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Evaluation of *in vitro* Antimicrobial Activity from the Leaves Extract of *Cassia fistula* Linn.

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The present study was undertaken with the objective of testing the antibacterial and antifungal activities of *Cassia fistula* leaves and identifies the bioactive compounds. The antimicrobial activity of leaves extract were determined in aqueous and organic extracts, and the minimum inhibitory concentration (MIC) against 6 species of pathogenic & non-pathogenic microorganisms: *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* using disc diffusion method. The chemical constituents of organic plant extracts were separated by thin layer chromatography (TLC) and the plant extracts were purified by column chromatography and were further identified by Gas chromatography-mass selection (GC-MS) analysis. Significant inhibitory activity was observed with methanol extracts of plants against the test microorganisms while less antibacterial activity was observed in hexane, acetone and aqueous extracts. The MIC of the plant extracts ranged between 1000μg to 2000μg/ml. The methanolic extract of *C. fistula* leaves had potent anti bacterial and antifungal activities against tested microorganisms. 12 compounds were identified in *C. fistula* leaves extract by GCMS analysis. The composition of *C. fistula* revealed the presence of aliphatic acids and alcohols.

Key words: Antimicrobial activity- Medicinal plants- MIC- TLC- GCMS.

Due to the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effect of certain anti-microbial agents, there is need to search for new antimicrobial and phytochemical constituents and using them for the treatment of microbial infections as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant[1]. Antibiotics are an essential part combating for harmful bacterial infections *in vivo*. During the last decade infectious diseases have played a significant role in the death of millions around the world, especially in developing countries like India. Because of the mutagenic nature of bacterial DNA, the rapid multiplication of bacterial cells, and the constant transformation of bacterial cells due to plasmid exchange and uptake, pathogenic bacteria continue to develop antimicrobial resistance and thus rendering certain antibiotics useless. An increased number of pathogens have also developed resistance to multiple antibiotics (Multiple Drug Resistance), threatening to develop complete immunity against all antimicrobial agents and therefore the search for novel antimicrobial agents is of the utmost importance[2]. It is therefore, desirable and essential to develop effective, safe and natural methods to

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control harmful pathogen growth. Use of plant products for the control of diseases has certain advantages such as biodegradability, availability, low toxicity and cost effectiveness. Plant produces complex mixture of natural compounds which also include terpenes. Many terpenes have been found to be active against a variety of microorganisms. Test has been performed on Gram-positive and Gram-negative bacteria and also on fungi [3].

Cassia fistula, a member of the Leguminosae family and is commonly known as Golden shower tree or Golden shower cassia. Other names also include Indian laburnum, golden shower and drumstick tree. It has been found throughout India, Ceylon, Malaya, China, South Africa and the West Indies. *C. fistula* is a medicinal plant used for varied purposes [4, 5]. The plant has a high therapeutic value and it exerts an antipyretic, analgesic and hypoglycemic activity and it is widely used as a mild laxative suitable for children and pregnant women. This plant has described to be useful in the treatment of haematemesis, pruritus, echinoderm, diabetes, skin diseases and in liver troubles [6]. In Aurvedic medicine, Golden shower tree is known as 'ARAGVADHA (disease killer) contains elevated quantities of anthraquinones and has been consequently mainly useful against gastrointestinal conditions (e.g. constipation or acid reflux). The leaves of *C. fistula* are laxative and indicated anti-rheumatic, anti-periodic, anti-tussive and wound healing properties [7, 8]. The leaf juice of this plant is used in folklore to treat cough in Tripura, India. Studies has been undertaken to evaluate the anti cough effect of the leaf extract against sulphur dioxide induced cough reflex in mice. According to the Ayurvedic and Unani system of medicine various parts of *C. fistula* has been highly useful in curing various diseases and as an antifertility, antitumor and hepatoprotective agent. *C. fistula* plant organs are known to be an important source of secondary metabolites, notably phenolic compounds [9]. So it would be essential to evaluate the phytochemical composition of this plant because of its widespread utilization as a folk medicine and to ensure that the phytoconstituitents are novel and may offer a possible role to play as effective antimicrobial agent. Therefore, the aim of the present study is to analyze the potential of *C. fistula* leaves extract for antimicrobial activity and detection of bioactive compounds by TLC, purification by column chromatography and characterization by GC-MS.

