

Original article

***In vitro* efficacy of amaltas (*Cassia fistula* L.) against the pathogens causing otitis externa**

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Abstract

Introduction and objective: Otitis externa, commonly called ear disease, is characterized by inflammation of the external ear canal. The aim of this study was to evaluate the antimicrobial potential of *Cassia fistula* flowers, leaves and bark extracts against *Staphylococcus aureus*, *Proteus mirabilis*, *Escherchia coli*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., and *Candida albicans*, pathogens causing otitis externa and their comparison with locally available ear drops.

Materials and methods: Methanol, ethanol, acetone, aqueous (hot and cold) extracts from the flowers, leaves and bark of *C. fistula* were tested for their antimicrobial activity against six ear pathogens causing otitis externa determined by agar well diffusion method.

Results: Organic flower and bark extracts displayed activity against all tested ear pathogens whereas leaf extract showed activity against four tested bacteria and aqueous extracts were unable to exhibit any antimicrobial activity. Of the three organic solvents evaluated, acetic flower extract was found to be best against *S. aureus* followed by bark extract and leaf extract. The acetic flower extract showed larger inhibition zones compared to the herbal ear drops with minimum inhibitory concentration (MIC) of 6.25mg/ml.

Conclusion: Acetic extract of *C. fistula* flower may be used to treat otitis externa especially caused by *S. aureus*. However, more detailed studies such as *in vivo* testing and pharmacokinetics properties are needed to determine its therapeutic potential.

Significance and impact of the study: Due to antimicrobial effects of amaltas organic extracts on ear pathogens, use of flower acetic extract in herbal ear drops is recommended.

Keywords: Otitis externa; *Cassia fistula*; Antimicrobial activity; Minimum inhibitory concentration; Organic and aqueous extracts

Introduction

Otitis externa refers to a spectrum of infections of the external auditory canal and auricle, usually associated with microbial infection of macerated skin and subcutaneous cellular tissue mainly caused by *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Proteus mirabilis* and *Candida albicans* [1-3]. It affects between 5 and 20% of the patients attending ENT clinics. Manifestations of otitis externa include pain, pruritus and erythema but as the disease progresses oedema, otorrhea and conductive hearing loss may also develop [4,5].

The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the spectre of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies [6,7]. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for the development of new drugs because of the unmatched availability of chemical diversity [8,9].

India is a varietal emporium of medicinal plants and is one of the richest countries in the world in regard to genetic resources of medicinal plants [10]. *Cassia fistula* L. (family *Fabaceae*), commonly called Amaltas, Golden shower tree and Indian labrum, a taxon of *Cassia* comprising 600 species, is an ornamental medium sized tree growing up to a height of 10-20m with beautiful branches of yellow flowers. It is the national flower of Thailand but natively belongs to southern Asia. This plant has a high therapeutic value as an antipyretic, analgesic, antioxidant, anti-inflammatory, hypoglycaemic and has been

used in the treatment of various disorders such as haematemesis, pruritus, leucoderma, rheumatism, skin diseases, tuberculosis, eye and liver ailments. [11-14].

Various parts of this plant are known to be important source of secondary metabolites, mainly phenolic compounds such as fistucacidin (3,4,7,8,4-pentahydroxyflavan), (-) epiafzelechin, (-) epiafzelechin-3-O-glucoside, (-) epicatechin, procyanidin B₂, biflavonoids, triflavonoids, rhein glucosides, sennoside A and B, chrysophenol and physcion isolated from the leaves [15,16]; Kaempferol, leucopelargonidin tetramer having a free glycol unit, bianthraquinone glycoside, rhein, fistulin, alkaloids, triterpenes from the flowers [17] and oxyanthraquinone and dihydroxyanthraquinone from the bark [18].

Literature search reveals conducting of a few studies on antimicrobial activity of various parts of this plant such as bark, pods, leaves and flowers [12-14,19,20]. However, antimicrobial studies against pathogens causing otitis externa are lacking. Therefore, the current study has been undertaken to evaluate the antimicrobial potential of various parts of this plant against the locally isolated microbes from the otitis externa patients and it was compared with locally available ear drops.

Material and methods

Plant collection

The bark, leaves and flowers of *C. fistula* were collected from the trees alongside the roads of University of Kurukshetra, Haryana. The taxonomic identity of the plant was confirmed by Dr. Vashishta of Botany Department, Kurukshetra University, Kurukshetra.

