Analysis of antimicrobial activity and phytochemical potential of *Cassia absus* Linn., *C. auriculata* Linn. and *C. fistula* Linn.

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Abstract

Dried seeds of *Cassia auriculata, C.absus* and *C.fistula* were collected from the forest of Javaathu hills, Tiruvannamalai District, Tamil Nadu, India for the comparative analysis of antimicrobial activity and phyto-chemical potential. The active bio-compounds from the seeds of above plants were extracted with ethyl acetate, hexane and methanol and found methanol as a suitable solvent, hence used for further analysis. The phytochemical screening of the seeds showed the presence of alkaloids, phenolics and flavanoids in all species; glycosides in *C.absus*; reducing sugars in *C.auriculata* and *C.fistula*; non-reducing sugars in *C.absus* and *C.fistula* and Saponins in *C.absus* and *C.auriculata* from trace to higher amounts. The quantitative determination of phenol and flavanoids of methanolic seed extracts from *C. auriculata*, *C.absus* and *C. fistula* revealed total phenolic content as 0.18, 0.15, 0.11 and flavonoids 0.08, 0.092, 0.087% respectively. The antibacterial activity of methanolic seed extract was tested using agar well diffusion method and broth dilution method for the inhibitory effect. In the test for antimicrobial activity, methanol extracts show inhibition against almost all tested pathogens. *C.absus* showed higher zone of inhibition (ZOI) as 21 mm against *Micrococcus luteus* and as 17 cm against *Klepsella pnuemoniae* at 1000 µg concentration. But *C.auriculata* and *C.fistula* had moderate inhibitory effect on all the bacterial spceies from 500 µg to 1000 µg concentrations. The antifungal activity of *C. absus, C.auriculata* and *C. fistula* seed extracts were tested by agar well diffusion method and found to have inhibitory effect against all the tested fungi *viz. Candida albicans, C.parapsilosis* and *C.tropicalis* from 11 mm to 21 mm ZOI. Our study provides the scientific validation for their traditional use in medicinal applications.

Keywords: Phytochemical potential, antibacterial testing, antifungal activity, *Cassia auriculata, Cassia absus and Cassia fistula*.

1. Introduction

Medicinal plants are the important source of bioactive compounds including antioxidant and antimicrobial activity. They serve as a source of many potent and powerful drugs in many countries [1]. According to the World Health Organization [2], the current estimate suggests that many developed countries have a great proportion of the population making use of traditional practice of health, especially the use of the medicinal plants. In China and India, the contribution is as much as 80%. Among various medicinal uses of plants, several species have potential antimicrobial and antiviral properties which play a major role in plant medicine [3, 4, 5]. The report on antibacterial activity of the *Taraxacum officinale* in different solvents like Methanol, Chloroform and distilled water used, it was found to be effective in methanol and chloroform extracts against bacterial pathogens tested [6]. The plant extracts of *Plumbago zeylanica* L. showed anti-microbial, antiviral, antioxidant, antifungal, anti-allergic properties and used in herbal formulations for centuries [7]. A large number of medicinal plants and their purified constituents have also shown beneficial therapeutic potentials which are due to the presence of flavones, isoflavones, flavonoids, anthocyanin, coumarin, lignans, catechins and isocatechins [8]. Phytochemical analysis of the *Ficus carica* leaf

extracts denoted the presence of flavonoids and phenols as antimicrobial compounds. These compounds were detected by Thin Layer Chromatography to evaluate for their potentials [9].

The bioactive compounds of plant origin mostly represent as secondary metabolites such as alkaloids, steroids, tannins, phenol compounds, flavonoids, resins, fatty acids and gums which are capable of producing definite physiological action on body [10]. The screening and evaluation of antibacterial activity of crude ethanol extract found minimum bactericidal concentration against these extracts both gram positive as well as gram negative bacteria [11]. In [12], reported that the Thai traditional plants are rich in phenolic contents which are antimicrobial function. The search for newer sources of antibiotics is a global challenge preoccupying research institution, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs [13]. Many plants are known for their antimicrobial traits chiefly due to secondary metabolites [14]. The phytochemical extraction and antimicrobial properties of different medicinal plants including *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan), *Azadirachta indica* (Neem), *Calotropis gigantea* and *Vinca rosea, Medicago sativa, Acalypa idnica* and *Canavalia ensiformis* were performed and found effective to control the growth in various bacterial and fungal strains [15,16,17, 18, 19, 20 and 21].

Based on the above scientific information and potentiality of the plants for the source of drugs, the present study has been carried out to analyse the antimicrobial potential of seeds of *Cassia* spp. collected from Javaadhu hills in India. Apart from the antimicrobial tests, the overall phytochemical profile and the active compounds present in the seed extracts have also been investigated.

