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Antibacterial and Analgesic Effects of the Stem Barks of *Calophyllum inophyllum*

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ABSTRACT: In the present study the methanol and chloroform extracts of the dried stem barks of "*Calophyllum Inophyllum*" were prepared and compared with Standard drug for their anti-bacterial and analgesic activities. The anti-bacterial activities were evaluated against number of different bacterial strains by detecting minimum inhibitory concentration and zone of inhibition. The minimum inhibitory concentration values were compared with control and zone of inhibition were compared with standard ciprofloxacin. The analgesic activities of both extracts were compared with standard drug Aspirin by Hot plates method using Swiss albino mice.
Key words: *Calophyllum Inophyllum,* Antibacterial, Analgesic.

INTRODUTION

Calophyllum inophyllum belongs to family clusiaceae (syn. Guttiferae) is a medium sized to large evergreen tree that average 8-20m in height with a broad spreading crown of irregular branches. The tree support a dense canopy of glossy elliptical leaves fragrant white flowers and large round nut. It grows along costal area and adjacent lower land forests although it occasional occur inland at higher elevation. A genus of evergreen trees, distributed in the tropics of Asia, mainly in the Indo-Malaysian region, Australia, Africa and tropical America. Along the East and West coasts of the Peninsula, Burma, the Andamans and Malay Peninsula, Ceylon. Essentially a littoral species East African Islands, Malaya, Australia, Polynesia[1].

In India it is distributed in the coastal regions of Maharashtra, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa, and the Andamans; often it runs wild; also reported to be growing in Arunachal Pradesh. About seven species occur in India. Some species are ornamental and others yield timber, commercially classified as POON and oil[2]. The following species are used medicinally in China, Indo China and the islands of the Indian Ocean C. *inophyllum* Linn. in Madagascar C. *laxiflorum Drake, C. parviflorum* Bojer., *C. tacamahaca* Willd. in the Antilles C. *calaba* Jacq. in Brazil C. *brasiliense* Camb[1].

In different parts of India, the plant is known by different vernacular names /local names are English - Alexandra Laurel, Alexandrian Laurel, Hindi - Sultanachampa, surpunka, undi, Sanskrit - Nagachampa, punnaga, surangi, Oriya - Polang, ponnang, Tamil- Pinnai, punnagam[1,2].

As per the ethnomedicinal information the various parts of calophyllum inophyllum possess medicinal properties. The fresh bark of *C. inophyllum* is used to treat diabetes[3]. The fresh fruit and its oil used externally against rheumatism, in topical infection and

seborrhea in human adult[4,5]. The dried leaf and its decoction used to cure rheumatism , skin-infections[6], cuts and sores[7-9].The fresh leaves infusion are used to cure bacterial infection, fungal infection and as vermifuge/ pediculicide[5].The resin are used orally as an emetic and purgative[10]. Dried seed extract are used against rheumatism in human adult[11]. The barks are astringent and useful in internal hemorrhages. In Cambodia the leaves are prescribed as an inhalation in migraine and vertigo. In New Caledonia the gum resins are applied to ulcerous wounds. In Java the trees are supposed to possess diuretic properties[1, 2].

The reported chemical constituents present in c. inophyllum are flavonoid compound Amentoflavone[12,13], steroid compound campesterol[14,15], Arachidic acid lipid[15], xanthone derivative Brasilixanthone- B and Buchanaxanthone[16,17], coumarin derivatives Calocoumarin-A, Calocoumarin-B, Calocoumarin-C and Apetalolide[18,19] , and Beta Amyrin a triterpene[20] .

All these above mentioned traditional uses indicate that their must be some antibacterial and analgesic properties lying with the plant. In the present investigation both the methanol and chloroform extracts were subjected for antibacterial and analgesic activities studies.

MATERIALS AND METHODS

Plant material

The plant specimen was identified by Prof. P.Jayaraman, Director, Plant Anatomy Research Centre, Chennai. After authentication, fresh stem barks were collected in bulk from young matured plants from the forest region of Similipal Biosphere Reserve of Mayurbhanj district Orissa in the month of august 2006. The stem barks were washed, shade dried and milled in to coarse powder by a mechanical grinder. The powder materials were passed through sieve number 40 and used for further studies.

