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Suman Joshi DSD
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Venkata Rao G
Department of Chemistry, SRR & CVR Govt. College, Vijayawada, Andhra Pradesh, India

Satya Prasad M
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Kishore Babu M
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Surya Narayana S
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Krishna Satya A
Assistant Professor, Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Correspondence
Krishna Satya A
Assistant Professor, Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510, India

Phytochemical screening and evaluation of antioxidant, antibacterial and antifungal activity of medicinal plant *Alphonsea sclerocarpa* Thaw

Suman Joshi DSD, Venkata Rao G, Satya Prasad M, Kishore Babu M, Surya Narayana S and Krishna Satya A

Abstract
Alphonsea sclerocarpa Thaw is a medicinal plant of Annonaceae family widely distributed in South India and Sri Lanka. With a view to study its potential medicinal properties the present work was initiated to evaluate phytochemical screening, antioxidant activity through scavenging of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and antibacterial and antifungal activities of Hexane, Chloroform, Ethyl acetate, Methanol and aqueous extracts of leaves from *Alphonsea sclerocarpa* Thaw. The phytochemicals like Alkaloids, Flavonoids, Saponins, Steroids and Tannins were identified in Ethyl acetate and Methanolic fractions. The Methanol and Ethyl acetate fractions showed high antioxidant activity compared to control Ascorbic acid. The Ethyl Acetate fraction showed high antibacterial activity than other solvents. The results revealed the leaf extract of *Alphonsea sclerocarpa* Thw. showed notable antibacterial and antifungal activity so as can be used as medicine.

Keywords: *Alphonsea sclerocarpa,* phytochemicals, antioxidant, antibacterial activity, antifungal activity

Introduction
Reactive Oxygen Species (ROS) viz., free radicals are involved in structural alterations of cellular molecules leading to cyto toxicity and cell death. A variety of biological phenomena such as ageing, inflammation, mutation, carcinogenesis, ischemia reperfusion injury, atherosclerosis, and neurodegenerative disorders etc. (Young and Woodside 2001) [11] are related to ROS and free radical generation. The free radicals are anticipated by antioxidants by nullifying them and protecting the cell. Plants are the richest source of antioxidants and other phytochemical constituents such as flavonoids and phenols which scavenge the free radicals. Since ancient time plants have proven medicinal properties with capabilities of curing various ailments to humans caused by either free radicals, nutritional defects or even by bacterial, viral, fungal infections. A large number of plants were screened for their potential therapeutic capable novel compounds. The search is still ongoing to explore more. It is evident from the past research that the therapeutic capabilities of plants are mainly due to presence of phytochemicals which are mainly subdivided as primary and secondary metabolites (Nostro A *et al.,* 2000) [8]. The proteins, carbohydrates, fats and chlorophyll are considered as primary metabolites while Alkaloids, Flavonoids, polyphenols, Terpenoids, Tannins, Saponins and Steroids etc., are considered as secondary metabolites. Alkaloids are the class of phytochemicals with nitrogenous organic skeleton and widely distributed with diverse medicinal functions. Flavonoids are the polyphenolic compounds with 15 carbon atoms, water soluble and commonly present in plants. The steroids are the organic compounds with four fused ring structure found in plants. The secondary metabolites are globally used as medicines either in pure form or in combination with other base materials in various medical practices such as Siddha, Unani and Ayurveda as these metabolites confers the side effects free and safe to use for all peoples of all age groups. All parts of the plants i.e., roots, stem, bark, leaves flowers and fruits are richest source of metabolites and some metabolites are present in all parts of the plant while some are confined to particular location. In the present study medicinal plant from Annonaceae family which is *Alphonsea sclerocarpa* Thaw was selected to study for screening various phytochemicals present and to explore its antioxidant, antibacterial and antifungal activities. Free radical scavenging activity was evaluated using 1,1-diphenyl-2-picryl hydrazyl (DPPH). *Alphonsea sclerocarpa* is commonly called as hard fruit *Alphonsea.* The plant grows rapidly and distributed widely in tropical areas especially in South India and Sri Lanka. It is a small tree, grows up to 6 m tall, leaves are simple, alternate and distichous.