2. Materials and methods

2.1. Chemicals and glassware

All the glassware (Borosil) were immersed in cleaning solution for 2 to 3 hours and washed thoroughly with tap water followed by detergent solution and finally rinsed with distilled water [22]. The cleaned glassware were dried in hot air drying chamber and stored for future use. All the chemicals used in the present study were analytical grade.

2.2. Culture media and maintenance of microbial culture

General laboratory techniques recommended by Purvis *et al* [23] was followed for the preparation of media, and culturing. For isolation, subculture and maintenance of bacterial cultures, Nutrient agar (NA) and Nutrient broth culture media and fungal for cultures, Potato dextrose agar (PDA) of slants and in petriplates were used respectively. Standard cultures of bacteria and fungi were used in this study for antimicrobial test.

2.3. Plant seeds collection and sample preparation

Dry seeds of *Cassia auriculata*, *C. absus* and *C. fistula* were collected from the fields located in Javaathu hills forest, Thiruvannamalai Dist, Tamil Nadu. The seeds were carefully washed with tap water, rinsed with distilled water, and air-dried in room temperature for 2 to 3 days for completion of drying. Then the seeds were ground with laboratory mixer in to fine powder and labeled then stored in the laboratory for further use.

2.4. Extraction of seeds with solvents - Direct extraction

Direct extraction was done with Hexane, Ethyl acetate and Methanol after the method of Eloff [24]. The seed sample was extracted with Hexane, ethyl acetate and methanol in the ratio of 1:10 in conical flask in shaking condition for overnight. The extract was filtered through the Whatmann No. 1 filter paper in a separate container. The process was repeated 3 times for the same plant material with fresh solvent. The extract was concentrated by evaporation in steam batch and the residues were re-dissolved in different solvents to yield 10mg/ml compound for further analysis. The quantity is expressed in percentage in relation to the weight of crude dried concentrated product.

2.5 Antibacterial activity

2.5.1. Well diffusion assay [24]

Nutrient agar was prepared and poured in the sterile Petri dishes and allowed to solidify. The 24 hr bacterial cultures of *Micrococcus luteus, Salmonella typhi, Staphylococcus aureus* and *Klebsiella pneumoniae* were streaked individually on different petridishes. Then wells (9mm diameter) were made on agar surface by using cork borer and different concentrations (250µg, 500µg, 750µg and 1000µg) of the seed extract were poured in the wells. Control with Dimethyl sulphoxide (DMSO) also was loaded in the wells for comparison with samples. Then the plates were incubated at 37 ^{o} C for 24h. After incubation the zone inhibition was measured.

2.5.2. Broth dilution assay [25]

Broth dilution assays for inhibition of bacterial cell suspension was done by standard method. Nutrient broth was prepared and about 5ml of broth were distributed in test tubes and sterilized. About 0.1ml of the 24 h growing cultures from *M. luteus, S.typhi, S. aureus* and *K. pneumoniae* were inoculated and the different concentrations of plant extracts as 100µg, 200µg….1000µg. The Dimethyl sulphoxide (DMSO) was dissolved and used as a standard for comparison. The tubes were incubated at 37 ^{O} C for 24 h. The optical densities were measured spectrometrically at 600 nm. The percentage of viable cells was calculated using the following formula.

$$\text{\% of inhibition} = \frac{\text{Control O.D - Test O.D}}{\text{Control O.D}} \times 100$$

2.5.3. Antifungal activity - Well diffusion assay [24]

Potato Dextrose agar was prepared and poured in the sterile Petri dishes and allowed to solidify. About 24 hrs fungal cultures (*Candida albicans*, *C. parapsilosis*, *and C. tropicalis*) were swabbed on it. The 5 wells (10 mm diameter) were made by using cork borer. The 4 different concentrations (250µg, 500µg, 750µg and 1000µg) of the sample, negative control (DMSO) were loaded in the wells. Then the plates were incubated at 37°C for 24h. After incubation, the zone of inhibition (ZOI) was measured.

2.6 Qualitative phytochemical analysis

The qualitative phytochemical tests were performed for establishing the profile of given plant extract for its chemical composition as per our earlier procedures [26}. The tests include for detection of Alkaloids, Phenolic compound (Ferric chloride test), glycosides (Borntrager's test), Flavonoids, Reducing sugars, Saponins, Proteins, Total phenols and Total flavonoid. Based on the colour intensity of the product in the reaction the scale 'high', 'moderate' and 'trace' value was assigned.

