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Research Article

Phytochemical Screening and Antimicrobial Activity of *Caesalpinia sappan* L. Leaves

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ABSTRACT

Objective: The aim of this work was preliminary phytochemical screening and determination of in vitro antimicrobial activity of *C. sappan* leaves (Leguminosae) collected from forest area of Tamilnadu, India. Methods: The crude drug was successively extracted by Soxhlet assembly using Petroleum ether, dichloromethane, ethyl acetate and methanol as solvents. Preliminary phytochemical screening of different extracts was carried out using several colour and precipitative chemical reagents as per described methods. Antimicrobial activity of the extracts was evaluated against fungal strains (*Aspergillus fumigatus* and *Candida albicans*), Gram +ve bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram –ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using agar wells dilution method. Nutrient agar medium at 37 ^{o}C and sabouraud dextrose e agar medium at 28 ^{o}C were used in antimicrobial activity evaluation and antifungal activity evaluation respectively. Results: Preliminary phytochemical screening of *C. sappan* leaves showed the presence of carbohydrates, glycosides, flavonoids, phenols, tannins and amino acids in the crude drug. *Caesapinia sappan* leaf extracts exhibited marked dose dependent antimicrobial activity in vitro against tested fungi and bacteria. Methanolic extract was found to be more potent particularly against *Streptococcus pyogenes* (Gram +ve bacteria). Conclusion: Various phytochemicals were found to be present in *C. sappan* leaves. Methanolic extract of *C. sappan* leaves exhibited better antimicrobial activity in vitro and can be used as a good therapeutic approach for infectious disease management and therapy. Further studies on isolation of phyto-constituents and both in vitro and in vivo evaluation of pharmacological activities of isolated bioactive constituents of the crude drug are recommended as future works.

Keywords: *Caesapinia sappan*, Leguminosae, phytochemical screening, antibacterial activity, antifungal activity.

INTRODUCTION

Back to the nature is not slogan. The last forty years are witnesses of ample researches carried out on exploration of new medicinal agents from natural resources including medicinal plants. This can be attributed to the fact that synthetic and presently available medicines are either too expensive or tend to bring out severe side effects. About 80% of the world population primarily in developing countries depend on traditional system of medicine for their primary health care needs[1].

Infectious diseases remain the leading cause of death worldwide and infections due to antibiotic resistant microorganisms have become move widespread in recent years[2]**.** Both resistance rates of bacteria against existent synthetic antibiotics and the toxicity of some modern synthetic antibiotics are considered as cosmopolitan challenges to the human as well as animals' health. Thus, search for novel antimicrobial agents to combat such resistant pathogens is considered as a crucial approach[25]**.** Screening of medicinal plants having antibacterial potential in order to explore their active constituents as new drugs with novel mechanisms of action, is of great interest of today's researches[4]. Natural products have provided a variety of lead structures, which serve as templates for the development of new drugs molecules[5,6]. Despite the increasing use of herbal medicines worldwide, there is still a significant lack of research data in field of herbal drugs[7]**.** Drugs of medicinal plants origin have good therapeutic potential with no or less side effects than those of synthetic medicinal plant[8].

The genus *Caesalpinia* belongs to family Fabaceae/Leguminosae. The genus comprises about 500 species distributed worldwide especially in tropical and subtropical zones[9]. Among them *Caesalpinia sappan* L. is known as Patang, Sumu (in Chinese herbal medicine) or Sappan. *Caesalpinia sappan* grows wildly in mountainous regions and as an ornamental tree it is cultivated in yard and gardens because of its yellow coloured flowers. *C. sappan* is widely distributed in Burma, Southwest China, Indonesia, Thailand, Vietnam, Myanmar, Sri Lanka, and India[10]. In India the tree is distributed in South India, Orissa, Madhya Pradesh, Tamilnadu, Kerala, Karnataka, Andhra Pradesh and West Bengal[5,12]**.**

C. sappan is a thorny and shrubby small to medium-sized tree up to 4 – 10 m tall. The compound bipinnate leaves of the tree are large up to 20 – 45 cm long and 10 – 20 cm