As the plant is less explored, it was selected to screen for various phytochemicals present in its leaves and to identify its potential antibacterial and antifungal activities. The plant image was represented in image-1.

Image-1 *Alphonsea sclerocarpa* Thaw

Materials and Methods

Plant collection: The *Alphonsea sclerocarpa* Thaw was collected from the Seshachalam forest region of Andhra Pradesh, authenticated by a taxonomist and a voucher specimen was deposited at herbarium of Sri Venkateswara University, Tirupati with voucher No. 871. The collected leaves were washed with double distilled water to remove dirt, shade dried for a couple of weeks, crushed and sieved with mesh size No.22, labeled as ASCRPA and stored in a cool, dry place until further use.

Extraction of phytochemicals: The dried leaves powder of 40 gms weighed and packed in soxhlet extractor and subjected to successive extraction with different solvents such as Hexane, Chloroform, Ethyl acetate, Methanol and water (Aqueous). Each extract was collected, Rotary evaporated and stored for further use with proper labeling.

Phytochemical screening

To carry out the different phytochemical screenings required reagents such as Benedicts reagent, Dragendorff's reagent, Fehling's solution A& B, Liebermann-Burchard reagent, Mayer's reagent and Molisch reagent were prepared as per standard procedures and protocols.

Test for flavonoids

The presence of flavonoids was tested using different tests such as a) Ferric chloride Test, b) Shinoda's test c) Sodium hydroxide test and d) Leadacetate test.

a) Ferric chloride Test

2mL of test solution was boiled with distilled water and filtered followed by addition of few drops of 10% ferric chloride solution. A greenish-blue or violet coloration indicates the presence of a phenolic hydroxyl group (Trease GE & Evans WC 2002) [1].

b) Shinoda's test

5g of each extract was dissolved in ethanol, warmed and then filtered. Small pieces of magnesium chips were then added to the filtrate followed by few drops of conc. HCl. The pink, orange, or red to purple coloration indicates the presence of flavonoids (Trease GE & Evans WC 2002) [1].

c) Sodium hydroxide test

0.2g of extract was dissolved in distilled water and filtered. To this, 2 mL of 10% aqueous sodium hydroxide solution was added to produce yellow coloration. A change in color from yellow to colorless on addition of dilute hydrochloric acid was the indication for the presence of flavonoids (Trease GE & Evans WC 2002) [1].

d) Lead acetate test

0.5g of extract was dissolved in distilled water and filtered. To the filtrate 3 mL of lead acetate solution was added and mixed well. Appearance of a buff-colored precipitate indicates the presence of flavonoids (Trease GE & Evans WC 2002).

Test for alkaloids

The presence of alkaloids was tested with a) Dragendorff's reagent test and b) Mayer's reagent test.

a) Dragendorff's reagent test

5g of crude extract was stirred with 1% aqueous HCl on water bath at 60 °C and then filtered. To the 1 mL of filtrate, few drops of Dragendorff's reagent was added. Orange- Red precipitate was taken as positive (Trease GE & Evans WC 2002) [1].

b) Mayer's reagent test

To 1 mL of filtrate, few drops of Mayer's reagent were added and appearance of buff- colored precipitate will be taken as presence of alkaloids (Brain, K.R., & Turner, T.D., 1975) [2].

Test for soluble starch

0.2g of extract was boiled in 1 mL of 5% KOH, cooled and acidified with H_2SO_4. Yellow coloration indicates the presence of soluble starch (Vishnoi, N.R., 1979) [3].

Test for Saponins

Saponins are the amphipathic glycosides with foaming characteristics. The presence of Saponins was tested by using Frothing test.

Frothing test

0.5g of extract was shaken with water in a test tube and it warmed in a water bath. The persistent froth indicates the presence of saponins (Sofowora, A., 1993) [4].