MATERIAL AND METHODS

Plant material

The major raw material used in this work was freshly harvested *C. fistula* leaves obtained from National Gir Foundation, Gandinagar (Gujarat). Fresh leaves were washed under running tap water followed by sterilized distilled water, air-dried and then powdered with the help of sterilized pestle and mortar. Most of the chemicals used were procured from Hi-media and Sigma chemicals.

Aqueous Extract Preparation

The air-dried fine powdered 10g leaves were boiled in 400 ml distilled water till one fourth of the extract initially taken was left behind after evaporation. The solution was then filtered using muslin cloth. Filtrate was centrifuged at 5000 rpm for 15 min. The supernatant was again filtered using Whatman Filter No. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles and stored at 4°C until further use.

Organic solvent extract preparation

Air-dried 10g powder was thoroughly mixed with 100 ml organic solvent (viz., Methanol, Hexane and Acetone). The mixture was placed at room temperature for 24h on shaker with 150 rpm. Solution was filtered through muslin cloth and then re-filtered by passing through whatman filter No. 1. The filtrate thus obtained was concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts for each type of organic solvent were prepared by mixing well the appropriate amount of dried extracts with dimethyl sulphoxide (DMSO) to obtain a final concentration of 100mg/ ml that was used for evaluation of antibacterial and anti fungal activities. Each solution was stored at 4°C after collecting in sterilized bottles until further use.

Microbial cultures

The test microorganisms used for the antimicrobial activity screening were *Staphylococcus aureus* (ATCC 25923), *Bacillus*

subtilis (ATCC 10707), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Life Sciences, Gujarat University and MG Institute of Science. All the cultures were maintained and sub cultured on nutrient agar medium.

Antibacterial assay

In vitro antibacterial activities of all aqueous and organic extracts of *C. fistula* were determined by standard agar well diffusion assay. Petri dishes (100mm) containing 25 ml of Mueller Hinton Agar (MHA) were seeded with 100µl inoculum of bacterial strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6CFU/ml). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria inoculated. Wells of 6mm diameter were cut into solidified agar media with the help of sterilized cup-borer. 100µl of each extract was poured in the respective well and the plates were incubated at 37°C for overnight. DMSO and sterilized distilled water were used as negative control while tetracycline antibiotic (one unit strength) was used as positive control. The experiment was performed in triplicate under strict aseptic conditions and the antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by the respective plant extract at the end of incubation period.

MIC for the bacteria

The antibacterial activity of the extracts was examined by determining the MIC in accordance with the Clinical and Laboratory Standard Institute (CLSI) methodology (CLSI 2005). The minimum bactericidal concentration (MBC) was taken as the lowest concentration of each extract that resulted in more than 99.9% reduction of the initial inoculum. All tests were carried out in Mueller Hinton broth (MBH) supplemented with dimethyl sulphoxide (DMSO) at a final concentration of 10% (v/v) to enhance their solubility. The extracts were dissolved in MHB. Test strains were suspended in MHB to give a final density of $5x10^5$ cfu ml^1 and these were confirmed by viable counts. Dilutions ranging from 100 to 2000mg/ml of the extracts were prepared in tubes including one growth control, MHB+ DMSO 10% (v/v) and one sterility control MHB+ DMSO 10% (v/v + test extracts). The MIC values were determined from visual examinations as being the lowest concentration of the extracts with no bacterial growth. Plates were incubated under normal atmospheric conditions at 37°C for 24h for bacteria.

MIC for the yeasts

The antifungal activity of the extracts was examined by determining the MIC in accordance with guidelines of British Society for Medical Mycology (BSMM) using Yeast Nitrogen Base Glucose (YNBG) medium supplemented with DMSO at a final concentration of 10% (v/v).The extracts were dissolved in YNBG medium. Yeast strains were cultured for 24-48 h at 35°C on SDA and then suspended in 4ml of sterile distilled water by adjusting to 1 McFarland using a nephelometer to give a final inoculum concentration of $1.5\pm1.0x10^3$ cfu ml^{-1}. Dilutions ranging from 100 mg ml to 2000 mg ml of the extracts were prepared in the tubes including one growth control, YNBG + DMSO 10% (v/v), and one sterility control YNBG 10% (v/v + test extracts). A 100 µl suspension of each of *Candida* strains in YNBG was added to individual wells and incubated at 35°C for 48h. The MICs of the extracts were defined as the lowest concentration that inhibited more than 80% of visible fungal growth. The final concentration of dimethyl sulphoxide in the assays did not interfere with the bacterial and candidal proliferation.

