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A STUDY ON THE ANTIBACTERIAL EFFECTS OF THE VARIOUS EXTRACTS OF *EXCOECARIA AGALLOCHA* L.

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ABSTRACT

The antibacterial activity of the root, stem and leaf extracts of *Excoecaria agallocha* L. was tested against the test organisms Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella spp., Shigella sonnei and Staphylococcus aureus. The activity was tested both by agar well diffusion and disc diffusion methods. It was found that the extracts were very effective in controlling the growth of all the organisms tested.

KEYWORDS: ***Excoecaria agallocha,*** **mangrove, antibacterial activity,** ***Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella spp., Shigella sonnei*** **and** ***Staphylococcus aureus.***

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INTRODUCTION

Bacteria form a diverse group of organisms found unanimously on earth. Some are beneficial whereas others are dangerous,causing severe disease conditions in plants and animals including man. Crop plants get severely affected by bacterial diseases like blights, soft rots, leaf spots, tumors and galls. They are a threat to mankind as they have a tremendous effect on the ecological balance and impose constraints on the cultivation, production and sales of crop plants. [1,2] They are extremely dangerous to humankind as they trigger highly contagious diseases like meningitis, typhoid, tuberculosis, anthrax, pneumonia, *etc*. They are highly infectious as many of the diseases that they cause are spread through food, water and air. Bacterial diseases are responsible for a variety of illness, disability and even death of people across the world. At times, it becomes difficult to contain these diseases because they cause some lethal illness resulting in outbreaks that may cross over to other geographical areas creating havoc. Thus, controlling bacterial diseases is a serious concern for agriculturists and medical practitioners. Resistance of bacteria in the available range of chemicals and antibiotics demand an alternate way for disease management.[3] This stipulates the need for renewed efforts to seek antibacterial agents from alternate sources other than the current chemicals and antibiotics. It is at this juncture that the control of bacterial diseases through plant derived compounds gained acceptance. Secondary metabolites from plants are found to be effective in controlling many bacterial diseases. [4, 5, 6, 7, 8] *Excoecaria agallocha* L. (Family Euphorbiaceae) is an evergreen, semi-mangrove plant seen abundantly in the coastal estuarine regions. They are one of the predominant species seen in the Pichavaram forests. Commonly known as “the blinding tree”, *Excoecaria agallocha* L. is widely used by traditional medical practitioners against various disease conditions. [9-18] The antibacterial property of various solvent fractions extracted from different parts of *Excoecaria agallocha* is evaluated in the present study.

MATERIALS AND METHODS

All the chemicals and reagents used were of high quality analytical grade. Nutrient agar was purchased from Hi-media, DMSO, petroleum ether, chloroform, ethyl acetate and n-butanol (all AR grade) were from SD Fine Chem Limited. High quality double distilled water (MilliQ) was used for all experimental purposes including aqueous extraction.

(i) Collection of plant material

Different parts (root, stem and leaf) of the plant *Excoecaria agallocha* L were collected from Wadakara, Calicut District, Kerala. The plant was identified and authenticated at CMPR, Kottakkal Arya Vaidya Sala, Kerala. All the plant parts were dried in shade and pounded in mechanical grinder to obtain moderately coarse powder and the phytochemicals were extracted in water. The samples of root, stem and leaf were further fractionated in organic solvents (petroleum ether, chloroform, ethyl acetate and n-butanol) in the increasing order of polarity.

(ii) Preparation of the extracts

About 5g each of the root, stem and leaf powders obtained was extracted separately with 400ml double distilled water and refluxed for 6 hours. The aqueous extract was then filtered and concentrated to dryness in a rotary evaporator under reduced pressure. The dried extract (about 4.5 g) was then refluxed with 100 ml petroleum ether for 6 hours. A quantity of 25ml of the individual extract was taken in a separating funnel with equal quantity of the solvent (PE) and kept undisturbed for some time. The organic layer was collected and the process repeated for further fractionation till the whole extract (100ml) was fractionated. Thus the PE fraction of root, stem and leaf are obtained. The extract was then concentrated to dryness in a rotavapour. The whole process was repeated to obtain the chloroform, ethyl acetate and n-butanol fractions of root, stem and leaf. The various solvent extracts thus prepared were re-extracted with DMSO and aliquoted and stored in 4°C till further use.