Test for terpenoids

Terpenoids or isoprenoids are the largest class of small organic molecules present plants with diverse functions. Terpenoids are derived from terpenes after structural modification.

5g of crude extract was dissolved in ethanol. To this, 1 mL of acetic acid was added followed by conc. H_2SO_4. A change in color from pink to greenish confirms the presence of terpenoids (Sofowora, A., 1993) [4].

Test for steroids

The presence of steroids was tested with four tests such as a) Salkowski test, b) Keller-Killiani test and c) Liebermann-Burchard test.

a) Salkowski test

0.2g of extract was dissolved in 2 mL of chloroform and added the conc. H_2SO_4. The development of reddish brown color at inter phase indicates the presence of steroids (Rim jhim Sheel *et al*., 2014) [5].

b) Keller-Killiani test
0.5mL of test solution was mixed with 2 mL of 3.5% $FeCl_3$, 2 mL of conc. H_2SO_4 and small amount of glacial acetic acid carefully. Appearance of reddish brown ring at inter phase is a positive indication for the presence of steroids.

c) Liebermann-Burchard test
0.2g of extract was mixed with 2 mL of acetic acid, cooled well in ice followed by the addition of conc.H_2SO_4 carefully. Color development from violet to blue or bluish- green indicates the presence of a steroidal ring (i.e. aglycone portion of cardiac glycoside).

Test for carbohydrates
Molisch's test
Extract was dissolved in distilled water and added with 2 mL of Molischs reagent and 1 mL of conc. H_2SO_4 was dispensed along the walls of the test tube. The mixture was allowed to stand for two minutes and then diluted with 5 mL of distilled water. Formation of a dull violet color at the inter phase of the two layers indicates the positive test for carbohydrates.

Fehling's test (for free reducing sugars)
The crude extracts were treated with 5 mL of Fehling's solution (A & B) and kept in boiling water bath. The formation of yellow or red color precipitate indicates the presence of free reducing sugars.

Fehling's test (for Combined Reducing Sugars)
0.5 g of Extract was hydrolyzed by boiling with 5 ml of dilute hydrochloric acid and the resulting solution neutralized with sodium hydroxide solution. To this, few drops of Fehling's solution were added and then heated on a water bath for 2 minutes at 60 °C. Appearance of a reddish-brown precipitate of cuprous oxide indicates the presence of combined reducing sugars.

Barfoed's test (for monosaccharide)
0.5g of the extract was dissolved in distilled water and filtered. To 1 mL of the filtrate, 1 mL of Barfoed's reagent was added and then heated on a water bath at 60 °C for 2 minutes. Reddish precipitate of cuprous oxide formation is the positive test for the presence of monosaccharides.

Anti-bacterial activity
Antibacterial activity was analyzed by agar well diffusion method. The antibacterial activity was carried out using 4 species of gram positive bacteria such as (1) *Bacillus subtilis* (MTCC No. 10407), (2) *Lactobacillus acidophilus* (MTCC No.10307) (3) *Staphylococcus aureus* (MTCC No. 6908) and (4) *Streptococcus mutans* (MTCC No. 890). Similarly, four species of gram negative bacteria include (1) *Escherichia coli* (MTCC No. 44), (2) *Pseudomonas aeruginosa* (MTCC No. 1034), (3) *Klebsiella pneumonia* (MTCC No. 9024) and (4) *Proteus vulgaris* (MTCC No. 744). All the bacterial cultures were procured from IMTECH, Chandigarh. Nutrient agar (NA) media was prepared, sterilized and poured into the petri plates and allowed it to solidify. 24 hrs cultures were spread on the plates with the help of L shaped rod and wells were made by using 6 mm cork borer on each plate. The wells were filled with 100 µL of 50 mg concentrated each extract separately. DMSO alone was used as control. The plates were incubated in an incubator at 37 °C. After 24 hours of incubation, each plate was examined for inhibition zones. Triplicates were maintained and mean values were recorded as zone of inhibition in mm.

