Antimicrobial Activity of *Cassia occidentalis* L (Leaf) against various Human Pathogenic Microbes

Vedpriya Arya, Sanjay Yadav, Sandeep Kumar, JP Yadav*
Department of Genetics, MD University, Rohtak – 124001, Haryana (India)

*Correspondence to: JP Yadav, yadav1964@rediffmaiLcom

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Abstract

Different organic and aqueous extracts of leaves of *Cassia occidentalis* L (Caesalpiniaceae) were screened for their antimicrobial activity against seven human pathogenic bacterial and two fungal strains by disk diffusion assay. The pattern of inhibition varied with the solvent used for extraction and the microorganism tested. Among these extracts, methanol and aqueous extracts showed significant antimicrobial activity against most of the tested microbes. The most susceptible microorganism was *P. aeruginosa* (18 mm zone of inhibition in aqueous extract) followed by *P. mirabilis* (15 mm zone of inhibition in methanol extract) and *Candida albicans* (8 mm zone of inhibition in methanol extract). Preliminary phytochemical analysis of different extracts revealed the presence of anthraquinones, carbohydrates, glycosides, cardiac glycosides, steroids, flavanoids, saponins, phytosterols, gums and mucilages while alkaloids were absent in all the tested extracts.

Keywords: *Cassia occidentalis* L; Antimicrobial activity; *Pseudomonas aeruginosa*.

Introduction

Despite of tremendous progress in human medicines, infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health. There impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance [1]. During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics [2] has lead to the search of new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structures, which overcome the above disadvantages [3]. Current research on natural molecule and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses [4].

Cassia occidentalis L called as Kasmard in Sanskrit, Kasondi in Hindi and Coffee Senna in English belongs to family Caesalpiniaceae. It is an ayurvedic plant with huge medicinal importance. Leaves of *C. occidentalis* plant have ethnomedicinal importance like paste of leaves is externally applied on healing wounds, sores, itch, cutaneous diseases, bone fracture, fever, ringworm, skin diseases and throat infection. Previous pharmacological investigations showed that *C. occidentalis* leaf extracts have antibacterial [5, 6], antimalarial [7], antimutagenic [8], antimutagenic [9], antiplasmodial [10] anticarcinogenic [11] and hepatoprotective [12] activity. Moreover, studies on this plant showed that the nature and amount of the phytochemicals varies according to the season and geographical location [12]. In India, habit of this plant varies greatly. It is found as annual plant in North (including Haryana), North-west India but as a perennial plant in South India. The previous results encouraged us to deepen the studies on antimicrobial properties of the leaves of *C. occidentalis* by evaluating the inhibition zone by using agar disk diffusion method. In the present study, an attempt was made to investigate the antimicrobial activity and preliminary phytochemicals from the leaves of *C. occidentalis* collected from Haryana region of India.

Methods

Plant material

Leaves of *C. occidentalis* were collected from the local areas of Rohtak district of Haryana on October 2008. The plant specimen was botanically identified and authenticated by comparing the herbarium specimen (MDU 2504) available in the Department of Genetics, M. D. University, Rohtak.

Preparation of the extracts

The leaves were cleaned with deionized water, oven dried at 50^0 C for 48 hours and powdered in a grinder. The plant material (200 gm) was sequentially extracted with different solvents (petroleum ether, benzene, chloroform, methanol and water) (2000 ml) according to their increasing polarity by using Soxhlet apparatus for 24 hours at a temperature not exceeding the boiling point of the respective solvent. The obtained extracts were filtered by using Whatmann No. 1 filter paper and then concentrated under vacuum at 40^0C by using a rotary evaporator. The extract was then lypholized (Allied Frost lypholizer) to powdered form at -55^0C under vacuum conditions. The extractive value of the extracts (percentage yield, water-soluble extractive and alcohol soluble extractive) was calculated. The residual extracts were stored in refrigerator at 4^0C in small and sterile plastic bottles.