Purification by column and identification by GC-MS analysis

The dried leaves (250gms) were powdered and exhaustively macerated with methanol for 1week in a shaker. The collected extracts were filtered and evaporated under vacuum. The residues were dried and weighed.

TLC of plant extract

TLC was used to select a suitable eluent from methanol, chloroform, toluene, petroleum ether and ethyl acetate singly and in combinations. Firstly, the sample dissolved in methanol were deposited on the TLC plate using a 0.5 mm glass capillary and dried with air stream. Methanol, chloroform, toluene, petroleum ether and ethyl acetate singly and in combination were used as

developing agents. Finally, these TLC plates were immersed into above solutions in wide-mouthed bottles. The colour was intensified under the condition of iodine vapor after drying. According to the results, a suitable eluent for separation was selected out which is given in table 3 and 4.

Column chromatography separation

Wet-process method was adopted to pack the column. Silica gel acted as a sorbent and mixture of Petroleum ether + ethyl acetate in ratio 80: 20 and Toluene + ethyl acetate in ratio 80: 20 one by one were the eluents. At the beginning, silica gel was added to the eluent. It was stirred and slowly poured into the column until the bed layer was solid. The temperature of the column was 25°C. The petcock was opened to decrease eluent level until its liquid surface and silica gel level were equal. Then the sample solution was poured slowly into the column along its wall. Finally, the eluent was added to the column, keeping the sorbent covered by the eluent. For both the mixtures five samples are collected. The separated compounds were collected in numbered test tubes and identified by TLC. The same tubes containing the compound were combined for condensing on a rotary evaporator.

GC-MS Analysis

For GC-MS analysis, a Hewlett-Packard-5890-II (Global Medical Instrumentation) gas chromatograph, equipped with a flame-ionization detector (FID) and coupled with an electronic integrator was used. Quantitative data were obtained by electronic integration of the FID-area data, without response factor correction. The gas chromatograph was fitted with a fused-silica capillary column (30m x 0.25 mm i.d., film thickness 0.25 µm). Helium was used as the carrier gas at 35.6 K Pa pressure with a flow rate of 1ml/ min; split ratio of 20.0; injection volume 1µl; injection temperature, 280°C at a rate of 9°C/ min; the final temperature of 280°C was held for 5 min. The mass spectrometer operated in EI mode at 70Ev, using a scanning speed of 1.5s over the range 40-300 amu and an ion source temperature

RESULTS AND DISCUSSION

It was observed that aqueous extract are not as effective as organic extract. Antimicrobial activity was observed in organic extract among which methanol extract has shown promising activity against tested organisms. No significant activity was observed in hexane and acetone extract and the results have been summarized in Table 1.

Data indicated that organic extract of leaves exhibited better anti-bacterial activities than those of aqueous extracts. Aqueous extract was found almost ineffective in inhibition of bacterial strains producing zone of inhibition ≤ 8mm. The organic extract of leaves shows the zone of inhibition ranging from 12mm to 17mm which is effective against tested microorganisms.

Among all the bacterial strains tested, *Staphylococcus aureus* was found most susceptible with maximum inhibition by methanolic extract producing zone of inhibition >16mm (Table 1). Extracts prepared in organic solvents consistently displayed better antimicrobial activity than that of aqueous extracts. Furthermore, extracts prepared in methanol was observed most inhibitory (diameter of zone ranging from 16mm to 12mm) followed by those prepared in hexane and acetone, respectively.

The present study also revealed that the use of organic solvents in the preparation of plant extracts provides more consistent antibacterial activity as compared to aqueous extracts. This observation clearly indicates that the polarity of antimicrobial compounds make them more readily extracted by organic solvents and using organic solvents does not negatively affect their bioactivity against bacterial and fungal species. This finding is also supported by several workers[10-12] that water extracts of plants do not have much activity against bacteria. Eloff [13] reported that methanol and ethanol were the most effective solvent for plant extraction than n-hexane and water. Present study indicated that methanol is ideal solvent to extract antimicrobial compounds found in leaves.