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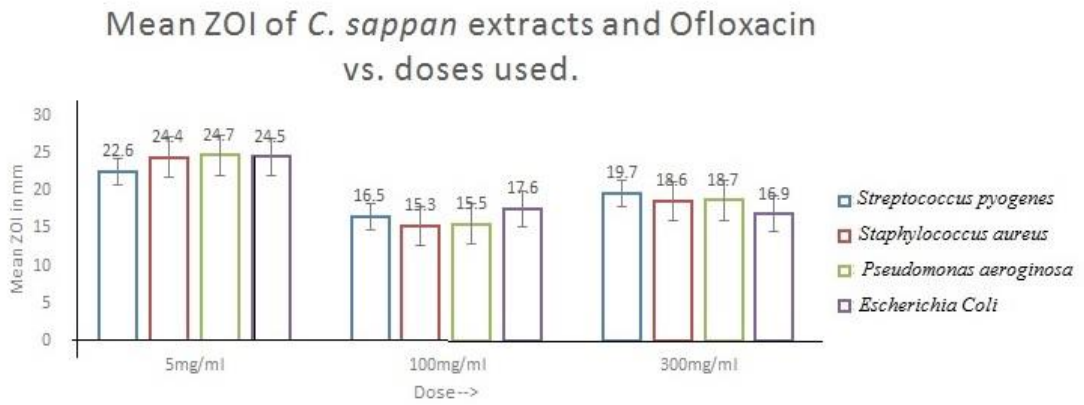

Figure 1: Mean ZOI of *C. sappan* extracts and Ofloxacin vs. doses used.

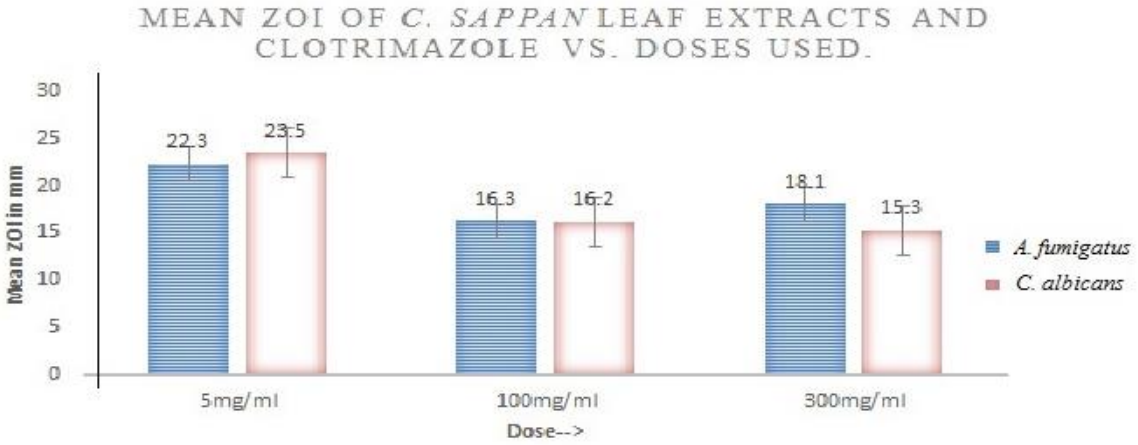

Figure 2: Mean ZOI of *C. sappan* leaf extracts and Clotrimazole vs. Doses used.

broad, each comprised of 8-16 pairs of up to 20 cm long pinnae. Each pinnae in its own turn bears prickles at the base and is composed of 10-20 pairs of leaflets. The leaflets are sub-sessile, oblong, 10-20 mm long and 6-10 mm broad, oblique at the base and rounded to emarginated at the apex. The yellow flowers are fragrant and aggregated in terminal and axillary panicles. The fruits are woody beaked pods, each contains 3-4 yellowish-brown seeds[6,12].

