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Biochemical Estimation and Antimicrobial Activities of the Extracts of *Caesalpinia Sappan* Linn.

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Abstract

Caesalpinia sappan Linn., a traditional Indian medicinal plant used widely in oriental medicine. The plant extracts were found to be a good source of secondary metabolites, vitamins and metals. The extracts were further tested against certain human pathogenic microbes. The methanol and ethyl acetate extracts of the heartwood was found to be effective against certain pathogenic microbes.

Key words: *Caesalpinia sappan*, Secondary metabolites, Vitamins, Antimicrobial activity

Introduction

Plants have always been a common source of medicaments due to their bioactive principles. Inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant material as a source of medicines for a wide variety of human ailments. Of the 2,50,000 higher plant species, more than 80,000 are medicinal. India is one of the world's 12 biodiversity centers with the presence of over 45000 different plant species. Of these, about 15000-20000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional communities. In India, drugs of herbal origin have been used in traditional systems of medicine such as *Unani* and *Ayurveda* since ancient times. The Ayurveda system of medicine uses about 700 species, *Unani* 700, *Siddha* 600, *Amchi* 600 and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed etc. some drugs are prepared from exudates of plant product such as gums, resins and latex. *Caesalpinia sappan* Linn. (Sappan lignum) heartwood is being used in Kerala as herbal drinking water for its antithirst, blood purifying, antidiabetic (Moon *et al.*, 1992; Kim *et al.*, 1997), improvement of complexion and several other properties. The plant is also being used worldwide for a large number of traditional medicinal purposes. Recent research confirms its anticancer, antitumor, antimicrobial (Kim *et al.*, 2004), antiviral, immunostimulant (Moon *et al.*, 1992; Mok *et al.*, 1998), antifungal activity (Reddy *et al.*, 2003) and several other activities. In addition

to medicinal and aromatic properties trunk wood, stem and leaves are also used as dyeing agent. Heartwood is also used as a colouring agent for wine, meat, fabric and it is well established as a safe natural colouring agent with good medicinal value for food products, beverages and pharmaceuticals. The woody part contains brazilin and brasilein and an essential oil consisting of D-a-phellandrene, ocimene, tannin, gallic acid and saponin. Brazilin is found to be the main constituent of the plant responsible for several of its biological activities (Mar *et al.*, 2003). In recent years, the extract of Sappan Lignum (the dried heartwood of *Caesalpinia sappan* L.) has been found to be a potential immunosuppressive agent. The reported main phenolic compounds in Sappan Lignum were divided into to four structural sub-types: i.e. brazilin, chalcone, protosappanin and homisoflavonoid. Among the protosappanin derivatives, such as protosappanin B and isoprotosappanin B, 10-O-methylprotosappanin B and 10-O-methylisoprotosappanin B, as well as protosappanin E1 and protosappanin E2 occur as pairs of epimers. Meanwhile, the homisoflavonoid epimers sappanol and episappanol, 4-O-methylsappanol and 4-O-methylepisappanol, 3'-O-methylsappanol and 3'-O-methylepisappanol were successively isolated along with a new compound, a 3-benzylchroman derivative 3'-deoxy-4-O-methylepisappanol (Fu *et al.*, 2008). Considering the medicinal importance of *C. sappan*, a study on the efficacy of *C. sappan* extracts on human pathogenic bacteria and fungus has been made anticipating to develop antibiotic in future.

Materials and Methods

Different parts of *C. sappan* such as leaves, pods, twigs and heartwood were used for the analysis. All the plant parts were sequentially extracted. Leaves were extracted with water, methanol and ethyl acetate and all other tissues were extracted in the order of water, ethyl acetate and methanol.

Estimation of secondary metabolites and vitamins

Plants secondary metabolites have recently been referred to as phytochemicals, which are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities (Akinmoladun *et al.*, 2007). Hence in this study the secondary metabolites and vitamins were estimated using the following methods.

Total phenol

Principle

Phenol reacts with phosphomolybdic acid in Folin-Ciocalteu reagent in alkaline medium and produce blue coloured complex (Molybdenum blue).

The method of Malick and Singh (1980) was followed. 0.5 ml of Folin-Ciocalteu reagent, diluted with an equal volume of distilled water before use was added to 1 ml of the alcohol extract in a test tube followed by 2 ml of 20% Na₂CO₃ and the mixture was heated on a boiling water bath for 1 minute. The blue colour was diluted to water and read at 650 nm. Reagent blank was maintained with 80% ethanol. Total phenol was calculated keeping pyrogallol as the standard.