Antifungal activity
The antifungal activity was carried out by using different fungal strains such as (1) *Aspergillus brasiliensis,* (2) *Aspergillus flavus,* (3) *Aspergillus niger* (MTCC No. 9687), (4) *Aspergillus solani,* and (5) *Candida albicans* (MTCC No. 4748). Into the sterile Petri plates, 1 mL of the fungal suspension was taken followed by addition of molten state potato dextrose agar medium and mixed well. After complete solidification, wells were bored with sterile cork borer of 6 mm diameter followed by filling with 100 µL of 50 mg concentrated extracts dissolved in DMSO. The drug Fluconazole with a concentration of 10 µg/mL was used as the standard antifungal agent (positive control). The petri plates were incubated at ~37 ^oC for 72 h.

***In vitro* antioxidant activity**
The antioxidant activity of the extracts was determined using DPPH. For this purpose 100 µg/ml concentration of extracts was dissolved in DMSO followed by addition of 4 ml of the 0.004% (w/v) DPPH (1,1-diphenyl-2-picrylhydrazyl) dissolved in methanol. The reaction mixture was kept for incubation in dark for 30 minutes. Ascorbic acid was used as standard. The absorbance was measured at 517 nm using Thermo scientific UV-Visible spectrophotometer. The DPPH scavenging activity (%) was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = [(A0-As)/A0] \text{ X } 100$$

Where, A_0 is the absorbance of the control, As is the absorbance of the plant sample

Results and discussion
The medicinal plant *Alphonsea sclerocarpa* was screened for presences of different phytochemicals are results were represented in Table No.1. Alkaloids and flavonoids are present in all the extracts where as other phytochemicals screened were confined to one or few extracts. The phytochemical results revealed that the plant is the richest source of various phytochemicals and there is no evidence for presence of terpenoids in either the Hexane, Chloroform, Ethyl acetate, Methanol and water extracts.

S. No	Phytochemical screened	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Aqueous extract
1	**Alkaloids**	+	+	+	+	+
2	**Carbohydrates**	--	--	--	--	+
3	**Flavonoids**	+	+	+	+	+
4	**Saponins**	--	--	+	--	+
5	**Soluble starch**	--	--	--	--	--
6	**Steroids**	+	+	+	--	--
7	**Tannins**	--	--	+	--	+
8	**Terpenoids**	--	--	--	--	--

Table-1. Phytochemical analysis of *Alphonsea sclerocarpa* different extracts

The result of antibacterial activity was represented in Image-2, Table-2 and graph-1. Out of four gram positive and gram negative bacteria screened all the bacteria showed good inhibitory response with Hexane, Chloroform, Ethyl acetate and Methanol fractions. Antibacterial activity was not tested with aqueous extract. Image-2 represents the petriplates with zone of inhibition. Table-2 gives the data on comparative zone of inhibition with standard drug. Ethyl acetate fraction showed maximum antibacterial activity followed by Methanol extract.