Based on literature review, *C. sappan* has an ancient background of usages in folk and traditional medicinal systems. The dried heartwood of the plant has a wide medicinal usages[13,14,15]. *C. sappan* is used for purifying blood, quenching thirst, treatment of jaundice, cough, respiratory ailments and wounds**,** curing blood pressure, heart diseases, amenorrhea, dysmenorrhea, blood stasis after delivery[5,6]. *C. sappan* is used as an ingredient in preparation of an indigenous drug named Lukol. The drug is orally utilized for treatment of non-specific leucorrhoea and bleeding following insertions of intrauterine device (IUD). The wood is also a component of Vicco vajradanti, a famous tooth paste and tooth powder in India[12]**.** According to Ayurveda, the heartwood is bitter, astringent, sweet, acrid refrigerant, vulnerary, depurative, constipating, sedative and haemostatic. It is useful in vitiated condition of *pitta*, burning sensation, wounds, ulcers, leprosy, skin diseases, diarrhoea, dysentery, epilepsy, convulsions, menorrhagia, diabetes and leucorrhoea[1,6].

Based on traditional usages and ethnobotanical importance of *C. sappan*, leaves of this plant were selected for this study. Preliminary phytochemical screening and antimicrobial activity of *C. sappan* leaves are reported in this paper.

MATERIALS AND METHODS

Procurement of plant materials

The leaves of *Caesalpinia sappan* were procured from Dhanlakshami Agro Plantations and Consultancy, Tamilnadu. The leaves were authenticated by Dr. H.B. Singh, Chief Scientist & Head of Raw Materials Herbarium & Museum (RHMD) at National Institute of Science Communication and Information Resources, New Delhi. The plant materials were dried under shade and ground to coarse powder.

Chemicals

All chemicals, reagents and solvents used in quantitative analysis and chemical investigation were of analytical grade and Lab grade procured from E. Merck, SD Fine and CDH Chemicals.

Microbial strains

Microbial strains used in this work were four bacterial strains namely *Staphylococcus aureus* (MTCC 389),

Table 1: Data showing the results of phytochemical analysis of *C. sappan* leaf different extracts.

Compounds	Tests	Extracts			
		Pet. ether	DCM	EA	MeOH
Alkaloids	Mayer's test	-	-	-	-
	Dragendorff's test	-	-	-	-
	Wagner's test	-	-	-	-
	Hager's test	-	-	-	-
Carbohydrates	Molisch's test	-	-	-	+
	Fehling's test	-	-	-	+
Glycosides	Modified Borntrager's test	-	+	-	+
	Legal test	-	-	-	+
Flavonoids	Lead acetate test	-	+	+	+
	Alkali reagent test	+	+	+	+
Phytosterols	Salkowski test	-	-	-	-
	Tshugajeu test	-	-	-	-
Phenols	Ferric chloride	-	-	-	+
Saponins	Foam test	-	-	-	+
Tannins	Gelatin test	-	-	+	+
Amino acids and proteins	Ninhydrin test	-	+	+	+

'+' = present, '–' = absent, DCM= dichloromethane, EA= ethyl acetate, MeOH=methanol

Table 2: Data showing the optimum mobile phase system for TLC of *C. sappan* leaf different extracts.

Extracts	Solvent systems	Ratio	No. of spots	Respective Rf values
Pet E	Hex : EA	8 : 2	6	0.23, 0.54, 0.61, 0.73, 0.80, 0.82
DCM	Hex : EA	7 : 3	4	0.6, 0.76, 0.82, 0.9
EA	CHCl3 : MtOH	7 : 3	6	0.37, 0.54, 0.57, 0.72, 0.78, 0.82
Me	EA : MeOH : FA : W	10 : 1.1 : 1.1 : 2.6	9	0.07, 0.29, 0.36, 0.45, 0.5, 0.65, 0.7, 0.75, 0.8

CHCl3=chloroform; DCM=dichloromethane extract; EA=ethyl acetate; FA=formic acid; Hex= hexane; Me=methanol extract; MtOH=methanol; Pet E=petroleum ether extract; Rf=refraction factor; W= water

Streptococcus pyogenes (MTCC 1924), *Pseudomonas aeruginosa* (MTCC 424), *E. coli* (MTCC 389) and two fungal strains *Aspergillus fumigatus* (MTCC 879) and *Candida albicans* (MTCC 183). Microbial strains were procured from IMTECH, Chandigarh, India.

