ORIGINAL ARTICLE

Antibacterial activities and phytochemical analysis of *Cassia fistula* (Linn.) leaf

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ABSTRACT

Cassia fistula Linn. which belongs to family Leguminosae is a medium-sized tree and its different parts are used in ayurvedic medicine as well as home remedies for common ailments. Sequential extraction was carried out using solvents viz. petroleum ether, chloroform, ethanol, methanol and water from leaf of the plant were investigated for preliminary phytochemical and antibacterial property. Results of the study showed that all the extracts had good inhibitory activity against Gram-positive test organism. Although all five extracts showed promising antibacterial activity against test bacterial species, yet maximum activity was observed in ethanol extract. The minimum inhibitory concentration ranged in between 94 to 1 500 μg/ml. Evaluation of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, protein and amino acids, saponins, and triterpenoids revealed the presence of most of constituents in polar extracts (ethanol, methanol, and aqueous) compared with nonpolar extracts (petroleum ether and chloroform). Furthermore, the ethanol extract was subjected to TLC bioautography and time-kill study against *Staphylococcus epidermidis.* All the findings exhibit that the leaf extracts have broad-spectrum activity and suggest its possible use in treatment of infectious diseases.

Key words: *Cassia fistula*, human pathogenic bacteria, minimum inhibitory concentration, Similipal Biosphere Reserve, TLC bioautography

INTRODUCTION

Cassia fistula Linn. (Leguminosae) is a very common plant and is widely known for its medicinal properties. In the Indian literature, this plant has been described to be useful against skin diseases, liver troubles, tuberculous glands and its use in the treatment of rheumatism, hematemesis, pruritus, leucoderma, and diabetes.[1,2] Besides, it has been found to exhibit anti-inflammatory and hypoglycemic activity and widely used as a mild laxative suitable for children and pregnant women.[3] Several reports are present on hepatoprotective,[4] antifertility,[5] and antioxidant properties of *C. fistula*.[6,7] Some studies have also been done on antimicrobial activity of *C. fistula* flower and seed along with some other Indian medicinal plants.[8-16] These studies give diminutive information on the antimicrobial property of the leaves of this plant.

Reports of the preliminary screening of this plant collected from Similipal Biosphere Reserve (SBR)[17] recorded of its antimicrobial activity against certain bacterial strains. The plant has diverse ethnomedicinal uses by the tribals of SBR. The bark paste is applied externally on the bite area for 2 to 3 times in a day at regular interval for 3 days. Half teaspoon juice extract is taken orally thrice daily to cure jaundice. The leaf paste along with neem is applied externally over all types of skin infections. Several reports are available on antimicrobial activities of *C. fistula* from bark, seed, flower, and fruits, but that on leaves are scanty.

In the present study, an attempt has been made to investigate the antibacterial activity of different solvent extracts of leaf of *C. fistula* obtained by sequential extraction method, against Gram-positive and -negative bacteria. In addition to this, TLC bioautography study and presence of phytochemical

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constituents in the respective extracts were also carried out.

MATERIALS AND METHODS

Plant Material

Leaves of *C. fistula* were collected in the month of April, 2007 from SBR, Mayurbhanj, Orissa and their identity was confirmed and the voucher specimen (NOU 25) was deposited in the Department of Botany, North Orissa University. The shed dried healthy leaves were powdered separately using mechanical grinder and then were passed through sieve in order to maintain uniform powder size.

Preparation of Extracts

Sequential extraction was carried out with the same powder using solvents of increasing polarity. About 250 g of dry leaf powder were sequentially extracted using petroleum ether, chloroform, ethanol, methanol, and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation. The concentrated extracts were freeze dried to remove the solvent at -2°C till further use. The yield of each extract was calculated and stored for further use.

Bacteria and Growth Media

Escherichia coli (MTCC 1098), *Escherichia coli* O157:H7 (RMRC, Bhubaneswar, India), *Salmonella typhimurium* (MTCC 3216), *Shigella sonnei* (NICED, Kolkata, India), *Bacillus subtilis* (MTCC 7164), *Bacillus licheniformis* (MTCC 7425), *Staphylococcus aureus* (MTCC 1144), and *Staphylococcus epidermidis* (MTCC 3615) were used as test microorganisms. All these bacterial cultures were grown at 37°C and maintained at 4°C on nutrient agar slants (Hi-media Pvt. Ltd., Mumbai, India).