Organoleptic properties determination

Organoleptic properties (color, texture and odour) of the plant extract were determined in respective solvents in wet as well as dry conditions.

Preparation of test samples

Test samples of the plant extract were prepared in DMSO (Dimethyl Sulfoxide) (400 mg/ml).

Tested microorganisms

Antimicrobial activity of leaves extract was investigated against seven registered bacterial isolates and two fungal strains, which were obtained from the Microbial Type Culture Collection (MTCC) from Institute of Microbial Technology, Chandigarh. These included two gram-positive bacteria including *Staphylococcus aureus* (MTCC96), *S. epidermidis* (MTCC435), five gram-negative bacteria *Proteus vulgaris* (MTCC426), *Pseudomonas aeruginosa* (MTCC424), *Klebsiella pneumoniae* (MTCC3384), *P. mirabilis* (MTCC425) and *E. coli* (MTCC433) *and* fungi *Aspergillus fumigatus* (MTCC343) and *Candida albicans* (MTCC227). The tested microorganisms were cultured on Nutrient agar (HiMedia, Mumbai) (for bacteria at 35$\pm$2^o C for 24 h) and on Sabouraud Dextrose Agar (HiMedia, Mumbai) (media) (for fungus at 28$\pm$2^{0}C for 48-72 h). The reference strains of bacteria and fungi were maintained on Nutrient agar (HiMedia, Mumbai) and on Sabouraud Dextrose Agar (HiMedia, Mumbai) slants respectively. The cultures were subcultured regularly (every 30 days) and stored at 4^0C as well as -80^0C by preparing suspensions in 10% glycerol.

Inoculumn preparation

A loopful of isolated colonies was inoculated into 4 ml peptone water and incubated at 37^0C for 4 h. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dehydrate with 99.5 ml 1% (v/v) sulphuric acid. This turbidity was equivalent to approximately 1-2×10^8 colony-forming units per milliliter (cfu/ml). This 2-h grown suspension was used for further testing.

Antimicrobial bioassay

The antimicrobial activities of the extracts were determined by the Kirby-Bauer agar diffusion method according to NCCLS standards [13,14]. Muller Hinton Agar (MHA) (HiMedia, Mumbai) was used for the antimicrobial activity test. Under aseptic conditions in the Biosafety chamber, 15 ml of MHA medium was dispensed into pre-sterilized petridishes to yield a uniform depth of 4 mm and inoculated by the bacterial and fungal culture, respectively. The sterile discs (Hi Media, Mumbai) (Diameter 6mm) were impregnated with 8 mg/disc concentration of the extract and dried for 10-15 minutes. The dried discs were placed on MHA agar surface with flamed forceps and gently pressed down to ensure contact with the agar surface. Streptomycin (HiMedia, Mumbai) (for bacteria) and Ketocanozole (HiMedia, Mumbai) (for fungus) (10μg) were used as positive controls and DMSO was used as a negative controL The discs were spaced far enough to avoid reflections wave from the edges of the petridishes and overlapping rings of inhibition. Finally, the petridishes were incubated for 18 to 24 hours at 35 ± 2^0C for bacteria and 28 ± 2^0C for 48 to 72 hours for fungus. The diameter of zone of inhibition (mean of triplicates$\pm$SD) as indicated by clear area which was devoid of growth of microbes was measured.

Determination of activity index

The activity index [15] of the crude plant extract was calculated as

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Determination of proportion index

The proportion index [16] was calculated as

$$\text{Proportion index (P.I.)} = \frac{\text{Number of positive results obtained for individual extract}}{\text{Total number of tests carried out for each extract}}$$

Statistical evaluation

The antimicrobial activity was determined by measuring the diameter of zone of inhibition that is the mean of triplicates$\pm$SD of three replicates.