Estimation of Tannins

Principle

Tannin like compounds reduces phosphotungstic molybdic acid in alkaline solution to produce a highly colored blue solution, the intensity of which is proportional to the amount of tannins. The intensity is measured in a spectrophotometer at 700nm.

The method of Schanderl (1970) was followed to estimate tannins. 0.1g of the sample was ground with 75ml of distilled water. Heated for 30 minutes, centrifuged (2000rpm-20min). The supernatant was used as sample after making up to 100ml. 1ml of the sample was added to 75ml water along with 5ml of Folin-Denis reagent, 10 ml of sodium carbonate solution and dilute to 100ml with water. A blank was pre-

pared with water instead of the sample. The absorbance was read at 700nm after 30 minutes. A standard graph was prepared by using 0-100µg tannic acid.

Quantification of Alkaloids

Principle

10mg of the sample were weighed into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added and covered to stand for 4 h. This was filtered and the extract was concentrated using a waterbath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed (Harborne, 1973; Obadoni and Ochuko, 2001).

Quantification of Flavonoids

Principle

10mg of the plant samples were extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper no. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed (Boham and Kocipai, 1994).

Vitamins

Determination of ascorbic acid (Vitamin C)

Ascorbic acid reduces the 2,6-dichlorophenol indophenol dye to a colourless leuco-base. The ascorbic acid gets oxidized to dehydroascorbic acid. The dehydroascorbic acid alone reacts quantitatively and not the other reducing substances present in the sample extract (Sadasivam and Manickam, 1996).

1g of sample was homogenized with 5ml of 4% oxalic acid. Centrifuge and collect the liquid. Transfer the supernatant in one test tube, add 5 drops of bromine water and make up the volume up to 10ml with 4% oxalic acid. 0.1 ml of brominated sample extract was taken and made up to 3 ml with distilled water. To this 1 ml of DNPH (Dinitrophenyl Hydrazine) reagent with 1-2 drops of thiourea was added. The tubes were incubated at 37°C for 3 hrs. After incubation 7 ml of 80% sulphuric acid was added to dissolve the orange-red osazone crystals. Measure the absorbance at 540nm.

Determination of riboflavin

Riboflavin is water soluble and photosensitive vitamin. 10mg of the sample was extracted with 10 ml of 50% ethanol solution and shaken for 1 hour. This was filtered into a 25 ml flask; 2 ml of the extract was pipette into 25 ml volumetric flask. 2 ml of 5% potassium permanganate and 2 ml of 30% Hydrogen peroxide were added and allowed to stand over a hot water bath for about 30 min. 0.4 ml of 40% sodium sulphate was added. This was made up to 10 ml mark and the absorbance measured at 510 nm in a spectrophotometer (Okwu and Josiah, 2006).

Determination of thiamin

Thiamin is one of the B-group vitamin whose deficiency causes beri-beri. 10mg of the sample were homogenized with ethanolic sodium hydroxide. It was filtered in to a 100 ml flask. 2 ml of the filtrate was pipette and the colour developed by addition of 2 ml of potassium dichromate and read at 360 nm. A blank sample was prepared and the colour also developed and read at the same (Okwu and Josiah, 2006).

Antibacterial and antifungal activity of the plant extracts

The extract of the dried heartwood samples were used for the study. The extract of different concentrations such as 50, 100 and 200 ppm was tested against many bacterial pathogens such as *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Citrobacter divergens* causing diarrhea, urinary tract infection, intravenous liver infection, nosocomial blood stream infection and neonatal meningitis respectively. The ethanolic and aqueous extracts were tested against different fungal pathogens such as *Aspergillus flavus*, *Aspergillus niger* and *Fusarium sps* causing lung and ear disease for their antifungal activity. It was demonstrated by well diffusion method described by Bauer *et al.*, (1966).

The disc diffusion method was used to screen the antimicrobial activity. In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained. The agar plates were prepared and were allowed to solidify for 5 minutes and 0.1 % inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. The different concentrations of extracts (1.25, 2.5 and 5 mg/disc) were loaded on 4 mm sterile disc. The loaded discs were placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 37°C for 24 hrs. The filter paper discs containing methanol

and ethyl acetate served as negative controls. Standards broad spectrum antibiotics like ampicillin, chloramphenicol, kanamycin, penicillin and Amphotericin B were used as positive controls.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined, using a common broth micro dilution method in 96-well microtiter plates (Camporese *et al.*, 2003; NCCLS, 2001). Two fold dilutions of each extract were carried out, starting from 16 to 0.062 mg/mL. 10 µL of the previously prepared different methanol microbial suspensions (105 CFU/ mL) were added to each well. Plates were incubated for 18 h at 37° C and then were examined with Elisa reader (Awareness Tech, USA) at 620 nm and the lowest concentration of each extract showing no growth was taken as its minimum inhibition concentration (MIC). The solution DMSO (100 µL/mL) served as the negative control. All the samples were tested in triplicates to confirm the activity.

