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Research Article

ANTIMICROBIAL SCREENING OF LEAF EXTRACTS OF CASSIA FISTULA LINN

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ABSTRACT

This study was carried out with an objective to investigate the antibacterial and antifungal potential of seeds of Cassia fistula Linn. The aim of the study is to assess the antimicrobial activity and to determine the zone of inhibition of extracts on some bacterial and fungal strains. In the present study, the microbial activity of chloroform extracts of seeds of Cassia fistula Linn. (An ethno medicinal plant) were evaluated for potential antimicrobial activity against medically important bacterial and fungal strains. The antimicrobial activity was determined in the extracts using agar disc diffusion method.. Crude extracts of Cassia fistula exhibited moderate to strong activity against most of the bacteria tested. Zones of inhibition of extracts were compared with that of different standards like Ampicilline, Ciprofloxacin, Norfloxacin and Chloramphenicol for antibacterial activity The extracts showed antibacterial activities comparable with that of standard against the organisms tested. The results showed that the remarkable inhibition of the bacterial growth against the tested organisms. The phytochemical analyses of the plants were carried out. The microbial activity of the Cassia fistula was due to the presence of various secondary metabolites. Hence these plants can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceutical research activities.

Keywords: *In vitro Antibacterial activity, antifungal activity, Cassia fistula, bacterial pathogens, secondary metabolites*

INTRODUCTION

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health related quality of human life since their introduction. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Systematic studies among various pharmacological compounds have revealed that any drug may have the possibility of possessing diverse functions and thus may have useful activity in completely different spheres of medicine. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary health care system.[1, 2] Herbs are widely exploited in the traditional medicine and their curative potentials are well documented.[3] About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful especially in the areas of infectious disease and cancer.[4] Recent trends, however, shows that the discovery rate of active novel chemical entities is declining.[5] Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action.[6, 7] The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world.[8] Much work has been done on ethno medicinal plants in India.[9]

In the recent years, research on medicinal plants has attracted a lot of attentions globally. Large body of evidence has accumulated to demonstrate the promising potential of Medicinal Plants used in various traditional, complementary and alternate systems of treatment of human diseases. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides etc, which have been found in vitro to have antimicrobial properties.[10, 11] Herbal medicines have been known to man for centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine. [12] Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The world health organization estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. [13] The harmful microorganisms can be controlled with drugs and these results in the emergence of multiple drug resistant bacteria and it has created alarming clinical situations in the treatment of infections. The pharmacological industries have produced a number of new antibiotics; resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents. [14]

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Therefore, actions must be taken to reduce this problem, such as to minimize the use of antibiotics, develop research of resistance, among microorganism and to continue studies to develop new drugs, either synthetic or natural to control pathogenic microorganism. In an effort to expand the spectrum of antibacterial agents from natural resources, Cassia fistula belonging to Leguminosae family has been selected.

In the Indian literature, this plant has been described to be useful against skin diseases, liver troubles, tuberculoses glands and its use in to the treatment of haematemesis, pruritus, leucoderm and diabetes has been suggested.[15, 16] It has been concluded that plant parts could be used as a therapeutic agent in the treatment of hypercholesterolemia partially due to their fiber and mucilage content.[17] Besides its pharmacological uses, the plant extract is also recommended as a pest and disease control agents in India.[18- 20] This plant is widely used by tribal people to treat various ailments including ringworm and other fungal skin infections.[21] Seeds are used to treat skin diseases. Cassia fistula plant organs are known to be an important source of secondary metabolites, notably phenolic compounds. [22]
Cassia fistula exhibited significant antimicrobial activity and showed properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents.[23] Thus C.fistula is well anchored in its traditional uses and has now found wide spread acceptance across the world.