Preliminary phytochemical analysis of leaves extract

Preliminary analysis of alkaloids, saponins, carbohydrates, glycosides, fixed oils and fats, aminoacids, flavanoids, anthraquinones, tannins and phenolic compounds were carried out by using the methods of Harbone [17] and Brindha [18].

Results

Physical characterization of herbal extracts

Extraction yield, alcohol soluble extractive and water-soluble extractive

Among all the plant extracts, water extract was found to have maximum extractive yield followed by the methanol and petroleum ether extract (Table 1). Methanol and aqueous extracts were found to have maximum alcohol soluble and water soluble extractive values.

Table 1. Extractive value of plant extract in different solvents.

Plant extract	Percentage yield of extract	Alcohol soluble extractive (%)	Water soluble extractive (%)
Petroleum ether	5.6%	1.2%	1%
Benzene	1.6%	1.0%	0.3%
Chloroform	2.4%	0.25%	0.2%
Methanol	6%	4.23%	4.5%
Aqueous	6.8%	2.55%	5.76%

Organoleptic properties

The color, texture and odor of the plant extracts in different solvents in both wet and dried conditions were characterized (Table 2). The methanolic extracts were better than corresponding aqueous and other organic extracts in retaining the natural fragrances of the plants. This may be due to the preservative ability of methanol (i.e. reducing breakdown of organic compounds by microorganisms), its enhanced extraction capability (i.e. more fragrant components extracted) or a combination of both. Dried extracts obtained by lypholization generally appeared darker and more turbid than the wet extracts.

Table 2. Organoleptic properties of leaves extract of *Cassia occidentalis*.

Plant extract	Wet extract			Dry extract		
	Color	Texture	Odor	Color	Texture	Odor
Petroleum ether	Greenish black	Water-like consistency	Odor of seaweed	Dark black	Oily	Unpleasant smelling
Benzene	Brownish black	Not foamy	Strong pungent smell	Dark brownish black	Sticky, resinous	Pungent, sourish smell
Chloroform	Dark black	Not foamy	Slightly pungent, fishy odor	Dark black	Waxy, thick	Slight fishy but sweet odor
Methanol	Reddish brown	Slightly foamy when shaken	Alcoholic odor	Reddish black	Sticky, resinous	Leafy smell
Aqueous	Brown	Highly foamy when shaken	Tobacco like	Dark brown	Slimy and Powdery	Slight sweet odor

Antimicrobial activities

Among all tested extracts, methanol and water extracts were found to be most active than corresponding organic extracts (Table 3, 4). Methanol extract was found to be active against six tested bacteria (*P. aeruginosa, K. pneumoniae, P. mirabilis, E. coli, S. aureus, S. epidermidis*). On the other hand, the aqueous extract was effective against three out of seven tested bacteria (*P. vulgaris, K. pneumoniae* and *P. aeruginosa*) and fungus (*C. albicans*). Aqueous extract was found to have maximum zone of inhibition against *P. aeruginosa* (18 mm) while the minimum zone of inhibition was against *K. pneumoniae* (3 mm). The benzene and petroleum ether extracts of the leaves of *C. occidentalis* were effective against *P. mirabilis* and *E. coli* respectively while chloroform extract was found to be very inactive against all tested bacterial and fungal and yeast strains. Similarly, activity index of the plant extracts varies from maximum 0.72 for aqueous (*P. aeruginosa*) to the minimum 0.13 for methanol (*K. pneumoniae*) extract. The proportion index for the antimicrobial activity among different extracts varied from 0 (chloroform) to 0.77 (methanol) (Figure 1). All active extracts were stable at 4^0C in both DMSO and in dry state up to several months and did not show any reduction of activity against the sensitive bacteria as compared to the activities of the first day.

Table 3. Antibacterial activity of leaves extract of *C. occidentalis*.