Bacteria were sub-cultured from the stock maintained in nutrient agar at 37° C and fungal strains were sub-cultured from the stock maintained in Sabouraud Dextrose Agar (SDA) medium at 28° C. The bacterial strains were grown on MacConkey agar plates at 37° C and maintained on nutrient agar slants while fungi were grown at 30° C and maintained in Sabouraud glucose agar plates.

Extraction of plant materials

The coarsely powdered dried *C. sappan* leaves (100 g) were successively extracted with 2.5 liters of four different solvents namely petroleum ether, dichloromethane, ethyl acetate and methanol with Soxhlet assembly. Extraction with pet ether was run at 60˚ C for 8 hours, with dichloromethane at 40˚ C for 8 hours, with ethyl acetate at 77˚ C for 4 hours and with methanol at 78˚ C for 3 hours. After extraction with each of the used solvents the plant material (marc) was dried at room temperature for overnight before being extracted with the next solvent. Each of the obtained extract was filtered using Wattman No. 1 and the filtrate was concentrated using rotatory evaporator (POPULAR, India). Concentrated extracts were further dried on water-bath (NAVYUG, India) to obtain the semisolid dried extracts. The dried extracts were kept in refrigerator at 4˚ C until being used timely.

Preliminary phytochemical screening

Different extracts of *C. sappan* leaves were subjected to phytochemical screening. The phytochemical investigation was performed using the standard chemical tests as per described in "Practical Pharmacognosy" by Kokate, in "The practical evaluation of phytopharmaceuticals" by Brain and Turner , Harborne (1998) and Siddiqui and Siddiqui[16,17,18,24]..

Development of TLC profile

Small amount of the dried extracts were separately dissolved in few ml of related solvents. Prepared solutions were then used for thin layer chromatography experiments. Laboratory made TLC plates prepared from silica gel G were used for qualitative work. The prepared plates were used after activation for 30 min at 110˚ C in hot air oven (NAVYUG, India). Final chromatograms were taken on pre-coated silica plates of aluminium plates, 0.25 mm thickness (E. Merck, Mumbai) after choosing optimized solvent systems (Table 2.). All TLCs had been performed

Table 3: Data showing antibacterial activity of *C. sappan* leaf extracts

Ext. / Std.	Conc. (mg/ml)	Mean ZOI ± SEM (mm)			
		Gram +ve bacteria		Gram –ve bacteria	
		Staphylococcus aureus	*Streptococcus pyogenes*	*Escherichia coli*	*Pseudomonas aeruginosa*
Ofloxacin	5	24.4±0.10	22.6±0.17	24.5±0.08	24.7±0.15
EA ext.	100	11.2±0.11	11.5±0.17	12.4±0.08	12.2±0.11
	200	12.5±0.14	14.6±0.14	14.5±0.13	13.5±0.05
	300	15.3±0.17	16.5±0.17	17.6±0.12	15.5±0.14
MeOH ext.	100	12.5±0.05	12.7±0.24	12.5±0.08	13.4±0,15
	200	14.4±0.05	15.7±0.15	15.3±0.10	15.5±0.20
	300	18.6±0.17	19.7±0.8	16.9±0.17	18.7±0.11

Ext. =extract, Std = standard drug, EA = Ethylacetate, ZOI=zone of inhibition, MeOH = methanol.19.7

according to methods proposed by Harborne and Wagner[16,27].

Optimization of mobile phase systems

TLC plates were developed in CAMAG TLC jars pre-saturated with the solvent systems. Different solvent systems with different ratios were used to find out the optimum solvent system for each extract. Developed TLC plates were air dried and observed visibly and under UV light (254 nm and 366 nm) using UV chamber (POPULAR, India). The spots were observed for their number and resolution. The number of separated spots and their Rf values were recorded for each TLC plate. Based on higher number of spots with better resolutions optimum mobile phase system was selected for every one of the tested extracts (Table 2.). The methanolic extract of *C. sappan* leaves was examined by TLC on silica gel G after optimization.