In general, the cell walls of Gram-negative organisms, which are more complex than Gram-positive ones, act as a diffusional barrier and making them less susceptible to the antibacterial agents than the Gram-positive bacteria [14]. In spite of this permeability difference, however methanol extracts of *Cassia fistula* leaves have still exerted some degrees of inhibition against Gram-negative *Salmonella typhimurium* as well. The antifungal substances contained in

the leaf extracts of *C. fistula* probably caused leaks in the cell wall or some alteration in the membrane permeability, resulting in the loss of the cytoplasm. These findings were consistent with many workers who observed that *Cassia* species leaves containing anti fungal properties. [15, 16, 17]

All antimicrobial activity occurred in a concentration dependent manner as suggested by MIC determination. However, the efficacy of extracts is less than to that of standard antibiotic, tetracycline.

Leaf extract (prepared in methanol) were subjected for determination of minimum inhibitory concentration (MIC) by standard two fold microbroth dilution method against susceptible bacterial species (Smith *et al* 2009). The present study shows that methanolic extract of *C. fistula* leaves had potent anti bacterial and antifungal activities against tested microorganisms (Table 2).

MIC of this extract was 2000µg/ml against *Staphylococcus aureus* and 1500µg/ml for *Bacillus subtilis* while it was 1000µg/ml for Gram-negative *Salmonella typhimurium.* The present study also shows that methanolic extract of *C. fistula* leaves had potent anti *C. albicans* activity with an MIC value of 2000µg/ml.

It can be concluded that polar protic solvents may be beneficial for augmenting antibacterial activity of both Gram negative and Gram positive microorganisms. It is worthwhile to pursue further research by taking polar protic solvent with different dielectric constant.

The methanol extract of *C. fistula* was subjected to purification by column chromatography with the combinations of solvents i.e. Petroleum ether + ethyl acetate, Toluene + ethyl acetate, Petroleum ether + chloroform and TLC purification of these fractions have been presented in Tables 3 & 4.

Table 1. Result of antimicrobial screening of aqueous and organic plant extracts of *C. fistula* determined by agar diffusion method (Zone of inhibition in mm diameter)

Plant type	Extraction Type	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*	*P. aeruginosa*	*C. albicans*
Cassia fistula	Methanol	18	16	6	14	5	17
	Hexane	4	5	5	5	4	6
	Acetone	7	6	6	5	5	11ND
	Aqueous	7	8	6	5	4	
Positive Control	Tetracycline	20	22	24	20	20	22
Negative Control	DMSO	-	-	-	-	-	-

Zone of inhibition (in mm diameter) includes the diameter of well (6mm) in agar well diffusion assay.
Assay was performed in triplicate and results are the mean of three values. In each well, the sample size was 100µL.
Tetracycline: One unit strength. ND- Not Detected

Table 2. Results of minimum Inhibitory Concentration (MIC) of methanol extract of *C. fistula*

Plant type	Extraction	Minimum inhibitory Concentration (µ g/ ml)					
	Type	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*	*P. aeruginosa*	*C. albicans*
Cassia fistula	Methanol	2000	1500	NI	1000	NI	2000
Positive Control	Broth+ T.O	G	G	G	G	G	G
Negative Control	Broth + C.E	NG	NG	NG	NG	NG	NG

T.O- Test Organisms, C. E- Crude Extract, G- Growth, NG- No Growth, NI- No Inhibition

These purified fractions were subjected to GCMS analysis which revealed that extract is a complex mixture of many constituents, 12 compounds have been identified in *C. fistula* leaves extract. The results were summarized in Table 5 and the test plants respective chromatogram was shown in Fig. 1.

The compositions of *C. fistula* extract revealed the presence of aliphatic acids and alcohols. Lupeol, stigmast-5-en-3-ol, beta-tocopherol, phytol, hexadeconic acids, hexadeconic acids methyl esters, Octadecadienoic acids and octa decatrienoic acid were the major components of this extract, lupeol, vitamin E acetate and stigmasterol were the most abundant one (30.95%, 17.54% and 17.28%). Others like hexadecanoic acid (10.13%), beta-tocopherol (6.82%) and phytol (3.75%) were also present in this extract.