Tested bacterial strains	Plant extract (8mg/disc)										
	Petroleum ether		Benzene		Chloroform		Methanol		Aqueous		Streptomycin (10µg)
	DIZ[a]	AI[b]	DIZ	AI	DIZ	AI	DIZ	AI	DIZ	AI	DIZ
P. aeruginosa	-	0	-	0	-	0	5±0.81	0.2	18±0.83	0.72	25
P. vulgaris	-	0	-	0	-	0	-	0	11±0.71	0.42	26
P. mirabilis	-	0	10±0.81	0.45	-	0	15±0.5	0.68	-	0	22
K. pneumoniae	-	0	-	0	-	0	3±0.25	0.13	3±0.25	0.13	23
S. aureus	-	0	-	0	-	0	12±0.71	0.44	-	0	27
S. epidermidis	-	0	-	0	-	0	8±0.23	0.36	-	0	22
E. coli	5±0.45	0.18	-	0	-	0	11±0.5	0.40	-	0	27

[a]DIZ = Diameter of zone of inhibition in mm (mean±SD); [b]AI = Activity Index

Table 4. Antifungal activity of leaves extract of *C. occidentalis*.

Tested bacterial strains	Plant extract (8mg/disc)										
	Petroleum ether		Benzene		Chloroform		Methanol		Aqueous		Ketocanozole (10µg)
	DIZ[a]	AI[b]	DIZ	AI	DIZ	AI	DIZ	AI	DIZ	AI	DIZ
A. fumigatus	-	0	-	0	-	0	-	0	-	0	12
C. albicans	-	0	-	0	-	0	8±0.62	0.8	5±0.34	0.5	10

[a]DIZ = Diameter of zone of inhibition in mm (mean±SD); [b]AI = Activity Index

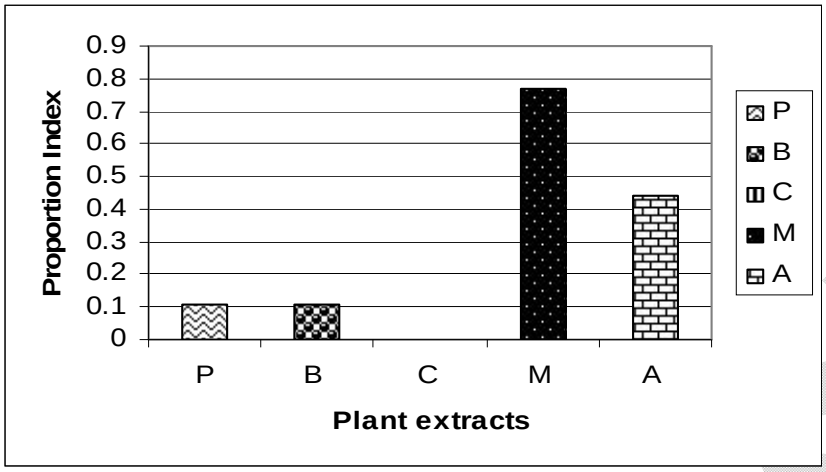


Figure 1. Proportion Index of antimicrobial activity of leaves extract in different solvents.

Preliminary phytochemical analysis of the extract

Preliminary phytochemical analysis of the plant extracts (Petroleum ether, benzene, chloroform, methanol, aqueous) showed the presence of anthraquinones, carbohydrates, glycosides, cardiac glycosides, aminoacids, phytosterols, fixed oils and fats, phenolic compounds, tannins, flavanoids, steroids, gum and mucilages and saponins while alkaloids are absent in all of the tested extracts (Table 5).

Table 5. Preliminary phytochemical analysis of leaves extract of *C. occidentalis*.