Extraction of plant material

The samples were carefully washed under running tap water followed by sterile

distilled water and air dried at 35-40°C for 4-5 days, homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. Four different solvents namely ethanol, methanol, acetone and aqueous (hot and cold) were used for extraction. A 10g of homogenized bark, leaves and flowers were separately soaked in conical flasks each containing 100ml of acetone, ethanol, methanol (95%) or sterile distilled water.

Also the equal amount (i.e. 10g) of homogenized bark and leaves were immersed separately in 100ml of hot sterile distilled water in conical flasks and allowed to stand for 30mins in a water bath (at 100°C) with occasional shaking followed by keeping all the flasks on rotary shaker at 200rpm for 24h [21,22]. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rotaevaporator. The dried extract, thus, obtained was sterilized by overnight UV-irradiation, checked for sterility on nutrient agar plates and stored at 4°C in labelled sterile bottles until further use [23].

Test microorganisms

Five bacteria namely *S. aureus* (HM626197)*, *Acinetobacter* sp. (HM626198), *P. mirabilis* (HM626199), *E. coli* (HM626200), *P. aeruginosa* (HM626201) and one yeast, *C. albicans*, were isolated from the patients having ear infection from the local ENT clinics of Kurukshetra [24]. Bacterial strains were identified on the basis of Gram staining, biochemical and molecular characteristics (16S rRNA sequencing) and on the basis of staining, morphological and cultural characteristics for the yeast [25,26].

The bacterial isolates were subcultured on Nutrient agar and Yeast on Malt yeast agar and incubated aerobically at 37°C. The media were procured from Hi Media

Laboratory Pvt. Ltd., Bombay, India. (*Nucleotide sequence of all the five bacteria have been submitted to GenBank database, which have provided GenBank accession number, HM626197-HM626201).

Ear drops

Three commonly prescribed ear drops by otolaryngologists, two allopathic ciprox (antibacterial), candid (antifungal), a herbal ear drop bilwa tel (antimicrobial), were procured from the local market in Kurukshetra.

Screening for antimicrobial activity

The acetone, methanol, ethanol, hot and cold aqueous *C. fistula* leaves, bark and flower extracts were used for evaluation of antimicrobial activity by the agar well diffusion method. In this method, a pure isolate of each microbe was grown on agar plates at 37°C for 24h. One plate of each microorganism was taken and a minimum of four colonies were transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10⁶cfu/ml (standardized by 0.5McFarland standard) and used as the inoculum for performing agar well diffusion assay.

One hundred microlitre (100µl) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8mm wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with molten agar medium. The dried extracts were reconstituted to 20% in dimethylsulphoxide (DMSO) for the bioassay analysis. A 100µl volume of each extract was propelled directly into the wells (in triplicates) of the inoculated agar plates for each test organism.

The plates were allowed to stand for 1h at 40°C for diffusion of the extract into agar

and incubated at 37°C for 24h. Sterile DMSO (20%) served as the negative control and ciplox (for bacteria), candid (for fungi) and Bilwa tel (antimicrobial) ear drops served as the positive controls. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone was greater than 8mm [23]. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones \pm standard deviations were calculated.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) for each test organism was determined by the modified agar well diffusion method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100mg/ml) in 20% DMSO followed by dilution in sterile distilled water to achieve a decreasing concentration range of 50mg/ml to 0.39mg/ml. A 100 μ l volume of each dilution was introduced into triplicate wells of the agar plates already seeded with 100 μ l of standardized inoculum (10⁶cfu/ml) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24h and observed for inhibition zones. MIC, taken as the lowest concentration of the test extract that completely inhibited the growth of the microbe, showed by a clear zone of inhibition (12mm), was recorded for each test organism [23,24].

Results

The antimicrobial activity of *C. fistula* bark, leaves and flowers extracts on the agar plates varied in different organic solvents (methanol, ethanol and acetone). Positive controls produced significantly sized inhibition zones against the tested bacteria (ranging between 11.6mm and 56.3mm) and the yeast (with zone of inhibition 21.3mm), and the negative control produced no observable inhibitory effect against any of the test organism (Tables 1 and 2). A perusal of the data in table 1 reveals that all the organic solvent extracts of both flower and bark possessed antimicrobial activity against all six tested ear pathogens while organic leaves extracts did not exhibit any activity against *P. aeruginosa* and *C. albicans*.