Agar Cup Method

The agar cup method (ACM) was used to study the antibacterial activity of the extracts.[18] Briefly, cultures of the bacteria from culture plates were scooped using a wire loop and separately mixed with normal saline and agitated with vortex mixer. A loop full was withdrawn and uniformly distributed on the surface of the agar plate by streaking using a sterile swab. Wells of approximately 6 mm in diameter and 2.5-mm deep were made on the surface of the solid medium using a sterile borer. The plates were turned upside down and the wells labeled with a marker. The extracts were reconstituted by dissolving in dimethyl sulfoxide (DMSO). Each well was filled with test sample. Sterile DMSO was used as negative control, while gentamicin and ciprofloxacin were used as positive control. The plates were incubated at 37°C for 24 hours. After 24 hours, the plates were removed and zones of inhibition measured with Himedia antibiotic scale and the results were tabulated. Extracts with zones of inhibition greater or equal to 8-mm diameter were regarded as positive. The mean±SD of the inhibition zone was taken for evaluating the antibacterial activity of the extracts.

Determination of Minimum Inhibitory Concentration

Although the results of the ACM cannot always be compared with the minimum inhibitory concentration (MIC) data, extracts which showed positive result were further evaluated for the determination of MIC. A broth microdilution technique was adopted using 96-well microtiter plates and tetrazolium salt, 2,3,5-triphenyltetrazolium chloride (TTC) was carried out to determine the MIC following the methods with modification, as described by Eloff.[19] In the plate, A_1 to H_1 were blank with MH broth only. A_3 to H_3 were having the stock solution of the test extract(s) and A_4 to H_4 till A_9 to H_9 were the wells in which the test extracts were serially diluted using MH broth. Wells A_{12} to D_{12} were controls having 20 µl of DMSO and E_{12} to H_{12} served as control over control. All wells were dispensed with 100 µl of MH broth. 20 µl of the herbal extract was transferred from stock test solution to the first well, that is, from A_4 to H_4 containing 100 µl of MH broth. 20 µl of the MH broth containing herbal extract was then transferred to the next well to create serial dilutions. 100 µl of the 0.5 McFarland adjusted activated culture in MH broth was then added to all the wells except the blank. 5 µl of 0.5% TTC was further added to all the dilutions, blank, control, and control over control. The final volume of all the wells was 205 µl. The microplate was sealed and incubated at 37°C at 130 rpm. 10 µl of the broth from each culture tube exhibiting MIC and control tubes were taken aseptically and were plated on one-day old Muller-Hinton (MH) agar plate as a point inoculum and allowed to dry for 10 minutes under the laminar air hood. The microplate was sealed and incubated at 37°C at 130 rpm and observed for growth of the microorganism. The lowest recorded MIC was further subjected to time kill kinetics using ethanol, methanol, and aqueous extracts of *C. fistula*. 20 µl of overnight broth culture of *S. epidermidis* was added to 180 µl of NB containing each extract. The microtiter plate was incubated at 37°C. The number of viable cells was determined after 0, 1, 2, 4, 8, 12, 16, 24, and 48 hours of incubation. A control culture without crude extract was incubated and assayed under the same condition.[20]

TLC Bioautography

TLC bioautography assay was performed by agar overlay bioautography technique. Plant extract samples (5 µl) were applied 2.5 cm from the base of the silica plate. After drying, the plates were developed using solvent chloroform : methanol (8.2 : 1.8) and chloroform : hexane (5.4 : 6.6). Developed TLC plates were carefully dried for complete removal of solvents. Aliquot of 50 ml of molten MH agar was prepared by adding 500 µl of bacterial inoculum (5×10^5 CFU). Now, the inoculum containing agar was overlaid on dried TLC plate under aseptic condition in laminar airflow. The plates were incubated at 37°C and examined for the zone of inhibition.