Antibacterial screening

In vitro antibacterial activity of *C. sappan* leaf (methanolic and ethylacetate extracts) was studied against two Gram +ve (*Staphylococcus aureus, Streptococcus pyogenes*) and two Gram –ve (*Escherichia coli and Pseudomonas aeruginosa*) strains by agar wells diffusion method ref. Nutrient agar was used as bacteriological medium. Both extracts were dissolved in sufficient volume of DMSO to get the different concentrations i.e. 100 mg, 200 mg and 300 mg/ml. Diameters of zones of inhibition produced by the extracts were compared with those produced by the standard antibiotic (Ofloxacin). The experiment was performed in triplicate to minimize the error.

The tests were carried out in triplicates and zone of inhibition was reported as mean ± SEM (N=3). (Table 3), (Figure 1.). Significance of the results were determining by application of one way analysis of variance (ANOVA) and student t-test ($p<0.05$).

Anti-Fungal screening

Two pathogenic fungi (*Aspergillus fumigatus* and *Candida albicans*) were used for the antifungal activity. Sabouraud dextrose agar medium (Hi-Media) was used as fungal growth medium. Both extracts (methanolic and ethyl acetate extracts) were dissolved in sufficient volume of DMSO to get different concentrations of 100 mg, 200 mg and 300 mg/ml. The in vitro antifungal activity was performed by agar wells diffusion method (Boyanova et al., 2005; Udgire and Pathade, 2013) Clotrimazole (0.1 ml at 5 mg/ml) was used as a standard.

The experiment was carried out at in triplicates and the zones of inhibition were reported as mean ± SD (N=3). Significance of the results were determining by application of one way analysis of variance (ANOVA) and student t-test ($p<0.05$).

RESULTS AND DISCUSSION

The results of qualitative phytochemical screening of *Caesalpinia sappan* leaves different extracts are tabulated in Table 1. Optimized mobile phase systems were developed for TLC profiling of *C. sappan* leaves different extracts (Table 2). The methanolic and ethyl acetate extracts of *C. sappan* leaves were tested for their antimicrobial activities. Both the extracts at dose of 300 mg/ml showed significant and dose dependent antibacterial and anti-fungal results against tested bacterial and fungal strains (Table 3 and Table 4), (Figure 1 and Figure 2).

The phytochemical screening revealed the presence of carbohydrates, flavonoids, glycosides, phenols, tannins, amino acids and proteins but absence of phytosterols, alkaloids and saponins in the crude drug (Table 1).

The optimum mobile phases developed for *C. sappan* leaves different solvent extracts can be used in TLC profiling of crude drug. Optimization was carried out by hit and trial method and trying several solvents systems with different ratios. The system/ratios giving higher number of well-resolved spots were selected as optimized mobile phase solvents (Table 2). The optimized solvent systems are hoped to be useful for fractionation and isolation of *C. sappan* leaves different phytoconstituents in future works.

C. sappan leaves extracts revealed dose dependent antibacterial and antifungal activities at doses of 100, 200 and 300 mg/ml. As shown in Table 3 and Figure 1, the methanol extract at dose of 300mg/ml against four bacteria namely *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *E. coli* showed zones of inhibition of 19.7±0.8, 18.6±0.17, 18.7±0.11 and 16.9±0.17 respectively. Ethyl acetate extract at the same dose 300 mg/ml showed zones of inhibition of 17.6±0.12, 16.5±0.17, 15.5±0.14 and 15.3±0.17 against *E. coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphyllococcus aureus* respectively. Antifungal potential of both methanolic and ethyl acetate extracts were tested in vitro against *Aspergillus fumigatus* and *Candida*

Table 4: Data showing antifungal activity of *C. sappan* leaf extracts.