MATERIALS

PLANT SAMPLE COLLECTION:

The materials used in this study consisted of dried leaves of Cassia fistula which are grown In Hyderabad India. These leaves were collected near my house,
At Rampally village, keesara mandala, Hyderabad Telangana India. Adhering dirt's were removed by washing and were cut into small pieces. The plant parts were dried at room temperature avoiding sunlight. The dried parts were then milled to form powder (2kg). Dry powder of leaves was soaked in ethanol (absolute alcohol) for 7 days in a glass container closed by glass cork with occasional shaking and stirring. The extract was filtered through cotton and filter paper and concentrated by rotary evaporator under reduced pressure (below 50°C) to afford semisolid masses.

Leaf material was homogenized into fine powder and stored in airtight container.



Fig-1: Fine dried leaf powder of cassia fistula

BACTERIAL SAMPLE:

Pathogenic strains of staphylococcus aureus and Lactobacillus were obtained from the Microbiology department from K.P .Labs Hyderabad, and were maintained on agar medium at 4 c for further experiments.

Antibacterial Screening

The ethanol extract (semisolid masses) were examined for their antibacterial potency by disc diffusion method against nine bacterial species (4 Gram- positive and 5 Gram-negative)

CULTURE MEDIA AND CHEMICALS:

Types of media was required for carrying out this study. Nutrient agar, peptone, sodium Chloride, beef extract .Also ethanol and methanol was used for extraction process and other Chemical etc. These chemical media and the solvent were used in the college (KGRITM) Hyderabad

ANTIBIOTICS & NON – ANTIBIOTIC DRUGS:

Antibiotics used include: Streptomycin.

These were collected from the APOLLO PHARMACY near kushaiguda ECIL X ROADS.

ANTIBIOTICS	ANTIBIOTICS POTENCY	MANUFACTURED BY
Streptomycin	250mg	Dr . Reddy's

TABLE 1: ABOUT THE STANDARD DRUG

METHOD

5.5.1 PREPARATION OF HYDROALCOHOLIC EXTRACTS (MACERATION):

Hydroalcoholic extract 50grams of leaves powder of CASSIA FISTULA was kept for maceration with 800ml of methanol, chloroform and petroleum ether separately for 5 days.
The extract was double filtered by using muslin cloth and Whatman no.1 filter paper



Fig-2: Maceration with Methanol, Chloroform and Petroleum ether

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5.5.2 DRYING OF EXTRACTS:

Extracts were dried separately. The chloroform, p .ether extracts were concentrated by evaporation on heating mantle at a temperature of 400 C. The extract was dried and used. The percentage yield of extract was found to be 45.89 grams. The methanolic extract was dried by evaporation until thick concentrated fluid was formed.

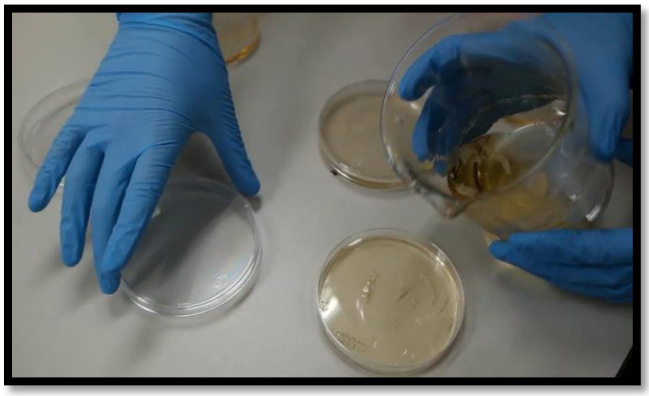
5.5.3 PREPARATION OF PLANT EXTRACTS AND DRUG STANDARD

CONCENTRATIONS:

1gram of each drug, hydroalcoholic extracts (each separately) was taken and the extracts were dissolved in 5ml of Di-Methyl Sulphoxide (DMSO).
Thus, 200mg/ml of stock was obtained as a standard concentration of alcoholic extracts and these were pasteurized for 15minutes at temperature 62^0C

PROCEDURE

Suspend 28grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. Mix well before pouring.
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.