(iii) Bioautographic screening

Bioautography is a very convenient and simple way of testing the plant extracts and pure substances for their effects on both human and plant pathogenic micro-organisms and can be successfully employed in the target directed isolation of active compounds. Two bioautographic screening methods are employed in the current study: agar diffusion, and disc diffusion. The various test strains and the details of the antibacterial assays are explained below.

(iv) Test bacterial strains

Cultures of *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella spp., Shigella sonnei* and *Staphylococcus aureus* were obtained from Pathology Department, PSG Medical College, Coimbatore and maintained as a subculture in slants in the Department of Botany, Avinashilingam University, Coimbatore.

(v) Antibacterial studies

Antibacterial studies were carried out by the standard agar well diffusion method and disc diffusion method.

(vii) Agar well diffusion method [19]

The agar well diffusion method described by Smania *et al*. (1995) was adopted for antibacterial assay. Each bacterial suspension was spread over the surface of nutrient agar plates with a cotton swab and four wells each of 0.125 mm diameter was made on the agar. The wells were filled with 25 µl each of the petroleum ether fraction of root, stem and leaf extracts along with proper control (in the fourth well) using a micropipette. The plates were incubated at 37°C for 24 h. The same procedure was repeated for chloroform, ethyl acetate and n-butanol fractions of the plant extracts. This procedure was repeated for each of the test organisms. The zone of inhibition was calculated by measuring the diameter of the clear zone formed around the well after reducing the diameter of the well.

(viii) Disc diffusion method [20]

The *in vitro* antibacterial activity of the sample solution was studied by disc diffusion method. Plates were prepared by pouring 20 ml of sterile nutrient agar (Hi-media) into the sterile petridishes and were inoculated with a loopful of broth culture of each test organism. Sterile paper disc (Whatman No: 0.25 mm diameter) impregnated with 10 µl quantity of dimethyl sulfoxide solution of the extract were air dried and placed on the agar plates. The plates were incubated at 37°C for 24 h. Control studies with chloramphenicol discs (2 mg/ml) and the solvent DMSO were done concurrently. The zone of inhibition was calculated by measuring the diameter of the clear zone formed around the disc after reducing the diameter of the disc.

RESULTS

The results of the antimicrobial activity of the extracts of *Excoecaria agallocha* on various test microbes are represented in the figures (Figure 1 and 2) and the zones of inhibition observed in all cases are represented in tables (Tables 1 to 4).

Effect of the different fractions of the extracts of Excoecaria agallocha against the test organisms by agar well diffusion method

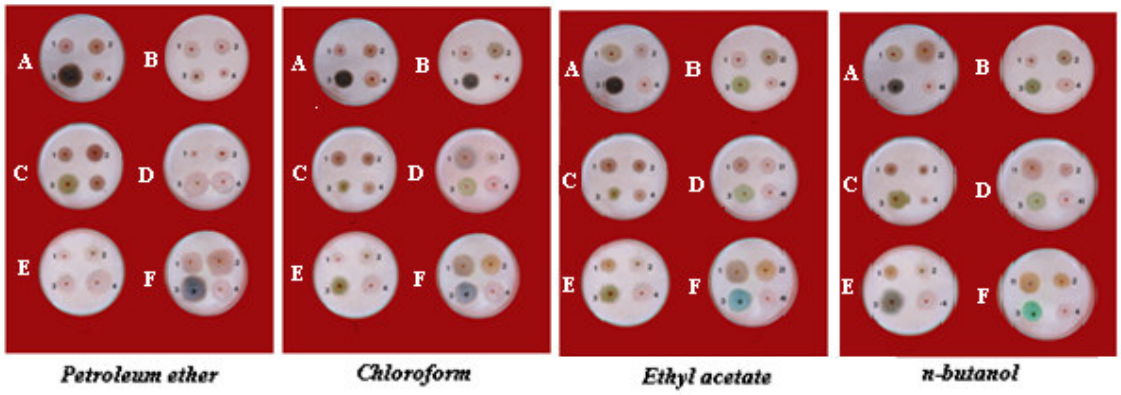

Figure. 1

Effect of various extracts of *Excoecaria agallocha* on the test organisms (by agar well diffusion method). In each plate, top left well had stem extract, top right had root extract, bottom left had leaf extract and bottom right had control of the respective solvents. A- *Bacillus subtilis,* B- *Klebsiella pneumonia,* C- *Escherichia coli,* D-*Salmonella spp.* E- *Shigella sonnei* and F- *Staphylococcus aureus.*