Preparation of extract

The dried powder barks were successively extracted in soxhlet apparatus by using different solvents (Petroleum Ether, Chloroform and Methanol) with increasing order of polarity in the ratio of drug to solvent (1:8) for 72 hours. . Each extracts were concentrated at reduced pressure using rotary evaporator and further subjected for antibacterial and analgesic activity studies. The type and extractive yield of different extracts of *C. Inophyllum* were observed and results of such observation are tabulated in Table no 1.

Preparation of the tested organisms

The lyophilized forms of different strains of microorganisms like *Bacillus licheniformis* (MTCC 429), *Escherichia coli* (MTCC 40), *Proteus vulgaris* (MTCC 426), *Pseudomonas aeruginosa* (MTCC 424), *Shigella flexneri* (MTCC 1457), *Bacillus subtilis* (MTCC441), S*taphylococcus aureus* (MTCC 87), S*taphylococcus epidermidis* (MTCC 2639) were obtained from the Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh, India and the bacterial strains *Shigella boydii*-8, *Salmonella typhi*-59, *Salmonella typhimurium* NCTC-74, *Vibrio cholerae*-811, *Vibrio cholerae*-854, *Klebsiella pneumoniae*-14 and *Klebsiella pneumoniae*-725 were collected from Division of microbiology, Jadavpur university, Kolkata. The bacterial cultures were maintained on Mueller-Hinton Agar (MHA) and were subcultured in the microbiology laboratory of the Royal college of Pharmacy and Health Sciences, Berhampur, Orissa, India. The average number of viable of organisms per ml of the stock suspensions was determined by means of the surface viable counting technique[21]. About (10^8-10^9) colony-forming units per ml was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

Inoculation:

One loopful of an overnight grown nutrient broth culture of each test organism served as the inoculums for such antimicrobial activity determination. The average size of inoculums was about 10^6 cells contained in 3mm diameter of standard loop[22].

Determination of the minimum inhibitory concentration (MIC)

Nutrient agar medium (250ml) was prepared and sterilized. Exactly 29 ml of media was dispersed in each of the 8 conical flasks, plugged with cotton and autoclaved. A Stock solution of *Calophyllum Inophyllum* extract of 9mg/ml in 1% di-methyl sulphoxide (DMSO) was prepared. Measured quantities of the stock solution of extract were poured to the molten nutrient agar media to prepare concentration of 25, 50, 100, 200, 300 and 400µg/ml and then poured in Petri dishes. The Petri dishes were marked accordingly. One sterile nutrient agar plate without extract but with equal volume of the solvent served as the control plate. These plates were refrigerated overnight for uniform diffusion of the extract throughout the media. The plates were dried at 37°C by keeping them in the incubator. One loopful (diameter-3mm) of an overnight grown peptone water culture of each test organism was placed in petridish marked by the checker board technique. The spot inoculated plate was incubated at 37ºC for 24 hours and the MIC value obtained[23-25]. The experiment was

repeated in triplicate and average values were disclosed in the Table no 2 and 3.

Determination of zone of inhibition

For the determination of zone of inhibition, pure ciprofloxacin was taken as a standard antibiotic for comparison of the results. Two sets of three dilutions (50, 100 and 200 µg/ml) of *C. Inophyllum* bark extract and ciprofloxacin (50, 100 and 200 µg/ml) were prepared in double distilled water in Mc Cartney bottles. Sterile nutrient agar plates were prepared and incubated at 37ºC for 24hrs to check any sort of contamination. Two sterile filter paper discs (Whatmann no.1) of 6mm diameter were soaked in two different dilutions of crude extract and placed in appropriate position on the surface of the flooded plate, marked as quadrants at the back of the Petri dishes. The Petri dishes were incubated at 37 ºC for 24 hrs and the diameter of the zone of inhibition were measured in mm. Similar procedure were adopted for the pure ciprofloxacin and the corresponding zone diameter were compared accordingly[26]. The experiment was repeated in triplicate and average values were written in the Table no 4.