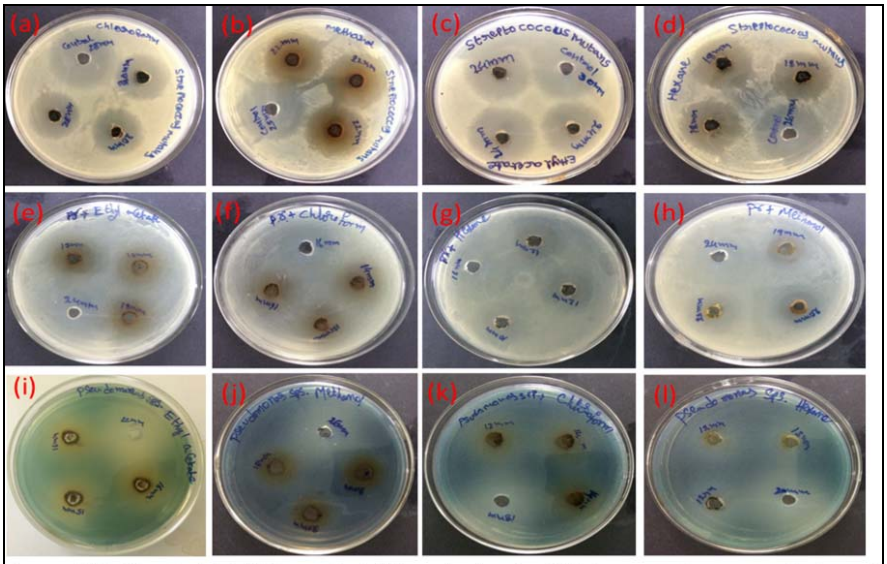
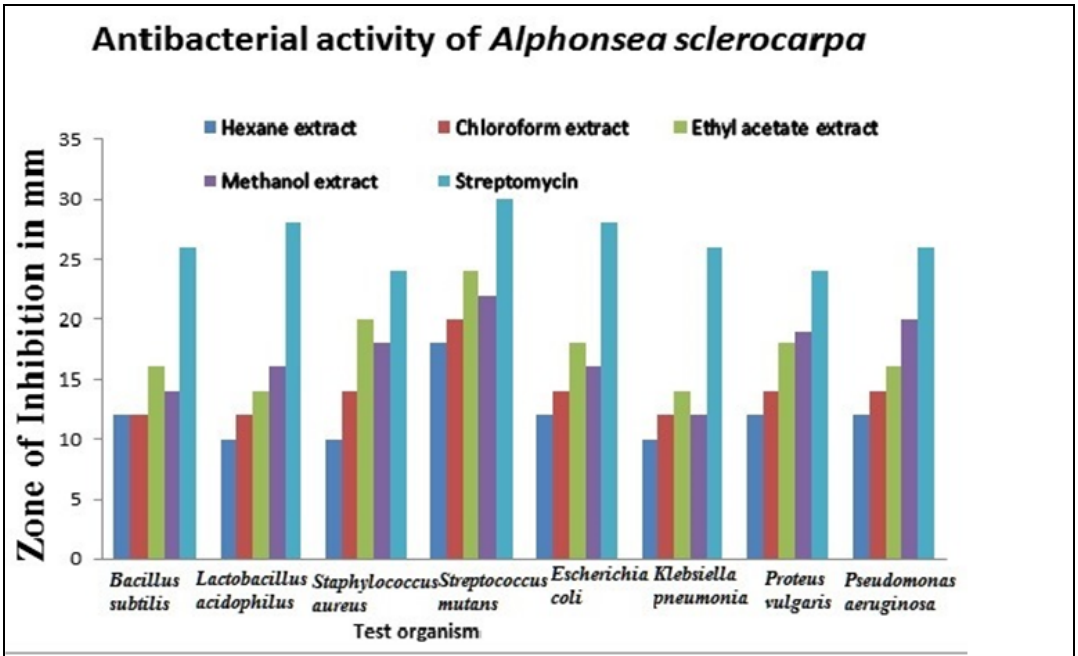


Image-2 Antibacterial Activity against different extracts of *Alphonsea sclerocarpa* - (a, b, c, d) *Streptococcus mutans*, (e, f, g, h) *Proteus vulgaris*, (i, j, k, l) *Pseudomonas aeruginosa*

S. No	Name of the culture	Zone of inhibition in mm				
		Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Control (Streptomycin)
1	***Bacillus subtilis***	12	12	16	14	26
2	***Lactobacillus acidophilus***	10	12	14	16	28
3	***Staphylococcus aureus***	10	14	20	18	24
4	***Streptococcus mutans***	18	20	24	22	30
5	***Escherichia coli***	12	14	18	16	28
6	***Klebsiella pneumonia***	10	12	14	12	26
7	***Proteus vulgaris***	12	14	18	19	24
8	***Pseudomonas aeruginosa***	12	14	16	20	26

Table:2 Antibacterial activity of different extracts and comparison with standard drug



Graph-1. Antibacterial activity with different extracts and comparison with standard

The antifungal activity of *Alphonsea sclerocarpa* was tested with different fungi and among all tested fungi maximum inhibition was shown to *Aspergillus brasiliensis*. Among the different extracts tested and compared with standard drug Fluconazole Ethyl acetate fraction showed more inhibition than other solvents.