Table 3. TLC results of different organic solvents when used singly

Elution Of Organic solvent	Result of Elution
Methanol	No separation
Chloroform	Spots are not clear
Toluene	Blur spot
Petroleum ether	Blur spot
Ethyl acetate	No separation

The present study evaluated the development of medicinal plant *C. fistula* which exhibited antibacterial activity against two Gram positive bacteria viz. *S. aureus*, *B. Subtilis* and against one fungus viz. *C. albicans*. The findings are consistent with those of Deepa *et al*. [18] who observed that Cassia species containing

Table 4. TLC results of different organic solvents when used in combinations

Elution of plant extracts (Ratio)	Result of Elution
P.E + E.A (40: 60)	One spot
P.E + E.A (50: 50)	One single clear spot
P.E + E.A (70: 30)	One spot
P.E + E.A (80: 20)	One single clear spot
P.E + E.A (60: 40)	ND
E.A + D.W (50: 50)	ND
BE + E.A (50:50)	No clear separation
BE + E.A (70:30)	No separation
TO + E.A (40:60)	No separation
TO + E.A (60:40)	ND
TO + E.A (80:20)	Quite clear spots are there
TO + E.A (70:30)	Not so clear spot
P.A + CH (60:40)	ND
P.A + CH (40:60)	ND

P.E- Petroleum ether; E.A- Ethyl acetate; BE- Benzene; TO- Toluene; CH- Chloroform; DW- Distilled Water, ND- Not Detected

Table 5. Compounds present in the *Cassia fistula* leaf extract

Compound	Retention time (min)	Amount (%)
2-Pentadecanone 6, 10, 14-trimethyl	33.331	1.05
5, 9, 13-Pentadecatrien -2-one, 6, 10, 14-	34.856	2.83
Hexadecanoic acid, methyl ester	34.948	2. 48
Hexadecanoic acid	35.823	10.13
9, 12-Octadecadienoic acid, (Z,Z)-, met	38.146	0.94
9, 12, 15-Octadecatrienoic acid, methyl	38.279	3.46
Phytol	38.498	3.75
5-methyl-5-(4, 8, 12- trimethyltridecyl) dil	42.657	3.35
beta-Tocopherol	52.804	6.82
Vitamin E acetate	53.874	17.54
Stigmast-5-en-3.-ol	56.274	17.28
Lupeol	57.414	30.95

The values in bold represent the main components found in leaf extract

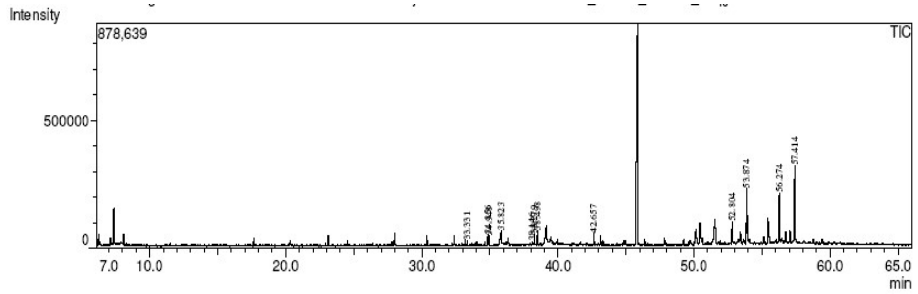

Fig. 1. Chromatogram of *C. fistula* leaf extract

anthraquinone, flavonoids and polysaccharides showed considerable activity against gram-positive microorganisms. The results are important since *S. aureus* is an important pathogen in man and animals, where resistance to other drugs is frequently reported. Methicillin resistant *Staphylococcus aureus* are widely distributed among hospitals and increasingly isolated from community-acquired infections [19]. The antimicrobial activity seems to depend on the contents of phenolic group in the plant extracts. High amounts of phenolic group present in the leaves of *C. fistula* implied that anthraquinone may be the active compound, which may be responsible for the antibacterial activity [20]. So the extracts of the leaves of *C. fistula* have high potential as antibacterial agent. It would be worthwhile to isolate the active fractions for further testing and use this plant for preparing a broad-spectrum drug.

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