It was observed that by this method, the highest inhibition towards *Bacillus subtilis* was obtained with the PE fraction of the leaf (with a zone of inhibition 26.54±5.20 mm) which was higher than that observed in control well, followed by the nB fraction of root (with a zone of inhibition 21.96 ± 3.15). Further, it was observed that the extracts were effective towards *Escherichia coli,* with PE stem extract showing highest zone of inhibition (19.63±2.88 mm) followed by the PE fraction of root (with a zone of inhibition 18.88±0.90 mm), EA fraction of stem (with a zone of inhibition 18.75 ± 0.82 mm) and leaf (with a zone of inhibition 18.50 ± 2.74 mm). In the case of inhibition of the extracts towards *Klebsiella pneumonia,* the maximum inhibition was observed by the PE leaf fraction (with a zone of inhibition 21.46±1.37 mm) followed by PE root and nB leaf fractions (with zones of inhibition 18.21±0.38 mm and 17.92 ± 0.75 mm respectively). *Salmonella spp.,* and *Staphylococcus aureus* were the most susceptible among the test organisms to the various extracts as they showed the highest zone of inhibition than any other test organisms. The PE leaf fraction showed almost similar results with both these bacterial strains (30.71±6.29 mm for *Salmonella spp.,* and 30.29±0.72 mm for Staphylococcus *aureus*). The growth of *Shigella sonnei* was inhibited by the *n*-B leaf extract 23.00 ± 2.25 mm followed by the leaf extract in ethyl acetate, which showed a zone of inhibition of 20.00 ± 0.45 mm.

Table 1

Diameter of zone of inhibition (mm) obtained when the various test microbes were treated with the petroleum ether and chloroform extracts of Excoecaria agallocha (by agar well diffusion method)

Organism	Diameter of zone of inhibition (mm)							
	Petroleum Ether				Chloroform			
	S	R	L	C	S	R	L	C
Bacillus subtilis	16.88± 0.66	14.88± 0.50	26.54± 5.20	12.13± 0.90	16.13± 3.31	15.54± 0.63	19.63± 0.90	16.79± 2.02
Escherichia coli	19.63± 2.88	18.88± 0.90	14.71± 1.01	11.96± 0.72	17.33± 1.63	15.75± 1.27	15.04± 0.40	8.00 ± 2.25
Klebsiella pneumoniae	17.04± 0.87	18.21± 0.38	21.46± 1.37	16.54± 0.52	16.25± 0.82	12.68± 0.31	12.29± 0.14	15.58± 0.51
Salmonella spp.	7.13± 0.90	15.63± 1.52	30.71± 6.29	25.29± 4.38	20.46± 2.44	15.00± 0.45	18.50± 0.45	17.21± 0.14
Shigella sonnei	12.71± 0.57	16.46± 1.37	15.79± 1.25	28.46± 4.78	12.50± 0.45	16.88± 2.05	17.92± 0.75	17.71± 2.50
Staphylococcus aureus	22.21± 3.59	31.54± 5.20	30.29± 0.72	22.71± 0.80	24.17± 5.22	19.38± 0.66	20.50± 3.80	23.83± 0.95

(The results are means of three readings ± S.D.)