RESULTS AND DISCUSSION

The amounts of crude material extracted per gram of powdered *C. fistula* leaf were 15.2, 24.5, 43.8, 68.2, and 158.6 mg, respectively, with chloroform, petroleum ether, methanol, distilled water, and ethanol solvents. Evaluation of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, protein and amino acids, saponins, and triterpenoids revealed the presence of most of the constituent in polar extracts such as ethanol, methanol, and aqueous extracts compared with nonpolar extracts (petroleum ether and chloroform). However, flavonoids, proteins and amino acids, tannins, and phenols were found to be universally occurring in all the extracts [Table 1].

Table 2 lists the plant extracts and their zone of inhibition against the test bacterium. Chloroform extract did not show any zone of inhibition against Gram-negatives, whereas Gram-positive bacteria were completely inhibited by all test extracts. Several techniques have been reported for the testing of antimicrobial activity of natural products including plant extracts at different time and stipulation. However, ACM is always employed for preliminary testing of antimicrobial activities of crude extracts, while advanced method like MIC is mostly used as secondary confirmation method. In ACM, all test bacteria showed zone of inhibition with petroleum ether, ethanol, methanol, and aqueous extracts. Among Gram-positives, *S. epidermidis*, *B. licheniformis*, and *B. subtilis* were absolutely inhibited by all extracts. Ethanol and methanol extracts of the leaf were most active inhibiting agent against both Gram-positive and -negative bacteria. On the other hand, petroleum ether extract had better antibacterial activity against most of the Gram-negative bacteria. Among Gram-negatives, the highest zone of inhibition was recorded for ethanol extract against *E. coli* O157:H7. From ACM, result was obtained that there was no significant difference among the test bacteria with respect to plant extracts, while there were marked differences between the activities of the plant extract and pure antibacterial drug (ciprofloxacin) [Figure 1]. Such significant differences are normally present when crude (unpurified) plant extracts are compared with pure drugs that are already in clinical use.[21] Also, the ACM is not always dependable for accurate assessment and comparison. This is because of the high degree of interference inherent in this method that arises from drug diffusion problems.[22]

Table 1: Phytochemical screening of *C. fistula* leaf

Name of the phytochemical	Qualitative test	PT	CH	ET	MT	AQ
Alkaloids	Mayer's reagent	-	-	+	+	+
	Dragendorff's reagent	-	-	-	-	-
	Hager's reagent	-	-	+	-	+
	Wagner's reagent	-	-	-	-	-
Carbohydrates	Molisch's test	-	-	-	-	-
	Fehling's test	-	-	-	+	+
	Benedict's test	-	-	-	+	+
Tannin and phenolic compound	With Ferric chloride	+	+	+	+	+
	With lead acetate	+	-	+	-	+
	With gelatin solution	-	-	-	-	-
Glycoside	Keller-Kiliani test	-	-	+	+	-
	Legal test	-	-	-	-	-
	Borntrager's test	-	-	-	-	-
Protein and amino acids	Biuret test	-	-	+	+	+
	Ninhydrin test	-	-	+	+	+
	Xanthoprotein test	-	-	-	-	-
	Millon's test	-	-	-	-	-
Gum and mucilages	Molisch's test	-	-	-	-	-
Flavonoids	With NaOH	+	+	+	+	+
	With H_2SO_4	-	+	+	+	+
	With Mg/HCl	-	-	+	+	+
Saponins	Honeycomb foam	-	-	-	-	-
	Foam test	-	-	+	+	+
Steroids and sterol	Salkowski's test	-	-	-	-	-
	Libermann burchard	-	-	-	-	-
Triterpenoids	Thionyl chloride test	+	-	+	+	-
Oils and fats	With filter paper	-	-	-	-	-
	With alkaline KOH	-	-	-	-	-
Vitamin C	With Indophenols	-	-	-	-	-
	Sodium nitroprusside	-	-	-	-	-

+: Presence; -: Absence

The MIC results showed that all the extracts were able to prevent the growth of all test organisms with selective activities. The growth of inhibition of test bacteria range from 94 to 1 500 μg/ml (w/v), with the lowest MIC value against *S. epidermidis* Table 3. The results of MBC showed that in a concentration of 1 500 μg/ml (w/v), most of the organisms from both Gram-positive and-negative bacteria were killed. These results clearly indicated that *S. epidermidis* was the most sensitive organism, which is inhibited with MIC values lower than 100 μg/ml recorded in 50% of the test cases. MIC values lower than 200 μg/ml were also