Phytochemicals tested	Test used	Leaves extract				
		Pet ether	Benzene	Chloroform	Methanol	Aqueous
Alkaloids	Mayer's test	-	-	-	-	-
	Wagner's test	-	-	-	-	-
	Dragendorff's test	-	-	-	-	-
	Hager's test	-	-	-	-	-
Anthraquinones						
Free anthraquinones		-	-	-	+	+
Combined anthraquinones		-	-	-	+	+
Carbohydrates	Molish's test	+	-	-	+	+
	Fehling's test	+	-	-	+	+++
	Barfoed's test	+	-	-	+	+

	Benedict's test	+	-	-	+	+
Glycosides	Borntrager's test	-	-	-	+	+
	Legal's test	-	-	-	+	+
Cardiac glycosides	Keller killiani's test	+++	++	+	-	-
Saponins	Froth test	-	-	-	+	++++
	Foam test	-	-	-	+	+++
Proteins and Amino acids	Millon's test	+	+	+	++	++
	Biuret's test	+	+	++	++	++
	Ninhydrin test	+	+	+	++	+++
Phytosterols	Libermann-Burchard test	-	-	-	-	++
	Salkowski's test	-	-	-	-	+++
Fixed oils and fats	Spot test	+	+	+	++	-
	Saponification test	+	+	+	++	-
Phenolic compounds and tannins	Ferric chloride test	-	-	-	+	+
	Gelatin test	-	-	-	+	++
	Alkaline reagent test	-	-	-	+	++
Flavanoids	Alkaline reagent test	-	-	-	+	++
	Lead acetate test	-	-	-	++	++
	Shimoda test	-	-	-	+	++
Steroids		+	+	+	+	+++
Gums and mucilages		-	-	-	++	++

+++ = Appreciable amount; ++ = Moderate amount; + = Trace amount; - = completely absent

Discussion

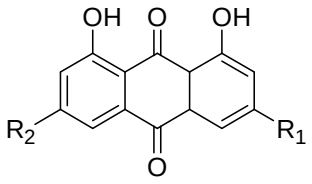
Methanolic and aqueous extracts of the leaves of *C. occidentalis* were most effective against the tested microorganisms. This is the first attempt to investigate the extract in different solvent on polarity basis, organoleptic properties and detailed preliminary phytochemical study of the leaf extract of this plant. Antimicrobial activity of the extracts of *C. occidentalis* was first time investigated against *P. aeruginosa, K. pneumoniae, P. vulgaris, P. mirabilis, S. epidermidis, A. fumigatus* and *C. albicans.* However, investigations have already been done on *E. coli* [5, 6, 19] and *S. aureus* [5]. However, our results showed remarkable variations in the effectiveness of the leaves extract against *E. coli*. In previous studies, for leaves extract, *E. coli* was found to be sensitive [5, 6] and in some experiments resistant [19]. Saganuwan and Gulumbe [6] observed that the *E. coli* was sensitive to methanol, hexane, chloroform and aqueous extract of leaves of *C. occidentalis* (collected from Kacha town, Niger state) at a concentration range 900-1000 mg/mL Similarly, Jain and his coworkers [5] observed that the metabolite rich fraction of (anthraquinones) leaves, pods, flowers and callus were effective against *E. coli* (Inhibition zone 22 mm). In contrast, our study revealed that the petroleum ether and methanolic extract of leaves of *C. occidentalis* (collected from Rohtak district, Haryana, India) was effective against *E. coli* at concentration of 400 mg/ml with 5 and 11 mm inhibition zone respectively. The inhibition activities were not observed in the chloroform and aqueous extracts of leaves of *C. occidentalis* against *E. coli* in the present study while Saganuwan and Gulumbe [6] observed the activities in these extracts. These differences in the plant extracts activities may be due to spatial and temporal variations of the plants. Infections caused by *P. aeruginosa*, especially those with multidrug resistance, are among the most difficult to treat conventional antibiotics. In our study, the growth of *P. aeruginosa* was remarkably inhibited by the aqueous extract of the leaves of *C. occidentalis*.