Table 2

Diameter of zone of inhibition (mm) obtained when the various test microbes were treated with ethyl acetate and n-butanol extracts of Excoecaria agallocha (by agar well diffusion method)

Organism	Diameter of zone of inhibition (mm)							
	Ethyl Acetate				n- Butanol			
	S	R	L	C	S	R	L	C
Bacillus subtilis	17.38± 1.65	13.42± 1.30	18.46± 1.26	17.71± 0.80	18.33± 1.40	21.96± 3.15	16.04± 3.26	11.33± 0.95
Escherichia coli	18.75± 0.82	16.67± 3.46	18.50± 2.74	12.79± 0.72	10.60± 0.48	10.50± 0.63	11.67± 1.02	15.50± 3.13
Klebsiella pneumoniae	15.00± 0.45	14.46± 1.91	12.58± 0.36	14.17± 1.02	10.50± 0.63	8.33 ± 1.16	17.92± 0.75	7.04 ± 0.88
Salmonella spp.	13.58± 1.58	13.37± 1.15	19.58± 2.22	17.92± 0.75	21.75± 2.72	11.33± 0.95	17.08± 1.56	16.17± 0.69
Shigella sonnei	15.50± 0.63	16.88± 1.89	20.00± 0.45	20.33± 1.63	14.25± 1.65	12.79± 2.53	23.00± 2.25	19.88± 1.25
Staphylococcus aureus	26.33± 4.25	22.38± 1.65	26.13± 3.31	18.83± 0.95	20.08± 1.30	14.04± 1.30	18.13± 1.32	13.79± 0.29

(The results are means of three readings ± S.D.)

Effect of the different fractions of the extracts *of Excoecaria agallocha* against the test organisms by disc diffusion method

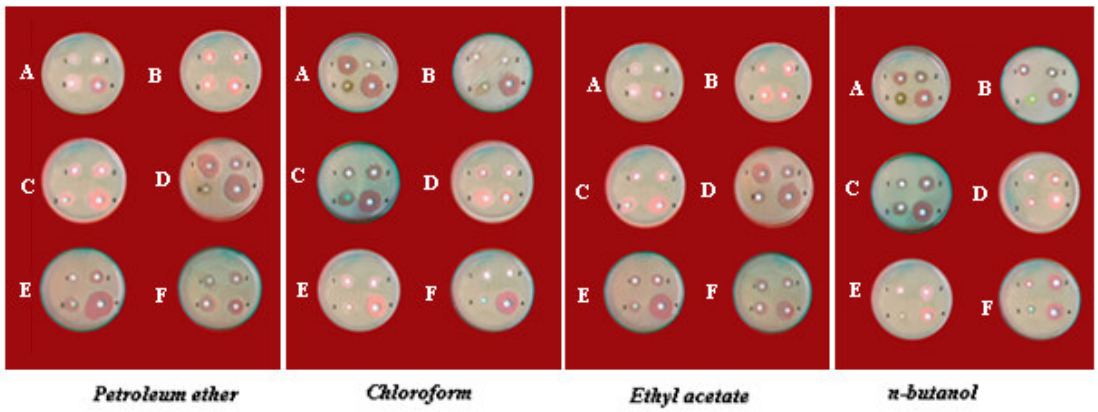

Figure. 2

Effect of various extracts of *Excoecaria agallocha* on the test organisms (by disc diffusion method). In each plate, top left disc had stem extract added, top right had root extract and bottom left had leaf extract of the respective solvents. Bottom right was control disc - Chloramphenicol (2mg/ml). A- *Bacillus subtilis,* B- *Klebsiella pneumonia,* C- *Escherichia coli,* D-*Salmonella spp.* E- *Shigella sonnei* and F- *Staphylococcus aureus.*

Compared to agar well diffusion method, the disc diffusion did not yield uniform inhibition when the test organisms were incubated with the extracts of *Excoecaria agallocha*. Some of the fractions (like the PE leaf fraction, chloroform root, leaf and stem fractions and the *n*-B leaf fraction) failed to show any noticeable inhibition in terms of zone of inhibition. *Bacillus subtilis* was inhibited most by chloroform stem extract (20.58 ± 1.57 mm) followed by the ethyl acetate leaf fraction (16.96 ± 0.85 mm). The chloroform fraction of leaf had no effect on the bacteria. *Escherichia coli* ethyl acetate stem fraction yielded the maximum inhibitory effect exhibiting a zone of inhibition of 23.71 ± 0.95 mm. The root and stem extract of chloroform was not effective in controlling the growth of *E. coli.* Root fractios of *n*-B and PE were effective against *Klebsiella pneumonia* as they showed similar results (17.67 ± 0.76 mm and 17.04 ± 2.01 mm respectively). Stem extract of PE fraction showed maximum inhibition of *Salmonella spp.* (25.08 ± 4.11 mm). The *n*-B root fraction was effective in controlling the growth of *Shigella sonnei.* It was observed that a zone of inhibition of 16.33 ± 1.88 mm was obtained when treated with the extract. The PE leaf extract showed a zone of inhibition of 16.63 ± 0.63 mm against *Staphylococcus aureus,* which was the highest against the test strain.