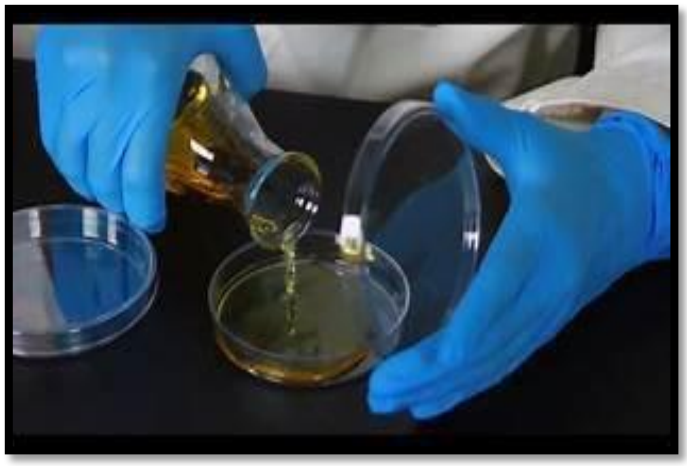


Fig 3 Pouring of Agar medium in to petri plates.

RESULTS

STERILITY TESTING OF PLANT EXTRACTS:

Methanolic, Chloroform and Petroleum ether extracts of CASSIA FISTULA was found to be free from bacterial and fungal contaminants as observed by streaking it on Nutrient agar plates after 1 week of incubation at room temperature.
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.

6.1 CUP PLATE METHOD:

After extraction of extract compounds from the solvent, Nutrient agar media was prepared and sterilized and inoculated within 24hours, culture of test organism and plated after solidification cups are made by a sterile rubber borer.
The compound concentrate was added into the cup by micropipette and kept for diffusion of the compound and incubated at 370C for 24 hours and observe the zones of inhibition in the plates.
The effectiveness of test and a range of antibiotics was determined against Staphylococcus aureus.
In this technique, an inoculum bacterium was added to melted, cooled agar. The agar inoculums mixture is then poured into a petriplates.
Up on solidification, isolated cells are trapped within the agar medium. These cells give rise to isolated pure colonies of bacteria. In an individual petri plate, single sample was taken and the activity is compared by the minimum inhibitory concentration and zone of inhibition studies.

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6.2 EVALUATION OF ACTIVITY:

MINIMUM INHIBITORY CONCENTRATION:

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.
ZONE OF INHIBITION (Kirby –Bauer Test):

In this, the size of the zone of inhibition indicates the degree of sensitivity of bacteria to a drug. In general, a bigger area of bacteria – free media surrounding an antibiotic disk means the bacteria are more sensitive to the drug the disk contains.

6.2.1 ACTIVITY AGAINST STAPHYLOCOCCUS AUREUS:

As shown in the table below the different zone of inhibition of chloroform, petroleum ether and methanolic extract, streptomycin was given. The zone of inhibition appeared as

S.NO	Concentration	Zone of inhibition (mm)
Methanolic extract	300µg /ml	28
Chloroform extract	300µg /ml	23
Petroleumether extract	300µg /ml	21
Streptomycin	300µg /ml	24