Results and Discussions

The plants form secondary metabolites, which are organic compounds for the protection against pests and dysfunctions in the human body, used as colouring agents, scents or attractants and as the plant's own hormones. A diet which is rich in plant foods contain a variety of secondary metabolites and contribute to protecting the body against cancer and cardiovascular illnesses (Crozier *et al.*, 2006).

Secondary metabolites of *C. sappan*

Quantitative determination of secondary metabolites of *C. sappan* has been made and the results are presented below in figure 1. High quantities of alkaloids were found in various tissues of *C. sappan* twig (14 mg/g) followed by the heartwood (10mg/g), flavonoid content was estimated to be 28 mg/g in heartwood sample followed by 21.3 mg/g in the pod. Alkaloids and their derivatives are used for analgesic, anti-spasmodic and antibacterial effects anticancer activity and anti-inflammatory activity (Del-Rio *et al.*, 1997; Okwu, 2004; Stray, 1998; Okwu and Okwu, 2004). The presence of such high proportion of alkaloid and flavonoids seem to contribute to the pharmacological and antimicrobial activity of the plant, as reported by many researchers (Moon *et al.*, 1990 and 1992, Nagai *et al.*, 1984, Hufford *et al.*, 1975). High values of phenolic compounds and tannins were estimated in *C. sappan* heartwood 150mg/g and 171mg/g respectively and thus it possesses potent anti-microbial activity (Xu *et al.*, 2005).

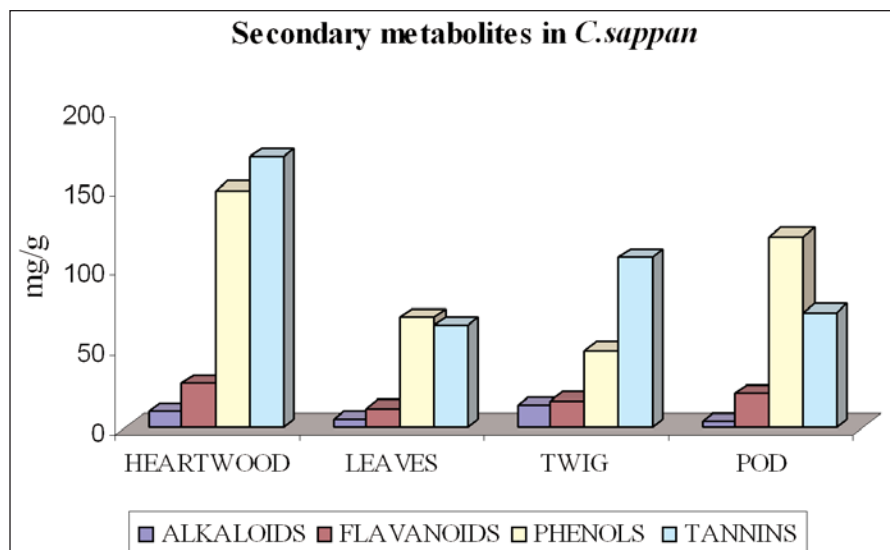


Fig. 1: Secondary metabolites of *C. sappan*

Vitamins of *C. sappan*

All the tissues of *C. sappan* plant are good sources of ascorbic acids, riboflavin, thiamine and niacin (Figure 2). The heartwood of *C. sappan* showed maximum amount of about 70.3 mg/g of ascorbic acid, 39.9 mg/g of niacin, 9.3 mg/g of thiamine and 8.3 mg/g of riboflavin. Vitamins are required for normal wound healing and can be efficiently used to reverse the adverse effects of deficiencies by proper dose administration of the extract (Okwu, 2003 and 2004). Intake of the aqueous extracts of *C.sappan* heartwood may suffice the daily recommended dose of vitamins required by man.