DISCUSSION

Presence of secondary metabolites / phyto chemicals like tannins, alkaloids, flavonoids, *etc.* account for the antimicrobial action of plant extracts.[21] The occurrence of these compounds could be the reason for other activities (like antioxidant, anticancer, *etc.*) as well. *Excoecaria agallocha* is a well studied a mangrove plant, with reports on its chemical constituents.[22] Investigations on the presence of metabolites from the plant revealed the presence of diterpenoids, [23, 24] triterpenoids, [25, 26] flavonoid, [27] glucoside, [28] polyphenols [29] and phorbol esters.[30] Preliminary phytochemical analysis of the extracts from *Excoecaria agallocha* L. showed the presence of many such compounds that could act as a potential mediator in antimicrobial studies. [31]

Table 3

Diameter of zone of inhibition (mm) obtained when the various test microbes were treated with the petroleum ether and chloroform extracts of Excoecaria agallocha (by disc diffusion method)

Organism	Diameter of zone of inhibition (mm)							
	Petroleum Ether				Chloroform			
	S	R	L	C	S	R	L	C
Bacillus subtilis	12.58± 0.80	12.08± 0.14	14.92± 1.01	18.17± 0.80	20.58± 1.57	-	8.54± 1.05	27.88± 3.13
Escherichia coli	14.46± 0.31	11.33± 1.38	18.50± 0.63	18.67± 0.29	-	-	11.54± 1.02	23.29± 1.30
Klebsiella pneumoniae	12.38± 0.45	17.04± 2.01	16.42± 1.30	25.79± 3.44	12.46± 0.36	16.63± 1.08	16.21± 3.44	26.42± 4.02
Salmonella spp.	25.08± 4.11	17.58± 2.50	-	34.75± 3.31	15.50± 0.45	11.54± 1.02	24.75± 6.25	16.21± 2.01
Shigella sonnei	13.08± 5.05	14.96± 1.30	13.29± 4.77	34.54± 3.21	11.83± 1.30	13.29± 0.95	10.79± 1.30	27.88± 3.13
Staphylococcus aureus	12.46± 0.36	13.92± 1.57	16.63± 0.63	17.58± 0.80	9.96 ± 0.36	9.64 ± 0.47	-	25.17± 2.37

(The results are means of three readings ± S.D.)

Table 4

Diameter of zone of inhibition (mm) obtained when the various test microbes were treated with ethyl acetate and n-butanol extracts of Excoecaria agallocha (by disc diffusion method)

Organism	Diameter of zone of inhibition (mm)							
	Ethyl Acetate				n- Butanol			
	S	R	L	C	S	R	L	C
Bacillus subtilis	10.29± 0.64	15.08± 0.31	16.96± 0.85	27.88± 3.13	14.85± 0.17	11.92± 0.80	13.71± 1.30	20.29± 1.75
Escherichia coli	23.71± 0.95	15.25± 0.66	14.04± 2.58	32.04± 4.77	10.87± 0.44	9.13 ± 2.72	11.00± 2.17	18.29± 0.36
Klebsiella pneumoniae	11.13± 0.82	13.88± 0.94	16.21± 2.01	21.17± 3.22	12.56± 0.31	17.67± 0.76	15.71± 1.35	22.25± 0.63
Salmonella spp.	20.92± 3.36	14.54± 1.57	11.63± 1.08	27.88± 3.13	14.96± 0.36	16.42± 0.72	9.33 ± 1.30	16.63± 1.65
Shigella sonnei	12.46± 0.36	15.06± 0.31	10.68± 1.37	34.54± 3.21	5.38 ± 0.63	16.33± 1.88	-	16.42± 1.91
Staphylococcus aureus	13.92± 1.30	11.52± 1.27	15.77± 0.92	20.08± 0.51	7.38 ± 0.22	15.46± 0.51	-	19.25± 1.21

(The results are means of three readings ± S.D.)