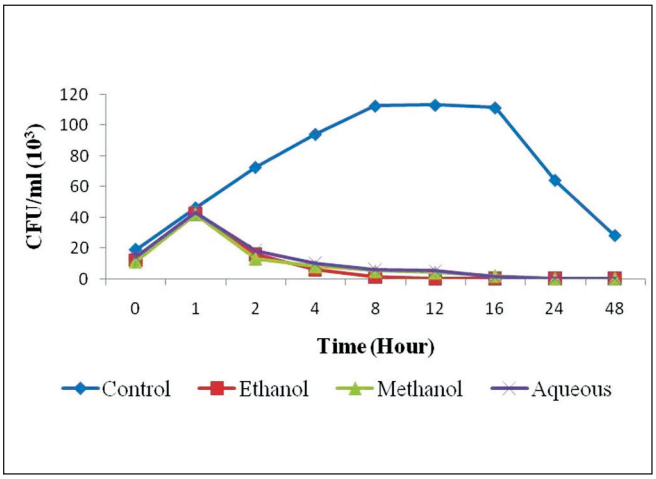

Figure 1: Time kill kinetics against *S. epidermidis*

Table 2: Antibacterial screening of different extracts of *C. fistula* leaf

Name of the strain	Zone of inhibition in mm					Ciprofloxacin
	PE	CH	ET	MT	AQ	
Gram-negative						
Escherichia coli	13.0±0.5	13.6±0.5	12.6±0.5	12.6±0.5	11.6±0.5	19.6±2.0
Escherichia coli O157:H7	12.6±0.7	-	14.6±0.7	12.6±0.7	13.3±1.4	23.0±2.0
Salmonella typhimurium	12.6±0.5	-	13.6±0.5	13.3±0.5	12.6±0.5	24.6±1.5
Shigella sonnei	12.3±0.6	-	15.0±1.0	14.3±0.5	12.3±1.5	26.6±1.1
Gram-positive						
Bacillus subtilis	14.6±0.7	12.3±0.7	14.6±0.7	12.6±0.7	13.6±0.7	16.6±1.5
Bacillus licheniformis	12.6±1.4	14.3±0.7	14.3±0.7	12.3±1.4	13.3±1.4	15.6±0.5
Staphylococcus aureus	10.6±0.5	10.6±0.5	13.6±0.5	13.3±0.5	12.6±0.5	29.0±2.0
Staphylococcus epidermidis	15.6±0.7	12.0±0.7	16.0±2.1	12.3±1.4	13.0±0.7	26.6±1.5

All values are mean zone of inhibition±SD (-) No zone of inhibition; Zone of inhibition including 6 mm borer; Extract concentration (30 mg/ml)

obtained with other extracts viz. ethanol, methanol, and aqueous against *B. subtilis*, *B. licheniformis*, and *S. epidermidis*. This activity could be considered very promising for two reasons, first, the test bacteria were resistant to the first line antibiotics and second, when compared with the reference drugs' (standard antibiotics) MICs (10 μg/ml). However, all lowest MICs exhibited by extracts with MBC value eight times of MIC in corresponding microorganisms highlighting their interesting antimicrobial potency. From these results, it can be observed that most of the tested samples exert a killing effect on the test organisms, as MBC and MFC values were recorded. However, when analyzing carefully the MIC and MMC results, it can be noted that MMC/MIC ratios lower than 4 was obtained with most of the studied samples, suggesting that killing effects could be expected.[23] The TLC results showed a total of 7 spots with R_f values 0.09, 0.43, 0.40, 0.375, 0.55, 0.64, and 0.72. Ethanol, methanol, and aqueous extracts had three similar R_f values (0.09, 0.43, 0.40) than other extracts, which was possible responsible for better antibacterial activity. Ethanol extract showed 5 distinct fractions with R_f value 0.72, 0.55, 0.43, 0.37, and 0.09. The same TLC plate of ethanol extract was subjected against *S. epidermidis* to study bioautography and result showed that the activity was limited to the fraction with R_f 0.72, which was evident as a bluish pink under exposure to UV light on the reference TLC plate with zone of inhibition 24 mm [Figure 2].