Metals of *C. sappan*

Some metals like copper, zinc, iron, magnesium, sodium, potassium and calcium are essential trace nutrient to all high plants and animals. In animals, including humans, metals are found primarily in the bloodstream, as a co-factor in various enzymes and in pigments like hemoglobin. They are needed in trace amounts for the normal metabolism of the cell. However in sufficient amounts; metals can be poisonous and even fatal to organisms. *C. sappan* has a fair amount of copper, mercury, zinc, iron, sodium and potassium of about 0.69 ppm, 1.16 ppm, 1.12 ppm, 2.02 ppm, 100 ppm and 1500 ppm

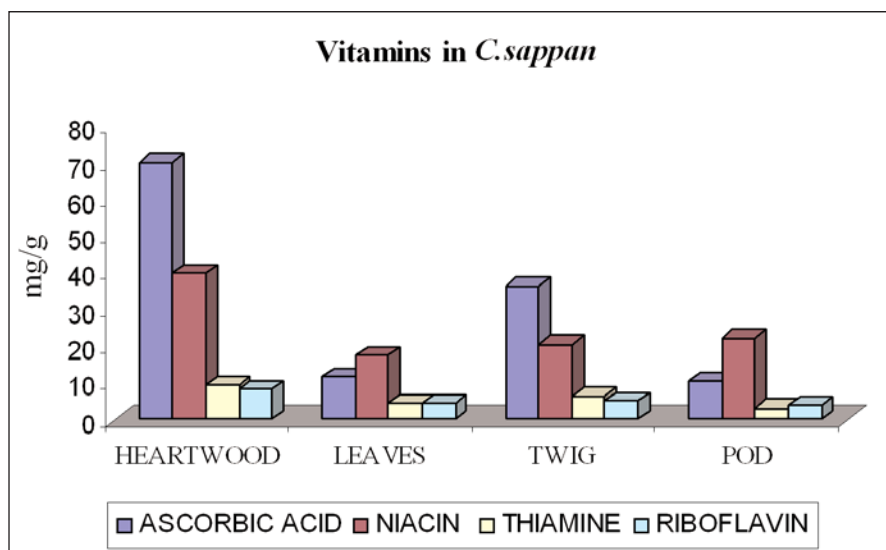


Fig. 2: Vitamins of *C. sappan*

respectively (Table I). Copper acts as a cofactor in various enzymatic actions. The antimicrobial effects of the *C.sappan* extracts may be attributed by the presence of copper ions in it. Mercury as the main ingredient is being prescribed by the physicians around the world for numerous ailments including constipation, depression, child bearing and toothaches (Mayell, 2001). Zinc is one of the most important minerals used by the body for various functions Iron is often incorporated into the heme complex. Potassium is important in neuron function and in influencing osmotic balance between cells and the interstitial fluid. Sodium ions are necessary for regulation of blood and body fluids, transmission of nerve impulses, heart activity and certain metabolite functions.

Table I: Metal composition of aqueous extract of *C. sappan*

Sl. No.	Metal	Amount Detected (ppm)
1	Copper	0.69
2	Mercury	1.16
3	Zinc	1.12
4	Iron	2.02
5	Manganese	ND
6	Sodium	100
7	Potassium	1500

Antimicrobial studies on *C. sappan*

Explant extracts derived from medicinal and aromatic plants especially phenols, phenolics and polyphenolic compounds have been claimed to have multiple biological activities including antioxidant, anti-inflammatory, antiviral, anticarcinogenic, antibacterial, antifungal, antioxidant effects etc (Halliwell *et al.*, 1995; Halliwell, 1997; Bisignano *et al.*,

1999). Different extracts of heartwood of *C. sappan* were examined for their antimicrobial property activity.

Methanolic and ethyl acetate extracts of the heartwood sample of *C. sappan* showed maximum zone of inhibition at 200 ppm against *E. coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Citrobacter divergens* and *Pseudomonas* sp. and inhibition zone from 1.1 - 2.7cm was observed against the above mentioned organisms (Table II). Egwaikhide and Gimba, (2007) evaluated the antibacterial activity of ethyl acetate extracts of *Plectranthus glandulosus* and observed the zone of inhibition ranging from 1.0-2.6 cm against *Bacillus subtilis* and *Staphylococcus aureus*. The ethyl extracts of *Parkia biglobosa* and *Parkia bicolor* were studied against *Bacillus cereus* and *Staphylococcus aureus* in varying concentrations such as 50mg/ml and 100mg/ml and it was concluded that higher the concentration of the plant extracts increased the antibacterial activity Ajaiyeoba and Fadare, (2002). It is evident from this study, that as the concentration of the various extracts of heartwood sample were increased, there is increase in the diameter of the inhibition zone irrespective of the organism used. The methanol and ethyl acetate extracts were used in this study as they were found to give better effect against the microorganisms, a study by Lin *et al.*, (1999) reported that the methanol extracts of the plant provide more consistent antimicrobial activity compared to those extracted by other solvents. A study was performed by Chaudhary and Tariq (2006), who indicated that *Coriander sativum* has inhibited the growth of *Micrococcus leuteus* and *Mycobacterium smegmatus* in their ethyl acetate extracts mainly due to the presence of phenolic compounds. High values of phenolic compounds and tannins were detected in the heartwood sample of *C. sappan* in our study which might have contributed to the anti-microbial activity of the various extracts.