Aqueous extracts, both hot and cold of bark, leaves and flowers of *C. fistula*, totally lacked antimicrobial activity. The acetonc flowers extract was found most effective against *S. aureus* (24.6mm) followed by *P. mirabilis* (18.6mm), *Acinetobacter* sp., *P. aeruginosa* (17.6mm), *E. coli* (16.6mm) and *C. albicans* (15.3mm). The ethanolic and methanolic flower extracts showed the inhibition zones of 18.3mm and 20.6mm against *S. aureus*. The zones of inhibition produced by the ethanolic and methanolic flower extract against the other tested pathogens were almost equal and ranged between 15mm and 17mm. *S. aureus* was found most sensitive pathogen having an MIC of 6.25mg/ml. Other tested pathogens were found to be less sensitive, having MIC values between 25mg/ml and 50mg/ml (Tables 1 and 3).

Table 1: Antimicrobial activity of *C. fistula* leaves, bark and flower extracts on pathogens causing otitis externa determined by agar well diffusion method

Solvent extracts (mg/ml)	Diameter of growth of inhibition zones (mm)																	
	Sa			Ac			Pa			Pm			Ec			Ca		
	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le
Methanol	20.6 ^a ±0.57 ^b	19.3 ±0.57	17.6 ±0.57	16±0	16.6 ±0.57	12.3 ±0.57	16.6 ±0.57	13±0	-	16.6 ±0.57	16.3 ±0.57	12±0	16±0	13.6 ±0.57	12.3 ±0.57	16.3±0	15±0	-
Ethanol	18.3 ±0.57	20.6 ±0.57	18.3 ±0.57	16.3 ±0.57	17±0	13.3 ±0.57	16.3 ±0.57	13.6 ±0.57	-	17.3 ±0.57	15.6 ±0.57	12.6 ±0.57	16.3 ±0.57	12.6 ±0.57	13±0	15.6 ±0.57	15.3 ±0.57	-
Acetone	24.6 ±0.57	22.6 ±0.57	18.6 ±0.57	17.6 ±0.57	17.3 ±0.57	13.6 ±0.57	17.6 ±0.57	14.3 ±0.57	-	18±0	16±0	13.3 ±0.57	16.6 ±0.57	13.6 ±0.57	13.3 ±0.57	16.6 ±0.57	15.6 ±0.57	-
Hot aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cold aqueous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Sa, *Staphylococcus aureus*; Ac, *Acinetobacter* Sp.; Pa, *Pseudomonas aeruginosa*; Pm, *Proteus mirabilis*; Ec, *Escherchia coli*; Ca, *Candida albicans*; Fe, Flower extract; Be, Bark extract; Le, Leaves extract.

-, No activity; ^a Values, including diameter of the well (8mm), are means of three replicates ^b ± Standard deviation

Table 2: Antimicrobial activity of ear drops against the locally isolated otitis externa pathogens determined by agar well diffusion method

Trade name	Batch no	Manufacturer	Diameter of inhibition zones (mm)					
			Sa	Ac	Pa	Pm	Ec	Ca
Ciplox ear drop	A93933	Cipla	56.3 ^a ±0.57 ^b	32.6±0.57	34±0	46.3±0.57	36±0	nt
Bilva tel ear drop	02	Shree	13.6±0.57	-	-	11.6±0.57	-	-
Candid ear drop	Y20880133	Baidyanath Majesta (A division of Glenmark)	nt	nt	nt	nt	nt	21.3±0.57

Sa, *Staphylococcus aureus*; Ac, *Acinetobacter* Sp.; Pa, *Pseudomonas aeruginosa*; Pm, *Proteus mirabilis*; Ec, *Escherchia coli*; Ca, *Candida albicans*; -, No activity; nt, not tested; ^a Values, including diameter of the well (8mm), are means of three replicates ^b± Standard deviation

Table 3: MIC of *C. fistula* leaves, bark and flower extracts on pathogens causing otitis externa determined by agar well diffusion method

Solvent extracts	MIC (mg/ml)																
	Sa			Ac			Pa			Pm			Ec			Ca	
	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be	Le	Fe	Be
Methanol	12.5	25	25	50	50	100	50	100	50	50	100	50	100	100	50	50	
Ethanol	25	25	25	50	50	100	50	100	50	50	100	50	100	100	50	50	
Acetone	6.25	12.5	25	25	25	100	25	100	25	50	100	50	100	100	50	50	

Sa, *Staphylococcus aureus*; Ac, *Acinetobacter* Sp.; Pa, *Pseudomonas aeruginosa*; Pm, *Proteus mirabilis*; Ec, *Escherchia coli*; Ca, *Candida albicans*; Fe, Flower extract; Be, Bark extract; Le, Leaves extract.