Table 6: Evaluation activity against staphylococcus aureus

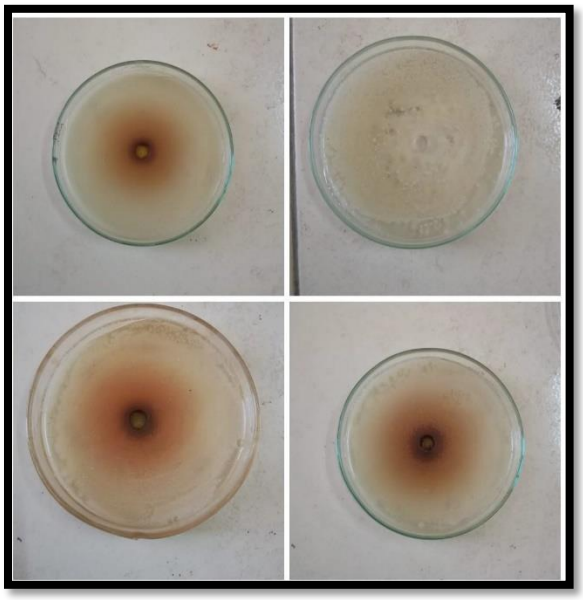


Fig-4: Activity against staphylococcus aureus

Preliminary phytochemical screening:

The extracts were subjected to preliminary phytochemical testing to detect the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, triterpenoids, steroids, glycosides, anthraquinones, cumarin, saponins, gum, mucilage, carbohydrates, reducing sugars, starch, protein, and amino acids.
Test microorganisms and growth media:

The following microorganisms: S. aureus (MTCC 96) was chosen.

Determination of zone of inhibition method In vitro antibacterial were examined for Hydro alcoholic and chloroform extracts. Antibacterial activitiy of plant extracts against four pathogenic bacteria (two gram positive and two gram negative) and three pathogenic fungi were investigated by the agar disk diffusion method. Antimicrobial activity testing was carried out by using the Agar cup method. Each purified extracts were dissolved in DMSO, sterilized by filtration using sintered glass filter and stored at 4°C. For the determination of zone of inhibition (ZOI), two gram positive, two gram negative and three fungal strains were taken as a standard antibiotic for comparison of the results. All the extracts were screened for their antibacterial and antifungal activities against the E. coil, P. aeruginosa, S. aureus, S. pyogenes, and the fungi C. albicans, A. niger, and A. clavatus. The sets of five dilutions (5, 25, 50, 100, and 250 µg/mL) of C. fistula extract and standard drugs were prepared in double distilled water using nutrient agar tubes. Muller Hinton sterile agar plates were seeded with indicator bacterial strains (108 cfu) and allowed to stay at 37°C for 3 h. The zones of growth inhibition around the disks were measured after 18 to 24 hours of in incubation at 37°C for bacteria . The sensitivity of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values.

Antibacterial activity of hydro alcoholic and chloroform extracts of Cassia fistula [zone of inhibition]

Microorganism	Zone of inhibition (mm) Concentration in µg/mL									
	Hydroalcoholic extracts (µg/ml)					Chloroform extracts (µg/ml)				
	5	25	50	100	250	5	25	50	100	250
E. coli	-	16	17	17	19	-	14	16	18	20
P. aeruginosa	-	13	14	17	18	-	13	15	17	18
S. Pyogenes	-	12	13	15	17	-	10	15	16	20
S. aureus	-	12	14	15	18	-	12	14	15	18

- = No zone of inhibition

Cellular Toxicity by Mtt Assay for the Ethanol, Methanol, Chloroform Extracts of Cassia Fistula Leaves

S.no	Concentration (ug)	Ethanol	Methanol	Chloroform
1	20	30.48	15.20	12.12
2	40	37.34	20.29	12.66
3	60	54.33	22.49	26.85
4	80	55.45	29.45	28.67
5	100	58.52	36.56	31.45
6	120	58.62	39.15	40.45
7	140	58.65	48.56	53.21

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8	160	61.92	61.92	61.24

TABLE 7

S.no	Concentration (ug)	%Inhibition			
		Ethanol	Methanol	P.Ether	Chloroform
1	5	51.31	40.17	22.65	4.20
2	25	13.26	41.30	51.18	16.36
3	50	60.70	43.42	25.03	15.14
4	100	20.15	44.30	11.90	35.66
5	250	25.03	31.06	35.16	10.63

% Inhibition of DPPH free radical scavenging activity for different extracts of Cassia fistula leaves in e.coli