Antibacterial compounds are important as bacteria are responsible for a wide variety of disease conditions[32] including many dental diseases (dental caries, bleeding gum, gingivitis *etc.*) and prevention of bacteria helps in controlling these diseases. Many of these results obtained in the present study are along the lines of those of the works previously reported. The chloroform and ethanol extracts of fresh root tubers of *Momordica cymbalaria* Hook were used to check the antimicrobial activity against various test organisms - *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Penicilium crysogenum, Tricho bacterium rubrum, Aspergillus niger, Aspergillus flavus, Rhizopus orizae* by agar well diffusion method. It was observed that *E. coli* and *S. aureus* were most susceptible to chloroform extract than ethanol extract. [33] Similar results were obtained when the antibacterial activity of six extracts (ethanol, methanol, ethyl acetate, chloroform, hexane and petroleum ether) from leaf, stem and root of *Mentha piperita* were tested against pathogenic bacteria (*Bacillus subtilis*, *Streptococcus pneumonia*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris and Klebsiella pneumonia*) by agar well diffusion method. [34] It was observed that the ethyl

acetate leaf extract showed pronounced inhibition than chloroform, petroleum ether and hexane. The leaf extract activity being more on *Bacillus subtilis*, *Staphylo-coccus aureus* and *Proteus vulgaris* than *Escherichia coli*, *Streptococcus pneumonia* and *Klebsiella pneumonia.* Ethyl acetate extract of the leaves of *Trifolium alexandrinum* exhibited the highest antibacterial activity i.e., higher inhibition zones against most of the bacterial strains studied in comparison to methanol, aqueous, dichloromethane and hexane extracts. [35] The ethyl acetate, chloroform, and petroleum ether fractions of *Caesalpinia bonducella* leaves were tested against different bacterial strains - *Salmonella typhi, Staphylococcus aureus, Shigella dysenteriae, Bacillus subtilis, Pseudomonas aeruginos, Bacillus cereus, Escherichia coli, Bacillus megaterium* and *Klebsiella spp* using disc diffusion method. Among the various extracts tested, the chloroform fraction showed maximum activity against almost all bacteria. [36] In the present study, the antibacterial effect of various extracts (of root, stem and leaf) from *Excoecaria agallocha,* extracted in various organic solvents (petroleum ether, chloroform, ethyl acetate and n-butanol, in the increasing order of polarity) was analyzed against six bacterial strains and it was found that all the extracts showed antibacterial effect in varying degrees. The differences observed in the inhibition of test organisms could be due to the presence of various compounds present in the sample and also the morphology of the test organisms. These preliminary investigations bring forth the importance of harnessing these compounds as potential antimicrobial agents.

CONCLUSION

To conclude, it was understood from the present study that the extract of *Excoecaria agallocha* contained many phytochemicals that are capable of inducing an inhibitory effect on the growth of the test organisms. To the best of our knowledge, this is first such extensive report on the antibacterial effects of *Excoecaria agallocha* tested by both agar well and disc diffusion methods. Though previous studies refer to the antibacterial effect of the extract from the same plant, this is the first ever, comprehensive report where the petroleum ether, chloroform, ethyl acetate and n-butanol fractions of all the three plant parts (leaf, stem and root) were studied. The two methods (agar well diffusion and disc diffusion) of antibacterial screening employed in the present study helped to advance our current knowledge on the behavior of the microbes when tested with different test samples. Further studies are on track to find the bioactivity of the extract on cancer cells and also to find out the mechanism of action of the extract/ compound. It is expected that the preliminary study reported here will open up new possibilities and prompt scientists to think about exploring the plant in more detail.

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