All three organic bark extracts showed their highest antibacterial activity against *S. aureus* with inhibition zone of 22.6mm for the acetonic extract followed by the ethanolic extract (20.6mm) and methanolic extract (19.3mm). *S. aureus* was again found to be the most sensitive pathogen, having an MIC of 12.5mg/ml (acetonic bark extract) followed by the methanolic and ethanolic extracts 25mg/ml. Organic bark extracts showed moderate activity against *P. mirabilis*, *Acitenobacter* sp. and *C. albicans* with zones of inhibition ranging between 16.6mm and 18mm and MIC of 50mg/ml.

The inhibition zones produced by organic solvents against *P. aeruginosa* and *E. coli* ranged between 13.6mm and 14.3mm and these two bacteria were found to be least sensitive having an MIC of 100mg/ml (Tables 1 and 3). In the case of leaves extracts of *C. fistula*, *S. aureus* was found to be the most active of the four bacterial pathogens that showed sensitivity to the organic extracts with zone of inhibition ranging between 17.6mm and 18.6mm and having an MIC of 25mg/ml. The zone of inhibition produced against the other three bacteria was almost equal and ranged between 12.3mm and 13.6mm and having an MIC of 100mg/ml (Tables 1 and 3). However, organic leaf extracts lacked antimicrobial activity against *P. aeruginosa* and *C. albicans*.

All organic flowers and bark extracts displayed much higher activity against the tested ear pathogens (with highest zone of inhibition of 24.6mm) compared to the ayurvedic herbal ear drop, bilwa tel. However, allopathic antibacterial ear drop ciplox, containing ciprofloxacin and antifungal ear drop, candid, containing clotrimazole, showed higher inhibitory activity against the ear pathogens (with highest zone of inhibition of 56mm and 21mm, respectively) (Tables 1 and 2).

Discussion

Cassia fistula has been used extensively in various parts of the world against a wide range of ailments [11]. The antimicrobial potential of this plant extracted in different solvents (e.g. aqueous, diethyl ether, ethyl acetate, dichloromethane, methanol, ethanol and chloroform) has been evaluated against different bacterial and fungal human pathogens and variable activities of different plant parts including flowers, leaves, pods, seeds, stem bark in different solvents has been reported [12,13,20,27].

A majority of the described antimicrobial effects of *C. fistula* extracts have been attributed to their secondary metabolites, notably phenolic compounds [11]. Our report is the first for antimicrobial activity of this plant against ear pathogens causing otitis externa. In our study, the organic flowers extracts of *C. fistula* were found to be the most active in inhibiting the growth of all the six tested microbial ear pathogens causing otitis externa followed by the bark extracts. The antimicrobial activity of flowers extracts against the tested ear pathogens may be due to the presence of secondary metabolites, Kaempferol, leucopelargonidin tetramer with free glycol unit, bianthraquinone glycoside, fistulin, alkaloids and triterpenes [17,28].

Recently 4-hydroxybenzoic acid hydrate isolated from the ethyl acetate flower extracts of this plant, has been found to have antimold activity, but not antibacterial or anti yeast activity [12]. The methanolic extracts of seeds and aqueous extracts of pods of *C. fistula* have been found to be nontoxic to humans [29,30]. Sangetha *et al.* [13] have reported the higher antimicrobial activity of the methanolic extract of stems followed by flowers, pods and leaves of this plant. Among the tested microorganisms, *S. aureus* was found to be the most sensitive

ear pathogen against the tested organic extracts. Jigna and Sumitra [31] have also reported the presence of stronger activity of plant extracts against Gram-positive bacteria than that of Gram-negative bacteria.

Of the nine organic extracts of three parts of this plants screened, the acetonic extract has been found to be most active and have a better antibacterial activity than the corresponding ethanolic and methanolic extracts (Table 1). Our results confirm the finding made by Cowan [32], Eloff [33], Nair *et al.* [34], who rated acetone as the best solvent. All the three aqueous extracts of this plant lacked antifungal and antibacterial activity against the tested ear pathogens.

Polarity of antimicrobial compounds that make them more readily extractable in the organic solvents and insufficient quantities of the active compound in the crude aqueous extract with the dose level employed may be the reasons for the absence of bioactivity of aqueous extract [13,20,23]. Interestingly, the organic extracts were more potent against the tested ear pathogens compared to the standard herbal ear drop (Bilwa tel) showing a great potential to be developed as an herbal ear drop to control the microbial ear infections.

Conclusion

Our results show that acetonic extracts of *C. fistula* have the best bioactivity against the six tested microbial ear pathogens causing otitis externa. This finding provides an insight into the usage of this plant as a traditional medicine for curing different ailments since generations.

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