The chemical constituents of plants vary depending on the species, variety and part of the plant, with conditions of growth (soil, water and temperature), and with the age of the plant. The phytochemistry also varies according to the geographical regions, season and time of collection and different climatic conditions [20]. The study of phytochemicals of *C. occidentalis* reveals that the nature and amount of phytochemicals varies according to climate. For example stems, leaves and root bark of the plant from Ivory coast, Africa contains small amount of saponins and no alkaloids, sterols, triterpenes, quinines, tannins and flavonoids. However, a large amount of alkaloids was found in stem, leaves and fruits from Ethiopia [12]. Similarly, in previous studies, flavanoids and steroids were absent in the leaves of *C. occidentalis* [6]. However, according to our investigation, methanolic and aqueous extract were found to contain both flavanoids and steroids. However, alkaloids were absent in all tested extracts. The antagonistic results of these findings may be attributed to different geographical locations and climatic conditions for the growth of the plant.

Some of the phytochemicals have already been isolated from the leaves extract of *C. occidentalis* like C-flavonoids of apigenin, chrysophanol, emodin (Figure 2) as well as their glycosides, free physcion, 1,1-bi-4,4´,5,5´-tetrahydroxy-2,2´-dimethyl anthraquinone, the flavon metterucinol-7-o-α-L-rhamnoside, tetrahydroanthracene derivatives, germichrysone and occidentallins A & B [12]. However, the phytochemicals responsible for the antimicrobial activity of the extract was unknown. Emodin, which is also isolated from the roots of this plant, is regarded as the possible antibacterial constituent from roots. Earlier studies have documented mutagenic/genotoxic effects of emodin in bacterial system [21]. Further, SI nuclease sensitivity analysis studies revealed that emodin induced dose dependent DNA damage in *H. pylori* [22] and inhibited respiration driven solute transport at micromolar concentrations in membrane vesicles of *E. coli* [23]. It may be possible that emodin is also responsible for the antimicrobial activity of the leaves of *C. occidentalis*. However, there is a strong need to investigate the exact mechanism of action of emodin isolated from leaves of this plant.

At the same time, mechanistic aspects of antimicrobial nature of *Cassia occidentalis* was also observed [24]. The mechanism of action of the antimicrobial activity of the family Casalpiniaceae to which cassia belongs may be explained in terms of their ability to induce leakage of these ions [25]. Ethanolic and hot water extract of *C. occidentalis* was investigated for their capacity to release sodium and potassium ions for some selected pathogenic bacteria in the genera

Bacillus subtillus, Staphylococcus, Echerichia, Streptococcus, Klebsiella, Pseudomonas and *Salmonella* using flame photometer. It was found that the aqueous extract was most effective in the leakage of Na and K ions than the ethanolic extract of all organisms except *Salmonella*. The aqueous extract released 2.66 ppm Na ions on *Pseudomonas aeruginosa*, whereas ethanolic extract released 13.3 ppm while the K ions released are 9.282 and 49.980 ppm for ethanolic and aqueous extracts respectively [26]. The antimicrobial efficacy of the *C. occidentalis* may result from damages and inactivation of enzymes due to their ability to induce leakage of these ions [27, 28]. Sodium ions and potassium ions have been known to affect osmotic balances in the cell and their leakage might cause cell lyses and eventual death. These ions are also known to activate enzymes, which are organic catalyst that mediate biochemical reactions [29]. Most cell activity including respiratory and biosynthetic function are under the control of enzymes.



Trivial Name	R_1	R_2
Rhein	COOH	H
Aloe-emodin	CH2OH	H
Chrysophanol	CH_3	H
Emodin	CH_3	OH
Physcion	C_{H3}	OCH_3

Figure 2. Phytochemicals isolated from the leaf of *C. occidentalis* plant.

Conclusion

Overall, the present study indicates the antimicrobial properties of leaves extract of *C. occidentalis* and provides some idea about phytochemical evaluation on *C. occidentalis*. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity. Further studies should be undertaken to elucidate the exact mechanism of action by which extracts exert their antimicrobial effect.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed equally during the collection of plant material, experimental data analysis and in the preparation of manuscript.

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