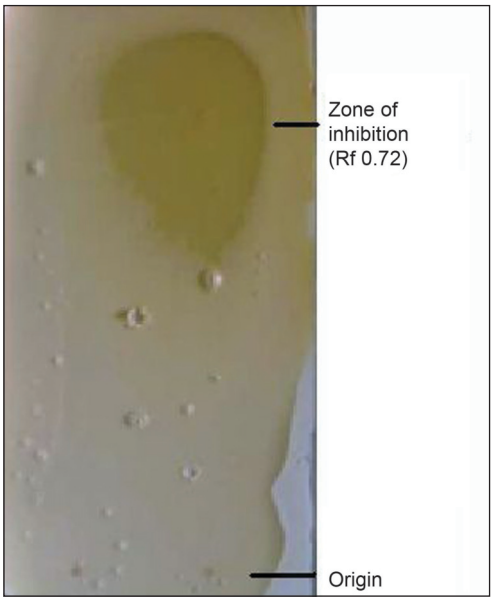

Figure 2: TLC Bioautography with *S. epidermidis*

Extracts of ethanol, methanol, and aqueous were further tested for antimicrobial activity with time kill curve experiment against selective organisms. Results show that ethanol, methanol, and aqueous extracts exhibit lytic activity against selective strain. This suggested membrane disruption as the likely mechanism of action. In case of *S. epidermidis*, ethanol, methanol, and aqueous extract were bactericidal in nature, exhibiting 50% survival after 4 hours of incubation. After 8 hours of incubation, ethanol and methanol exhibited a prominent kill with 1.9 and 2.1% survival, respectively. This was followed by aqueous extract which exhibit 5.26% survival after 8 hours incubation. This concludes that ethanol is a potent bacteriostatic agent as compared with that of methanol and aqueous.

Kumar *et al*.[9] reported antimicrobial activity of fruit of *C. fistula* by agar dilution streak method at a concentration of 500 μg/ml. Only *E. coli* was moderately inhibited, whereas no inhibition was found in case of *B. subtilis* and *S. epidermidis*. However, in our study, *B. subtilis* was completely inhibited at concentration of 375 μg/ml by ethanol, methanol, and aqueous extract, while *S. epidermidis* was inhibited at concentration of 187.5 μg/ml by the same extract. Valsaraj *et al*.[15] studied the antibacterial activity of *C. fistula* seeds by broth dilution method and antifungal activity by ACM. According to their observation at 12.5 mg/ml concentration, *E. coli* and *B. subtilis* were inhibited while *S. aureus* was inhibited at a concentration 6.25 mg/ml. Perumal Samy *et al*.[12] reported moderate antibacterial activity of *C. fistula* against a wide spectrum of bacteria such as *E. coli, Bacillus mycoides, B. subtilis, Mycobacterium smegmatis, Klebsiella aerogenes, Pseudomonas aerogenes,* and *Proteus vulgaris.* A polar compound including 5-nonatetra contanone,