Acute toxicity and lethality (LD50) test

The Acute toxicity and lethality (LD50) test of methanol and chloroform extracts of *Calophyllum inophyllum* was determined in albino mice by administering the extracts orally to different groups at the dose level of 250, 500, 1000 and 2000 mg/kg body weight. All animals were observed for toxic symptoms and mortality for 72 hrs[27]. No mortality was observed up to a dose level of 2000 mg/kg body weight. As per the ranking system European Economic Community (EEC) for acute oral toxicity, the LD_{50} dose of 2000 mg/kg and above is categorized as unclassified (EC Directive 83/467/EEC, 1983).

Determination of analgesic activity of the extracts by Hot plates Model

Swiss albino mice weighing between 20 to 25 gm of either sex were used and were maintained at 25±3°C. They were kept in a well ventilated animal house under the natural photo periodic condition in polypropylene cage and were feed standard pellet diet and water *ad libitum.* The animal experiment was conducted with prior approval of Institutional Animal Ethics Committee of Royal College of Pharmacy and Health Sciences, Berhampur, Orissa. The analgesic activity of *C. Inophyllum* extracts were assessed using the Hot plate method[28]. Thirty six mice of either sex were divided in six groups (n = 6). Group-1 animals treated with 1% DMSO (10 ml/kg, p.o.) as control and Group-2 with morphine sulphate (5 mg/kg, s.c.) as reference. Remaining groups (Group-3, 4, 5 and 6) were administered with chloroform and methanol extracts (in dose of 100 mg/kg and 200 mg/kg, p.o.) as test groups. At 30, 60, 120 and 180 min after administration, animals were lowered onto the surface of a hot plate (50±1.0^{0}C) enclosed with cylindrical glass and the time for the animal to jump or lick the fore limb was noted as the reaction time (RT). Cut off time in the absence of a response was 15 sec to prevent the animals from being burnt[29].

RESULTS AND DISCUSSION

The results regarding the antibacterial activity of methanol and chloroform extracts obtained from stem barks of *Calophyllum Inophyllum* are indicated in Table-2, 3 and 4. The minimum inhibitory concentration (MIC) and Zone of inhibition values (ZOI) were carried out by using fifteen different bacterial strains of both Gram +ve and Gram –ve microorganism. The MIC of test compound compared with control group and ZOI values with standard ciprofloxacin. From the results of MIC values it indicates that the methanol extract of *Calophyllum Inophyllum* showed significant antibacterial properties against Gram +ve and Gram –ve bacteria by agar dilution technique compared to chloroform extract. As per the MIC results obtained in Table-2 shows that *Pseudomonas aeruginosa* MTCC No-424, *Staphylococcus aureus* MTCC No-87 and *Staphylococcus epidermidis* MTCC-2639 were inhibited at the concentration of 25µg/ml and were highly sensitive to the extract. The strains *Bacillus Licheniformis* MTCC. No-429, *Bacillus subtilis* MTCC No-441, *Escherichia coli* MTCC NO-40 and *Klebsiella pneumoniae*-14 were inhibited at the concentration of 50 µg/ml and were moderately sensitive. The remaining eight bacterial strains were found to be inhibited within the concentration range of 100-300µg/ml and were less sensitive to the extract. All the bacterial strains were inhibited within the concentration range of 25 to 300 µg/ml. Basing on MIC results, the methanol extract was selected for further antibacterial activity studies carried out by ZOI and the result was depicted in table no 4. It indicates that the antibacterial activity of *C. inophyllum* methanol extract was reduced in the order of *Bacillus Licheniformis* MTCC No-429 > *Bacillus subtilis* MTCC No-441 > *Pseudomonas aeruginosa* MTCC No-424 > *Escherichia coli* MTCC NO-40 > *Staphylococcus aureus* MTCC No-87 > *Staphylococcus epidermidis* MTCC-2639 > *Klebsiella pneumoniae*-14 > *Proteus vulgaris* MTCC No-426 > *Shigella flexneri* MTCC No-1457 > *Shigella boydii*-8 > *Vibrio cholerae*-811 > *Klebsiella pneumoniae*-725 > *Salmonella typhi*-59 > *Salmonella typhimurium* NCTC-74 > *Vibrio cholerae*-854.