3. Results

3.1. Phytochemical screening

The qualitative phytochemical screening of *C. absus, C. auriculata* and *C. fistula* has revealed that the presence of alkaloids, phenolics and flavanoids in all the three plants. Whereas, the absence of reducing sugars in *C. absus*, absence of glycosides and non reducing sugars in *C.auriculata* and absence of glycosides and saponins in *C.fistula.* However, higher amount of alkaloids, flavanoides and saponins in *C.absus*; terpinoids and tannins were present in moderate amount. Glycosides and saponins were also present in trace amount. Protein constituent was completely absent in methanolic extracts (Table 1) in all the samples.

3.2. Quantitative determination of total phenols and flavanoids

Based upon the preliminary phytochemical test, quantitative determination of phytoconstituents was carried out for the extracts of *C. auriculata, C. absus* and *C. fistula.* It was found that the total flavonoids 0.080%, 0.092% & 0.087 and phenol 0.182%, 0.151% & 0.106% was present in methanolic extract (Table 1).

Table 1: Comparative Phyto-chemicals analysis among Cassia spp.

Phytochemical detection	*Test*	*Test Results in different species*		
		C.absus	*C.auriculata*	*C.fistula*
Alkaloids	Mayer's test	+++	++	+
Phenolic compound	Ferric chloride test	+ 0.182%	+++ 0.151%	+ 0.106%
Glycosides	Borntrager's test	+++	-	-
Flavonoids	Shinoda's test	++	++	+++
	Alkaline reagent test	++ 0.080%	+++ 0.092%	+++ 0.087%
Detection of reducing sugars	Fehling's test	-	++	++
Detection of non reducing sugars	Benedict's test	++	-	++
Detection of saponins	Saponification test	+++	+++	-
Test for proteins	Millon's test	-	-	-

+++: Present in good amount; ++: Present in moderate amount; +: Present in trace amount; - :Completely absent

3.3 Antibacterial activity against human pathogens – Well diffusion assay

In vitro preliminary screening of the antibacterial activity of plant seeds extracts of *C. auriculata, C. absus and C. fistula* was studied against bacterial cultures using diffeent solvent extracts. The methanol extract shows high level of inhibition**.** *Cassia* spp. exhibited the inhibitory activity. However, *C.absus* showed higher ZOI (21 cm) against *M.luteus* at 1000 µg concentration. But *C.auriculata* had moderate inhibitory effect on all the bacterial spceies at 500 µg to 1000 µg concentrations.

Table 2: Antibacterial activity of Cassia spp. The methanolic seed extracts were tested by Well diffusion assay

Test microorganisms	Zone of inhibition (mm) at different Concentrations (µg)			
	250µg	500µg	750 µg	1000 µg
C. absus				
M. luteus	13	16	18	21
S. typhi	-	-	-	13
S. aureus	-	13	14	14
K. pneumonia	-	14	15	17
C. auriculata				
M. luteus	-	12	14	15
S. typhi	-	11	12	13
S. aureus	-	11	13	16
K. pneumonia	-	12	14	15
C. fistula				
M. luteus	-	12	15	17
S. typhi	-	-	11	13
S. aureus	-	11	13	15
K. pneumonia	-	-	12	14

Thus, the antifungal activity of *C. auriculata, C. absus* and *C. fistula* seed extracts showed positive results against all the test fungi screened. Among different solvents used, the methanol extract shows maximum inhibition as the ZOI in the range from 11 cm at 500 µg to 21 cm at 1000 µg concentration compare to other solvents (data not provided) (Table 2).

3.4 Determination of inhibitory concentration 50 (IC_{50}) using broth dilution methods

On broth dilution methods used, the methanolic crude extract observed maximum inhibitory effect at the concentration of 1000 µg/ml of all test pathogens (Table 3, 4, 5). The IC_{50} concentration of crude extract of *C. auriculata, C. absus and C. fistula* ranged from 300 - 800µg/ml tested on dose dependent manner with different test pathogens. It was inferred that the methanolic extract showed maximum inhibition with IC_{50} against *M. luteus* of 300 µg/ml followed *S.typhi* with IC_{50} of 400 µg/ml, *S. aureus* with IC_{50} of 500 µg/ml and *K. pneumoniae* with $IC_{50}$of 600 µg/ml respectively (Table 3).