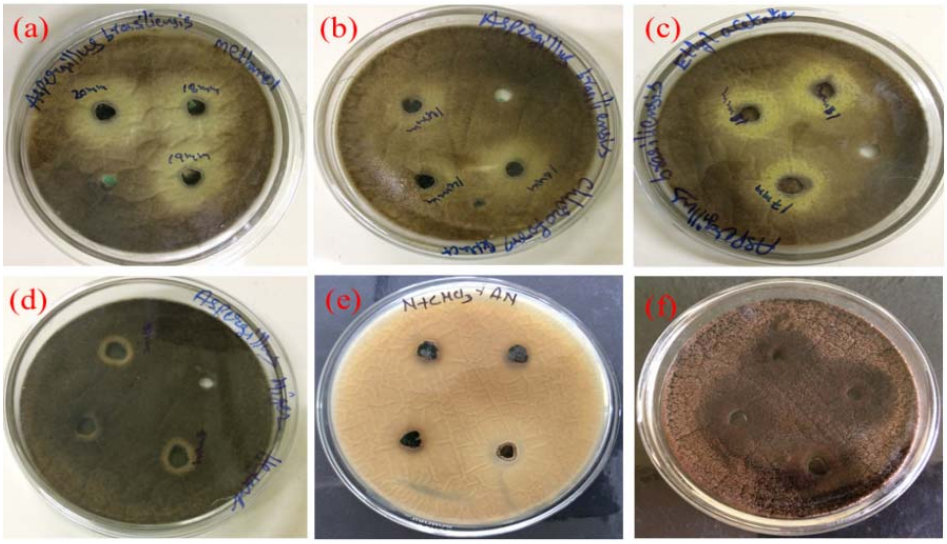
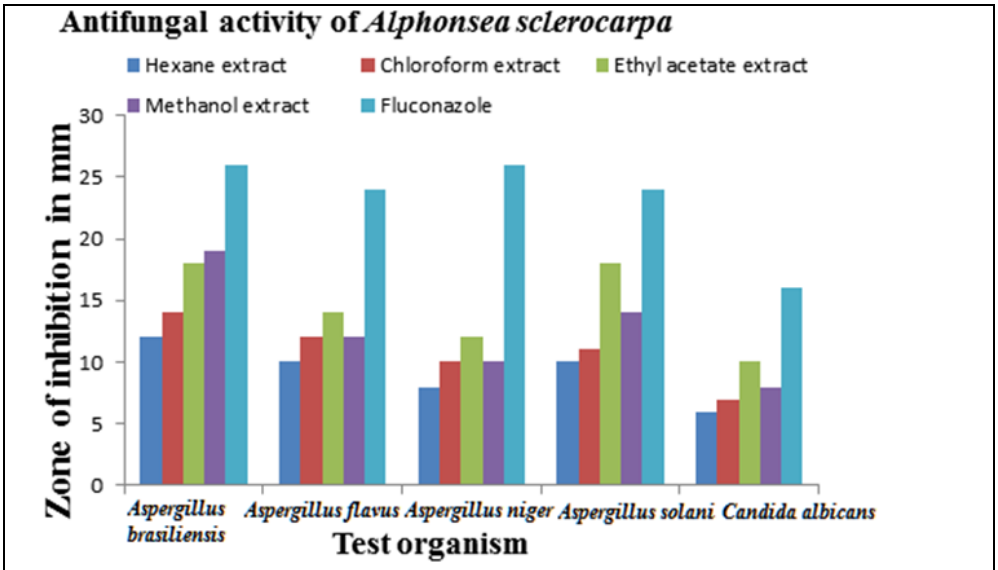


Image-3 Antifungal activity against different extracts of *Alphonsea sclerocarpa* (a, b, c,) *Aspergillus brasiliensis,* (d) *Aspergillus niger,* (e) *Candida albicans* (f) *Aspergillus solani*