Table II: Antibacterial activity of heartwood of *C. sappan*

S.No.	Name of the bacterial pathogen	Control	Zone of inhibition (mm)					
			Methanolic extract (ppm/well)			Ethylacetate extract (ppm/well)		
			50	100	200	50	100	200
1	<i>Escherichia coli</i>	20 ^C	06	08	11	07	10	13
2	<i>Proteus vulgaris</i>	30 ^A	11	19	26	-	-	-
3	<i>Staphylococcus aureus</i>	25 ^P	-	-	-	-	-	-
4	<i>Pseudomonas aeruginosa</i>	22 ^K	-	-	-	13	20	25
5	<i>Klebsiella pneumonia</i>	28 ^K	12	16	26	14	21	27
6	<i>Citrobacter divergens</i>	27 ^A	17	18	25	09	13	19

Standards antibiotic discs of C-Chloramphenicol, A-Ampicillin, P- Penicillin and K-Kanamycin, Concentration 30 µg/disc.

Table III shows the inhibitory zone profile of heartwood sample against fungal pathogens. *Fusarium* sps. was found to be resistant to the extracts of heartwood sample, it might be susceptible to the extracts of the other tissues of *C. sappan*. From this study it may be recorded that the methanolic and ethyl acetate extracts of the heartwood of *C. sappan* have least impact on the human pathogenic fungi.

vided some biochemical basis for the ethnomedicinal values of extracts from *C. sappan* in the treatment and prevention of infections like diarrhoea. As rich source of phytochemicals, minerals and vitamins *C. sappan* could be a potential source of useful drugs. HPLC analysis reveals the presence of anthocyanin in *C. sappan* which is also a rich source of polyphenols and hence it can be widely used in food indus

Table III: Antifungal activity of heartwood of *C. sappan*

S.No.	Name of the bacterial pathogen	Control	Zone of inhibition (mm)					
			Methanolic extract (ppm/well)			Ethylacetate extract (ppm/well)		
			50	100	200	50	100	200
1	<i>Aspergillus flavus</i>	28 ^A	-	-	-	-	20	30
2	<i>Aspergillus niger</i>	16 ^C	-	-	06	-	-	-
3	<i>Fusarium sps</i>	20 ^A	-	-	-	-	-	-
4	<i>Ganoderma lucidum</i>	22 ^C	8	13	17	-	-	-

Standards antibiotic discs of *C-Chloramphenicol* (30µg/disc), *A- Amphotericin B* (25µg/disc)

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined for the methanol and ethyl acetate extracts against bacterial strains (Table IV). *E. coli* had an MIC of 50µg/ml for both the methanol and ethyl acetate extracts. The methanol extract was effective on *P. vulgaris* at 150µg/ml; *P. aeruginosa* was sensitive to the ethyl acetate extract and exhibited an MIC of 100µg/ml. *K. pneumonia* and *C. divergens* were sensitive to both the extracts and had an MIC from 50-150µg/ml.

Table IV: Minimum inhibition concentration values

Sl. No.	Name of the bacterial pathogen	Minimum inhibitory concentration (MIC) (µg/ml)	
		Methanolic extract	Ethylacetate extract
1.	<i>Escherichia coli</i>	50	50
2.	<i>Proteus vulgaris</i>	150	-
3.	<i>Pseudomonas aeruginosa</i>	-	100
4.	<i>Klebsiella pneumonia</i>	100	150
5.	<i>Citrobacter divergens</i>	100	50

Conclusion

Caesalpinia sappan is one of the trees found to possess many medicinal values. The present study therefore has pro-

vided some biochemical basis for the ethnomedicinal values of extracts from *C. sappan* in the treatment and prevention of infections like diarrhoea. As rich source of phytochemicals, minerals and vitamins *C. sappan* could be a potential source of useful drugs. HPLC analysis reveals the presence of anthocyanin in *C. sappan* which is also a rich source of polyphenols and hence it can be widely used in food indus

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