Table 3: Antibacterial activity of Cassia absus by broth dilution assay

Concentrations (µg)	Bacteria (inhibition is represented in % value)			
	M. luteus	*S. typhi*	*S. aureus*	*K. pneumonia*
100	24	13	18	-
200	37	24	23	14
300	**51.07**	37	30.40	23
400	55	**52**	33	30
500	57	55	**53.29**	36
600	60	62	55.21	**50**
700	66	66	64	50
800	72	71	72	57
900	74	77	84	65
1000	84	81	86	75

Table 4: Antibacterial activity of Cassia auriculata by broth dilution assay

Concentrations (µg)	Bacteria (inhibition is represented in % value)			
	M. luteus	*S. typhi*	*S. aureus*	*K.pneumonia*
100	19	9	22	-
200	27	17	29	15
300	35.6	25	35	26
400	48.8	36	41	33
500	**52.71**	41	47	39
600	57	**54**	50	44
700	62	59	**56.29**	49
800	68	65	68	**53.2**
900	72	71	71	63
1000	78	80	82	70

Table 5: Antibacterial activity of Cassia fistula by broth dilution assay

Concentrations (µg)	Bacteria (inhibition is represented in % value)			
	M. luteus	*S. typhi*	*S. aureus*	*K.pneumonia*
100	18	16	15	19
200	25	24	19	24
300	35.9	33	22.98	33
400	40	39	29	37
500	47	44	36	41
600	**53.12**	48	42.6	49.21
700	65	**56**	49.7	**51**
800	70	67	**58.02**	59
900	76	72	67	64
1000	82	79	74	77

3.5 Antifungal activity

The antifungal activity of *C. auriculata, C. absus* and *C. fistula* seed extracts show positive results against all the test fungi screened. Among the different solvents used, the methanol extract shows inhibition of all the 3 species of fungi tested in the range of 11 cm at 500 µg to 21 cm at 1000 µg concentration. The elevation in the inhibitory effect was observed with increasing concentrations of sample. The result of antifungal activity was shown in Table 6.

Table 6: Antifungal activity of Methanol extracts of different species of Cassia by well diffusion assay

Fungal species	Zone of inhibition (mm) at different Concentrations (µg)			
	250µg	500µg	750 µg	1000 µg
Cassia absus				
Candida albicans	-	15	19	18
Candida parapsilosis	-	16	17	18
Candida tropicalis	-	14	16	21
Cassia auriculata				
Candida albicans	-	12	13	12
Candida parapsilosis	-	12	14	12
Candida tropicalis	-	13	13	17
Cassia fistula				
Candida albicans	-	-	12	14
Candida parapsilosis	-	11	12	17
Candida tropicalis	-	12	14	18

4. Discussion and Conclusion

As a result, some natural products have been approved as new antimicrobial drugs, but there is still an urgent need to identify novel substances that are active towards pathogens with high resistance [27]. It is well observed that from the extracts of hexane, ethyl acetate and methanol in the present study, the methanol extract showed the significant effect of antimicrobial property and the presence of bioactive compounds which is in accordance with the report of Sohail *et., al,* [6]. The importance of phytoactive bio-compounds in various phytomedicines is elaborated by Mendonça [28] and found it antimicrobial and antioxidant property. Pohrel (2010) [29] evaluated the antimicrobial activity of medicinal plants of Nepal and isolated pure antimicrobial compound from *Bauhinia variegata.* The antibacterial activity of the present study shows that all the species of *Cassia viz. C. absus, C. auriculata and C. fistula* having moderate level of antibacterial and antifungal activity. Enormous reports on study of anti-bacterial and antifungal activity of various plant parts of different plant species are available for reference to the present study [30,31,32,33]. The antifungal activity of the plant species studied in the present experiments also observed as moderate level of inhibition on various *Candida* species. The study conducted by Abhisek et. al. [34], Prince and Prabakaran [35] show the plant compounds isolated from medicinally important plants which exhibited antifungal activity which are belongs to human pathogenic species. Isolation of antifungal proteins from medicinal plant species were performed and proved the antifungal activity against few fungi by Ameerjamil *et al*.[36]. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources and one of such resources is folk medicines or plant sources. Systematic screening of them may result in the discovery of novel effective compounds [37]. Since the emergence of multi-drug resistance pathogenic bacteria as well as undesirable side effects of certain antibiotics have triggered immense response in the search for new antimicrobial drugs of plant origin.

The results of the present study indicate that methanol extract of *Cassia auriculata, Cassia absus and Cassia fistula* seeds are high in phenolic contents and these extract exhibit strong antimicrobial activities and not the protein component. The moderate level of antibacterial and antifungal effect of all the three plant species like *C. auriculata, C. absus and C. fistula* also indicate the use of these plants in control of microbes in various field of medicinal applications. This study supports the contention that traditional medicines remain a valuable source in the potential discovery of natural drugs and antibiotics in pharmaceuticals. Significant antimicrobial activity showed by *Cassia auriculata, Cassia absus and Cassia fistula*, provide a scientific validation for the traditional use of these plants. Further studies are needed to explore *in vivo* studies are needed for better understanding their mechanism of action.

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