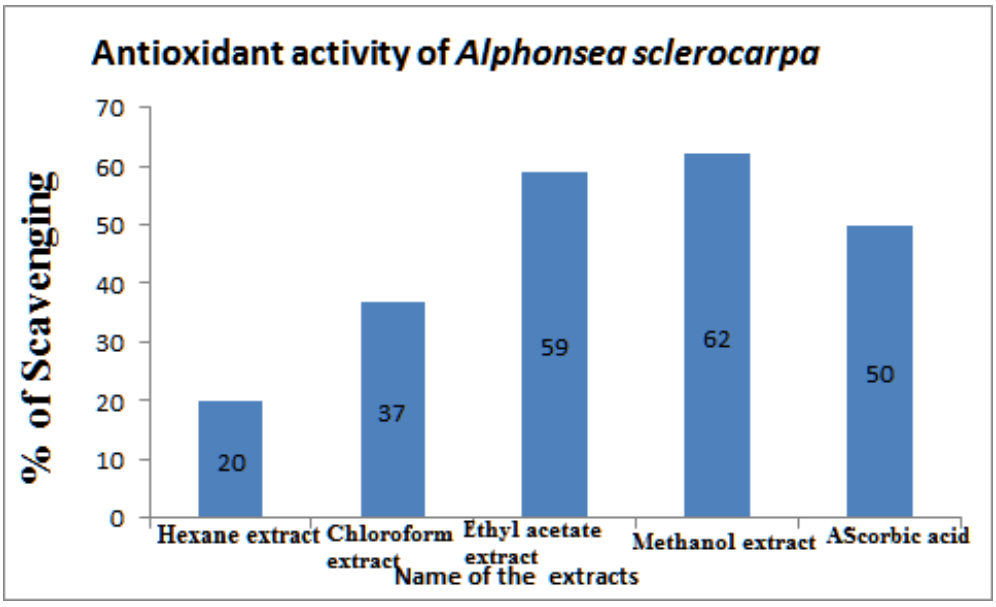
S. No	Name of the culture	Zone of inhibition in mm				
		Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Control (Fluconazole)
1	*Aspergillus brasiliensis*	**12**	**14**	**18**	**19**	**26**
2	*Aspergillus flavus*	**10**	**12**	**14**	**12**	**24**
3	*Aspergillus niger*	**8**	**10**	**12**	**10**	**26**
4	*Aspergillus solani*	**10**	**11**	**18**	**14**	**24**
5	*Candida albicans*	**6**	**7**	**10**	**8**	**16**

Table:3- Antifungal activity of different extracts and comparison with standard drug



Graph-2. Antifungal activity of *Alphonsea sclerocarpa* and comparison with standard

The results of Antioxidant activity was represented in Graph -3. The Methanol extract showed highest antioxidant activity followed by ethyl acetate fraction.



Conclusion

Now a day there is search for potential alternative sources to curing diseases with efficient and side effects free nature, plants are becoming sole source with wide number of secondary metabolites capable of curing various ailments especially related to bacteria and fungi (Munoz-Mingarro NAD *et al.,* 2003, Coelho de Souza *et al.,* 2004) [6, 7]. From our research it is evident that the plant *Alphonsea sclerocarpa* has potential phytochemicals with notable antifungal and antibacterial activities. There has been an upsurge of interest in the therapeutic potential medicinal plants as antioxidants in reducing oxidative stress-induced tissue injury (Pourmorad *et al.,* 2006) [12]. So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts (Cook and Samman, 1996) [9]. The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties (Rice-Evans *et al.,* 1997) [10]. Thus from the above research it is evident that the phytochemicals present in medicinal plant *Alphonsea sclerocarpa* Thaw have potential phytochemicals with great medicinal applications. This is giving scope to further studies on isolation and characterization of phytochemicals to get potential drugs from plants.

Conflict of interest: The authors declare conflict of interest as none

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