The acute toxicity studies revealed an oral LD50 greater than 2000 mg/kg. The methanol extract of *Calophyllum Inophyllum* stem barks significantly ($P < 0.05$) and dose-dependently protected the mice against thermally induced pain stimulus. The reaction time after two hours at the dose of 100mg/kg and 200 mg/kg was found to be 11.1±0.22 and 12.8±0.20 respectively where as for standard drug, morphine sulphate (5mg/kg) found to be 13.9±0.14, while the reaction time in control group was 4.8±0.11 sec. In comparison to chloroform extract the effect of methanol extract found to be more protective (Table-5).

Preliminary Phytochemical analysis of the methanol extract of *Calophyllum Inophyllum* revealed the presence of tannins, polyphenolic compounds, flavonoids, saponins and terpenoids by using standard procedure for detection of phytoconstituents[30, 31]. Notably, both tannin and phenolics have been reported to possess antibacterial activity [32, 33].

Table-1: Types and % yield of different extracts of *Calophyllum Inophyllum*

Sl.no.	Plant part	Solvent used for extraction	Colour of the extracts	Physical appearance of the extracts	%Yield in (w/w)
1	Stem bark	Petroleum ether	Yellowish brown	Sticky mass	6.43
2	Stem bark	Chloroform	Dark brown	Dried powder	2.01
3	Stem bark	Methanol	Reddish brown	Dried powder	21.08

Table-2: Determination of the minimum inhibitory concentration (MIC) of methanol extract of *Calophyllum inophyllum*

SI. no	Name of the Bacteria	Concentrations of methanol extract (µg/ml).						
		0	25	50	100	200	300	400
1	*Bacillus Licheniformis* MTCC. No-429	+	+	-	-	-	-	-
2	*Bacillus subtilis* MTCC No-441	+	+	-	-	-	-	-
3	*Proteus vulgaris* MTCC No-426	+	+	+	-	-	-	-
4	*Pseudomonas aeruginosa* MTCC No-424	+	-	-	-	-	-	-
5	*Shigella flexneri* MTCC No-1457	+	+	+	-	-	-	-
6	*Shigella boydii*-8	+	+	+	-	-	-	-
7	*Escherichia coli* MTCC NO-40	+	+	-	-	-	-	-
8	*Staphylococcus aureus* MTCC No-87	+	-	-	-	-	-	-
9	*Staphylococcus epidermidis* MTCC-2639	+	-	-	-	-	-	-
10	*Salmonella typhi*-59	+	+	+	+	+	-	-
11	*Salmonella typhimurium* NCTC-74	+	+	+	+	-	-	-
12	*Vibrio cholerae*-811	+	+	+	-	-	-	-
13	*Vibrio cholerae*-854	+	+	+	+	-	-	-
14	*Klebsiella pneumoniae*-14	+	+	-	-	-	-	-
15	*Klebsiella pneumoniae*-725	+	+	+	-	-	-	-

'0' stands for plain nutrient agar without the drug serving as control '+' stands for growth and '-' stands for no growth.

Table-3: Determination of the minimum inhibitory concentration (MIC) of Chloroform extract of *Calophyllum inophyllum*

SI. no	Name of the Bacteria	Concentration of chloroform extract (µg/ml)						
		0	25	50	100	200	300	400
1	*Bacillus Licheniformis* MTCC. No-429	+	+	+	+	+	+	+
2	*Bacillus subtilis* MTCC No-441	+	+	+	+	+	+	+
3	*Proteus vulgaris* MTCC No-426	+	+	+	-	-	-	-
4	*Pseudomonas aeruginosa* MTCC No-424	+	+	+	-	-	-	-
5	*Shigella flexneri* MTCC No-1457	+	+	+	+	+	+	-
6	*Shigella boydii*-8	+	+	+	+	+	-	-
7	*Escherichia coli* MTCC NO-40	+	+	+	+	+	+	+
8	*Staphylococcus aureus* MTCC No-87	+	+	+	+	+	-	-
9	*Staphylococcus epidermidis* MTCC-2639	+	+	+	+	+	-	-
10	*Salmonella typhi*-59	+	+	+	+	+	-	-
11	*Salmonella typhimurium* NCTC-74	+	+	+	+	+	+	-
12	*Vibrio cholerae*-811	+	+	+	+	+	+	+
13	*Vibrio cholerae*-854	+	+	+	+	+	+	+
14	*Klebsiella pneumoniae*-14	+	+	+	+	-	-	-
15	*Klebsiella pneumoniae*-725	+	+	+	+	+	-	-

'0' stands for plain nutrient agar without the drug serving as control '+' stands for growth and '-' stands for no growth.