Ext./Std.	Conc.(mg/ml)	Mean ZOI ± SEM (mm)	
		A. fumigatus	*C. albicans*
Clotrimazole	5	22.3±0.12	23.5±0.08
EA ext.	100	12.5±0.16	9.2±0.03
	200	14.4±0.05	14.1±0.05
	300	16.3±0.05	16.2±0.05
MeOH ext.	100	12.6±0.15	11.4±0.13
	200	15.4±0.10	13.7±0.06
	300	18.1±0.03	15.3±0.06

EA ext. = ethyl acetate extract, Ext = extract, Conc = concentration, MeOH ext. = methanol extract, Std= standard drug, ZOI= zone of inhibition.

albicans. The methanolic extract at dose 300 mg/ml exhibited better potential against *Aspergillus fumigatus* than that of ethyl acetate extract. In case of *Aspergillus fumigatus* zone of inhibition for 300 mg/ml dose of both methanolic and ethyl acetate extracts were measured to be 18.1±0.03 mm and 16.3±0.05 mm respectively. As our data (Figure 1, Figure 2, Table 3, Table 4) show, the methanolic extract of *C. sappan* leaves revealed better antimicrobial activity than that of ethyl acetate extract against all tested bacteria and fungi. Exceptionally, in case of *E. coli* (Gram –ve) and *C. albicans* ethyl acetate extract was slightly potent than methanol extract. During this work, it was found that among the tested bacterial strains, *S. pyogenes* (Gram +ve) was more sensitive to methanolic extract of *C. sappan* leaves. The stronger activity of methanolic extract can be attributed to the polar compounds of the crude drug which have more affinity to be extracted with polar solvents (e.g. ethanol). Flavonoids, phenolics and tannins which are extractable compounds with methanol were present in methanol extract of *C. sappan* leaves. Our results of antimicrobial activity tests were in compliance with those of previous literature which reported the potent antimicrobial effects of methanolic extract of *C. sappan* and other medicinal plants[1,2,24]. The ethanolic and methanolic extracts of *C. sappan* bark have shown potent antibacterial activity[1]. Methanolic extract of the bark exhibited stronger antibacterial activity than that of ethanol extract. Among the six tested bacteria *Staphylococcus aureus* (Gram+ve) was found more susceptible to the extract[1]**.** When ethanol, chloroform and ethyl acetate extracts of *Cucumis anguria* fruits were studied against some human pathogenic bacteria and fungi, the ethanol extract showed more potent antimicrobial activity[21]. Both methanolic and ethanolic extracts of *C. sappan* heartwood and leaves have shown strong inhibitory effect in vitro when tested against some human pathogenic bacteria and fungi[23]. Brazilin a neoisoflavonoids has been isolated from ethanolic and methanolic extracts of *C. sappan* heartwood[15]. Brazilin exhibited strong antibacterial activity against multi drug resistant bacteria (dimethoxy phenyl-penicillin-resistant *Staphylococcus aureus*) and vancomycin-resistant *enterococcus*[2,24]**.** Among the two categories of bacteria, Gram +ve strain was more susceptible to methanolic. This may refer to the complexity of the cell wall structure of Gram –ve bacteria than that of Gram+ve bacteria. Our study concluded that metabolites with potent antimicrobial property present in *C. sappan* leaves can be extracted efficiently with methanol. *C. sappan* heartwood is very well proved and known for its folkloric and ancient wide therapeutic usage in different systems of medicines. We claim that the methanol extract of the leaves can also be used in curing both bacterial and fungal infections. However, we recommend performing further researches on *C. sappan* leaves for exploring further screening of pharmacological activity of the crude drug both in vitro and in vivo. Since it is evident that medicinal plants are the power-plants of synthesizing pharmacologically potent leads that can serve as strong curing agents.

CONCLUSION

Although a diverse number of modern medicines are used for curing acute and several illnesses, herbal medicines have maintained their importance. Our survey of literature concluded that *Caesalpinia sappan* is used traditionally since ancient times for many medicinal purposes. During the present work methanolic and ethyl acetate extracts of *C. sappan* leaves revealed antimicrobial properties. Methanolic extract showed better antibacterial activity in vitro, and can be developed as antibacterial agent for curing infectious diseases. However, further works are required to explore other pharmacological activities of *C. sappan* leaves both in vitro and in vivo.

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Conflict of interest

Authors declare that they do not have any conflict of interest.

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