhibitory effect.

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