Table-4: Determination of Zone of inhibition of methanol extract of *Calophyllum Inophyllum* stem bark

SI.No	Name of the Bacteria	Methanol extract (µg/ml)			Ciprofloxacin (µg/ml)		
		50	100	200	50	100	200
1	*Bacillus Licheniformis* MTCC. No-429	7.50	9.00	10.50	8.00	9.50	11.50
2	*Bacillus subtilis* MTCC No-441	7.00	9.00	11.00	7.50	9.50	12.00
3	*Proteus vulgaris* MTCC No-426	7.00	8.00	9.50	7.50	8.50	11.00
4	*Pseudomonas aeruginosa* MTCC No-424	8.00	9.50	11.50	8.50	10.00	13.00
5	*Shigella flexneri* MTCC No-1457	7.50	8.50	9.50	8.00	9.00	11.50
6	*Shigella boydii*-8	7.00	8.00	9.00	8.50	9.50	11.00
7	*Escherichia coli* MTCC NO-40	7.50	8.50	11.00	8.00	9.00	12.00
8	*Staphylococcus aureus* MTCC No-87	7.50	9.00	11.00	8.00	9.50	12.50
9	*Staphylococcus epidermidis* MTCC-2639	8.00	9.50	11.00	8.50	10.00	13.00
10	*Salmonella typhi*-59	7.00	8.50	9.50	7.50	9.00	10.00
11	*Salmonella typhimurium* NCTC-74	6.50	7.50	8.50	7.50	8.50	9.50
12	*Vibrio cholerae*-811	7.00	8.50	10.00	8.00	9.00	11.50
13	*Vibrio cholerae*-854	7.00	8.00	9.00	8.00	9.00	10.00
14	*Klebsiella pneumoniae*-14	7.50	8.50	10.50	8.00	9.00	11.50
15	*Klebsiella pneumoniae*-725	7.00	8.00	9.50	7.50	8.50	10.50

Values are Zone of inhibition (mm); tests were done in triplicate.

Table-5: Evaluation of analgesic activity of methanol and chloroform extracts of *collophyllum inophyllum* bark by hot plate method.

Groups	Treatment	Dose (mg/kg body wt)	Reaction time(in sec.) after administration of drugs in minute				
			Basal reaction time	30	60	120	180
1	Control (1% DMSO)	10 ml/kg	4.5±0.12	5.0±0.07	4.9±0.09	4.8±0.11	5.1±0.05
2	Morphine sulphate	5 mg/kg	4.7±0.09	9.7±0.13*	12.8±0.18*	13.9±0.14*	13.4±0.07*
3	Chloroform extract	100mg/kg	4.9±0.10	5.7±0.21	6.9±0.14	8.5±0.09	8.9±0.08
4		200mg/kg	4.7±0.21	6.6±0.15	8.6±0.19	9.5±0.20*	9.9±0.13*
5	Methanol extract	100mg/kg	4.8±0.15	7.1±0.11*	10.4±0.27*	11.1±0.22*	11.1±0.19*
6		200mg/kg	4.5±0.22	8.3±0.24*	11.6±0.25*	12.8±0.20*	12.5±0.26*

*P< 0.05 compared to Morphine sulphate and control respectively. All values are expressed in Mean ± Standard deviation, n=6

CONCLUSION

Herbs are an integral part of nature. Plants contain natural substance that can promote health. From the present investigations it can be concluded that the antibacterial and analgesic activities of *Calophyllum Inophyllum* stem barks may be due to the combined or individual effect of the phytoconstituents found, which can be further confirmed by the extensive studies. The antimicrobial and analgesic activities of this plant highlighted the importance of the extracts in traditional preparations. Basing on the above results we can further conclude that the *Calophyllum inophyllum* plant may be helpful in treating various kinds of diseases in future days.

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