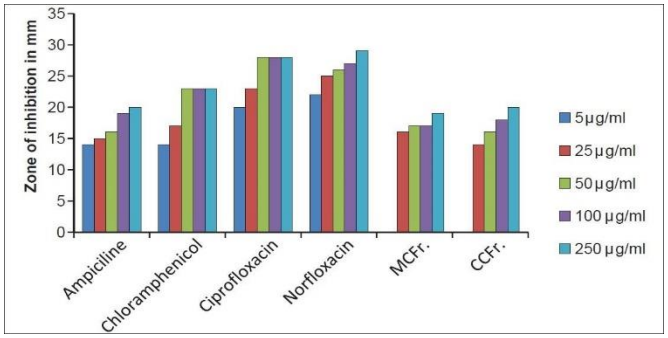


Fig: 5

Antibacterial activity against P. aeruginosa (MTCC 424) MCF: Methanolic extract of Cassia fistula leaves, CCF: Chloroform extract of Cassia fistula

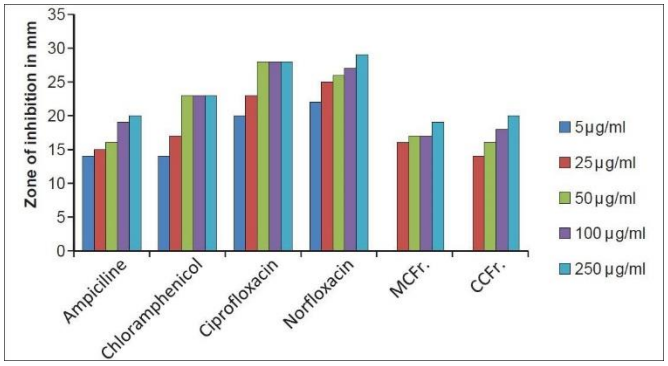


Fig: 6

Antibacterial activity against pyrogens MCF: Methanolic extract of Cassia fistula leaves, CCF: Chloroform extract of Cassia fistula

S.no	Concentration (ug)	%Inhibition			
		Ethanol	Methanol	P.Ether	Chloroform
1	5	50.44	42.17	22.67	4.25
2	25	12.30	42.32	52.15	16.36
3	50	60.72	44.36	25.06	15.24
4	100	22.45	45.66	12.09	35.96
5	250	27.34	31.06	36.87	10.62

TABLE 8

In-vitro antibacterial activity of (2S)-7-hydroxy-5-hydroxypropyl-2- (2-hydroxypropyl)chromone (compound 1) and Ampicillin

Text organism			
	Compound 1 (5ug/disc)	Compound 1 (10ug/disc)	Ampicillin (2ug/disc)
	Gram-positive bacteria		
Staphylococcus aureus	6.86 +/-0.02	11.73+/-0.04	16.56+/-0.01
	Gram-Negative bacteria		
Escherichia coli	6.65 +/- 0.03	11.53 +/- 0.04	17.10 +/- 0.02
Pseudomonas aeruginosa	5.56 +/- 0.02	11.93 +/- 0.04	17.13 +/- 0.01

TABLE 9

Antibacterial activity (Zone of inhibition)					
Drug	Concentration (µg/ml)	Zone of inhibition in mm			
		E. coli	*P. aeruginosa*	*S. pyogenes*	*S. aureus*
Ampicillin	5	14	14	11	10
	25	15	15	14	13
	50	16	15	16	14
	100	19	18	18	16
	250	20	20	19	18
Chloramphenicol	5	14	14	10	12
	25	17	17	13	14
	50	23	18	19	19
	100	23	19	20	20
	250	23	21	20	21
Ciprofloxacin	5	20	20	16	17
	25	23	23	19	19
	50	28	24	21	21
	100	28	26	21	22
	250	28	27	22	22
Norfloxacin	5	22	18	18	19
	25	25	19	19	22
	50	26	21	20	25
	100	27	23	21	26
	250	29	23	21	28

The antibacterial and antifungal activities of the extracts increased linearly with increase in concentration of extracts (µg/ml). As compared with standard drugs, the results revealed that in the extracts for bacterial activity, S. pyogenes and S. aureus were more sensitive as compared with E. coli and P. aeruginosa, and for fungal activity, C. albicans shows good result

as compare with A. niger and A. clavatus. The growth inhibition zone measured ranged from 11 to 20 mm for all the sensitive bacteria, and ranged from 14 to 20 mm for fungal strains.