Table 3: MIC and MBC of different extracts of *C. fistula* leaf

Plant extract	Test organism	Leaf				Antibiotic	
	Gram-negative	MIC µg/ml	MBC µg/ml	MBC/ MIC	TA mg/ml	MIC µg/ml	MBC µg/ml
Petroleum Ether	*Escherichia coli*	1 250	5 000	4.0	19.6	10	10
	Escherichia coli O157:H7	1 250	5 000	4.0	19.6	10	10
	Salmonella typhimurium	1 250	5 000	4.0	19.6	10	10
	Shigella sonnei	625	1 250	2.0	39.2	10	10
	Gram-positive						
	Bacillus subtilis	750	1 500	2.0	32.6	10	10
	Bacillus licheniformis	750	1 500	2.0	32.6	10	10
	Staphylococcus aureus	625	5 000	8.0	39.2	10	10
	Staphylococcus epidermidis	375	1 500	4.0	65.3	10	10
Chloroform	*Escherichia coli*	1250	>5 000	-	12.2	10	10
	Escherichia coli O157:H7	-	-	-	-	10	10
	Salmonella typhimurium	-	-	-	-	10	10
	Shigella sonnei	-	-	-	-	10	10
	Bacillus subtilis	750	>3 000	-	20.3	10	10
	Bacillus licheniformis	750	3 000	4.0	20.3	10	10
	Staphylococcus aureus	1250	>5 000	-	12.2	10	10
	Staphylococcus epidermidis	750	>5 000	-	20.3	10	10
Ethanol	*Escherichia coli*	625	5 000	8.0	253.8	10	10
	Escherichia coli O157:H7	312	2 500	8.0	507	10	10
	Salmonella typhimurium	1250	5 000	4.0	127	10	10
	Shigella sonnei	312	1 250	4.0	507	10	10
	Bacillus subtilis	187	375	2.0	848	10	10
	Bacillus licheniformis	187	375	2.0	848	10	10
	Staphylococcus aureus	312	625	2.0	507	10	10
	Staphylococcus epidermidis	94	375	4.0	1687	10	10
Methanol	*Escherichia coli*	625	5 000	8.0	69.4	10	10
	Escherichia coli O157:H7	-	-	-	-	10	10
	Salmonella typhimurium	625	2 500	4.0	69.4	10	10
	Shigella sonnei	312	625	2.0	139.1	10	10
	Bacillus subtilis	375	750	4.0	115.7	10	10
	Bacillus licheniformis	375	750	2.0	115.7	10	10
	Staphylococcus aureus	625	2 500	4.0	69.4	10	10
	Staphylococcus epidermidis	94	375	4.0	461.7	10	10
Aqueous	*Escherichia coli*	1 250	>5 000	-	54.6	10	10
	Escherichia coli O157:H7	1 250	5 000	4.0	54.6	10	10
	Salmonella typhimurium	625	5 000	8.0	109.1	10	10
	Shigella sonnei	1 250	5 000	4.0	54.6	10	10
	Bacillus subtilis	375	1 500	4.0	181.9	10	10
	Bacillus licheniformis	375	750	2.0	181.9	10	10
	Staphylococcus aureus	125	250	2.0	54.6	10	10
	Staphylococcus epidermidis	187	750	4.0	364.7	10	10

2-hentriacontanone, triacontane, 16-hentriacontanol, and sitosterol along with oil showing antibacterial activity had also been isolated in *C. fistula* pods.[10] Recently, Vimalraj *et al*.[16] tested the antibacterial activity of aqueous and alcoholic extract of stem bark of *C. fistula* with disc diffusion and MIC methods. Alcoholic extracts recorded greater inhibition against *S. aureus* compared with aqueous extract. Zones of inhibition of alcoholic and aqueous extracts were in the range of 7.0 to 12.0 mm and 7.0 to 11.6 mm, respectively. MIC values of the alcoholic extracts against *S. aureus* were in the range of 0.78 to 6.25 mg/ml . Our finding was almost coinciding with this study.

Though reports (Kumar *et al*., Perumal Samy *et al*., and Valsaraj *et al*.)[9,12,15] are available on antimicrobial activities of seeds, pods, bark of *C. fistula*, no work has been conducted on potentiality of leaves of this plant. Our report is of the first for antibacterial activity on leaf extracts of *C. fistula*. *S. epidermidis* is a part of normal skin flora, and is often attached to the upper layer of the skin (epidermis) or mucosa, without causing any symptom. When the skin is injured (wounds, burns, intravenous drug addicts, etc), *S. epidermidis* may enter into deeper layers of the skin or even the blood and cause an infection. *S. epidermidis* is the most common causative agent of post-cataract surgery endophthalmitis.[24] Since this bacterium is completely inhibited by leaf extracts, the plant can be useful for the treatment of *S. epidermidis*.

The presence of antibacterial substances in the higher plants is well established. Plants are the source of inspiration for novel drug compounds as plant-derived medicines have made significant contribution toward human health. Isolation of bioactive compounds from plant material largely depends on the type of solvent used in the extraction procedure. The traditional healers use primarily water as the solvent. This study concludes that the plant extraction by ethanol provided more consistent antimicrobial activity compared with water extract.

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