The results show that the extracts of Cassia fistula were found to be more effective against all the microbes tested.

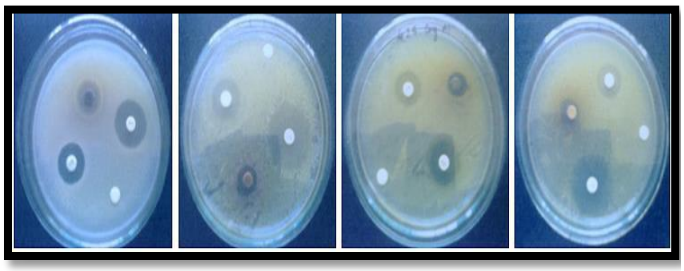
Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extracts obtained from Cassia fistula show strong activity against most of the tested bacterial and fungal strains. The results were compared with standard antibiotic drugs. In this screening work, extracts of Cassia fistula were found to be not inactive against any organism, such as Gram-positive, Gram-negative, and fungal strains were resistant to all the extracts of Cassia fistula.

The above results show that the activity of Hydro alcohol extracts of Cassia fistula shows significant antibacterial and antifungal activities. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals in the present investigation showed that the plant contains more or

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less same components like saponin, triterpenoids, steroids, glycosides, anthraquinone, flavonoids, proteins, and amino acids. Results show that plant rich in tannin and phenolic compounds have been shown to possess antimicrobial activities against a number of microorganisms.

Methanolic extract of cassia fistula against gram positive and gram negative bacteria



SUMMARY AND CONCLUSION

- Anti-microbial agent: Any chemical or biological agent that either destroys or inhibits the growth of microorganisms is known as anti-microbial agent.
- It consists of dried leaves of CASSIA FISTULA belonging to family Leguminosae.
- C. fistula has been shown to possess anti-oxidant, antidiabetic activity, hypolilidemic activity, hepatoprotective activity, antipyretic activity, antitussive activity, anti- inflammatory activity, antitumor activity, antiulcer, wound healing activity.
- The extraction of cassia fistula leaves was done by Maceration for methanol, chloroform, petroleum ether extract.
- To all above mentioned extracts phytochemical tests are performed.
- Nutrient agar medium was prepared and growth of pathogenic strains of staphylococcus aureus was induced by pour plate method.
- By cup plate method the effectiveness of test and a range of antibiotics was determined against staphylococcus aureus by MIC and zone of inhibition.
- The activity was observed along with the standard drug like Streptomycin.
- The zone of inhibition was appeared as methanolic extract 28mm, chloroform extract 23mm, petroleum ether extract 21mm. The test methanolic extract of Cassia fistula leaves shows maximum zone of inhibition then standard drugs.
- Antimicrobial resistance is a global problem. Emergence of multidrug resistance has limited the therapeutic options. Hence, monitoring resistance is of paramount importance. Hence, this study was aimed to focus the antimicrobial properties of C. fistula on gram positive, gram negative, and fungal organisms. In the current investigations, the hydro alcoholic and chloroform extracts of C. fistula were found to be active on some isolated microorganism and fungi as compared to standard drugs.
- This study has justified the traditional use of fruit pulp in infectious conditions. However, before use in human being isolation of pure compound, toxicological study, and pharmacological activity should be carried out thereafter. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antimicrobial agents.

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