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Comparative study of *in vitro* antibacterial activity of leaves, bark, heart wood and seed extracts of *Caesalpinia sappan* L.

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ABSTRACT

Objective: To evaluate and compare the maximum antimicrobial activity by screening different parts of *Caesalpinia sappan* L. extracted in different solvents against *Salmonella ebony* (MTCC 3384) (*S. ebony*), *Klebsiella pneumoniae* (MTCC 432) (*K. pneumoniae*), *Escherichia coli* (MTCC 443) (*E. coli*) and *Bacillus subtilis* (MTCC 10619) (*B. subtilis*).

Methods: Dried plant parts were extracted with petroleum ether, methanol, chloroform and water by Soxhlet extraction method. The extracts were tested against *S. ebony*, *K. pneumoniae*, *E. coli* and *B. subtilis* by using the agar well diffusion method.

Results: Among the above solvents, the methanol and chloroform and aqueous extracts of leaves, seeds, bark and heart wood showed strong antibacterial activity. The inhibition zone for heart wood extracts against *K. pneumoniae* was (30.333 ± 0.330) mm in methanol, chloroform (28.166 ± 0.730) mm and aqueous extract (28.166 ± 0.170) mm; for *B. subtilis*, that was (27.333 ± 0.440) mm in methanol, (27.166 ± 0.170) mm in chloroform, (26.166 ± 0.440) mm in aqueous extract and least in petroleum ether (12.660 ± 0.170) mm. The leaf extracts in methanol showed maximum antibacterial activity against *S. ebony* as seen by the inhibition zone (16.000 ± 0.290) mm and *K. pneumoniae* (13.000 ± 0.290) mm. The maximum antibacterial response of the seed extract against *K. pneumoniae* was observed in chloroform solvent (14.000 ± 0.580) mm followed by aqueous extract (13.833 ± 0.600) mm. No response was observed in petroleum ether and methanol.

Conclusions: The heart wood extracts showed the highest antibacterial activity having a minimal inhibition concentration of 2 mg/mL in all three solvents against the four bacterial strains, except petroleum ether where MIC was 5 mg/mL against *E. coli* and *B. subtilis*. The aqueous and methanolic extracts of bark showed minimal inhibition concentrations of 5 mg/mL and 2 mg/mL against *K. pneumoniae* respectively whereas aqueous extract of bark showed a minimal inhibition concentration of 5 mg/mL against *E. coli*.

1. Introduction

The growth of new infectious diseases and emergence of several infections appears to have been controlled, but the increase in multidrug resistance bacteria is followed which poses a serious threat to human and animal and creats the necessity for studies directed towards the development of new antimicrobials. Since the 1930s to 1960s, 20 classes of antibiotics were available in market but in the last four decades only three new classes of antibiotics have been introduced[1-3]. Considering the failure to acquire new molecules with antimicrobial properties from microorganisms, the screening and identification of antimicrobials from natural plant sources is of great importance. Phytochemicals derived from plant secondary metabolites serve as the prototype to develop less toxic and more effective medicines in controlling the growth of microorganism[4,5]. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or virus. *Caesalpinia sappan* L. (*C. sappan*) or East Indian red wood tree (Caesalpiniaceae) is a medicinal plant having a lot of pharmaceutical and commercial importance. The heart wood of *C. sappan* is traditionally used in India (Ayurveda) for wound healing, treatment of ulcers, diarrhea, epilepsy, diabetes, *etc*. An herbal drink of the heart wood is used for its blood purifying effect and anti-diabetic effect[6,7]. In traditional Chinese medicine, it is used as analgesic and anti-inflammation for the treatment of traumatic disease and menstrual disorders. The seeds are used as a stomachic in Indo-China. The tree is also a source of the commercial redwood or Brazil wood. Flavonoids[8-10] and phenolic[11,12] such as 4-O-methylsappanol, protosappanin B[13], protosappanin A[14], protosappanin E, caesalpin J[15], brazilin[16], brazilein, triterpenoid and steroid (such as camp sterol, stigmasterol, β-sitosterol) were isolated from the wood. The hepatoprotection[6],

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immunomodulation[16], hypoglycemia[17], anticomplementary[18], anticonvulsant[19], anti-inflammatory, anti-oxidation[20-22] and other biological activities of *C. sappan* have been reported. The antibacterial activity has been reported earlier by using different strains and solvent extracts of different parts of the plants[23-26], but only a limited solvent extracts were used for antibacterial activity assay. The objective of this study was to evaluate and compare the maximum antimicrobial activity by screening different parts of *C. sappan* extracted in different solvents against *Salmonella ebony* (MTCC 3384) (*S. ebony*), *Klebsiella pneumoniae* (MTCC 432) (*K. pneumoniae*), *Escherichia coli* (MTCC 443) (*E. coli*) anaad *Bacillus subtilis* (MTCC 10619) (*B. subtilis*).

2. Materials and methods

2.1. Collection of plant material

The seed, bark and heart wood of *C. sappan* used in this study were obtained from a commercial supplier (Farm Trust India, Palakkad, Kerala, India), and leaf materials were collected from a 4-year-old plant grown from the above seeds in the green house facility of Yogi Vemana University and it was confirmed by Dr A. Madhusudhana Reddy, Asst. Prof., Department of Botany, Yogi Vemana University. The plant materials were washed, chopped, shade dried and powdered and then packed in polythene bags for further analysis. The voucher specimen (Yogi Vemana University Net house (KDP) AMR4721YVUH) was deposited in herbarium facility of Department of Botany, Yogi Vemana University.

2.2. Preparation of plant extracts

Soxhlet extraction method was used for the extraction of seed, bark, heart wood and leaves of *C. sappan*[27]. Twenty grams of plant powder was packed in Whatmann No. 1 filter paper and placed in extraction chamber which was suspended below the round bottom flask containing the solvent (200 mL) and above the condenser. The flask was heated and the solvent evaporated, moved into the condenser where it was converted into liquid that dropped down into the extraction chamber containing the plant materials. The extraction chamber was with solvent surrounding the sample, and designed as when solvent exceeded a certain level it overflowed and dropped into

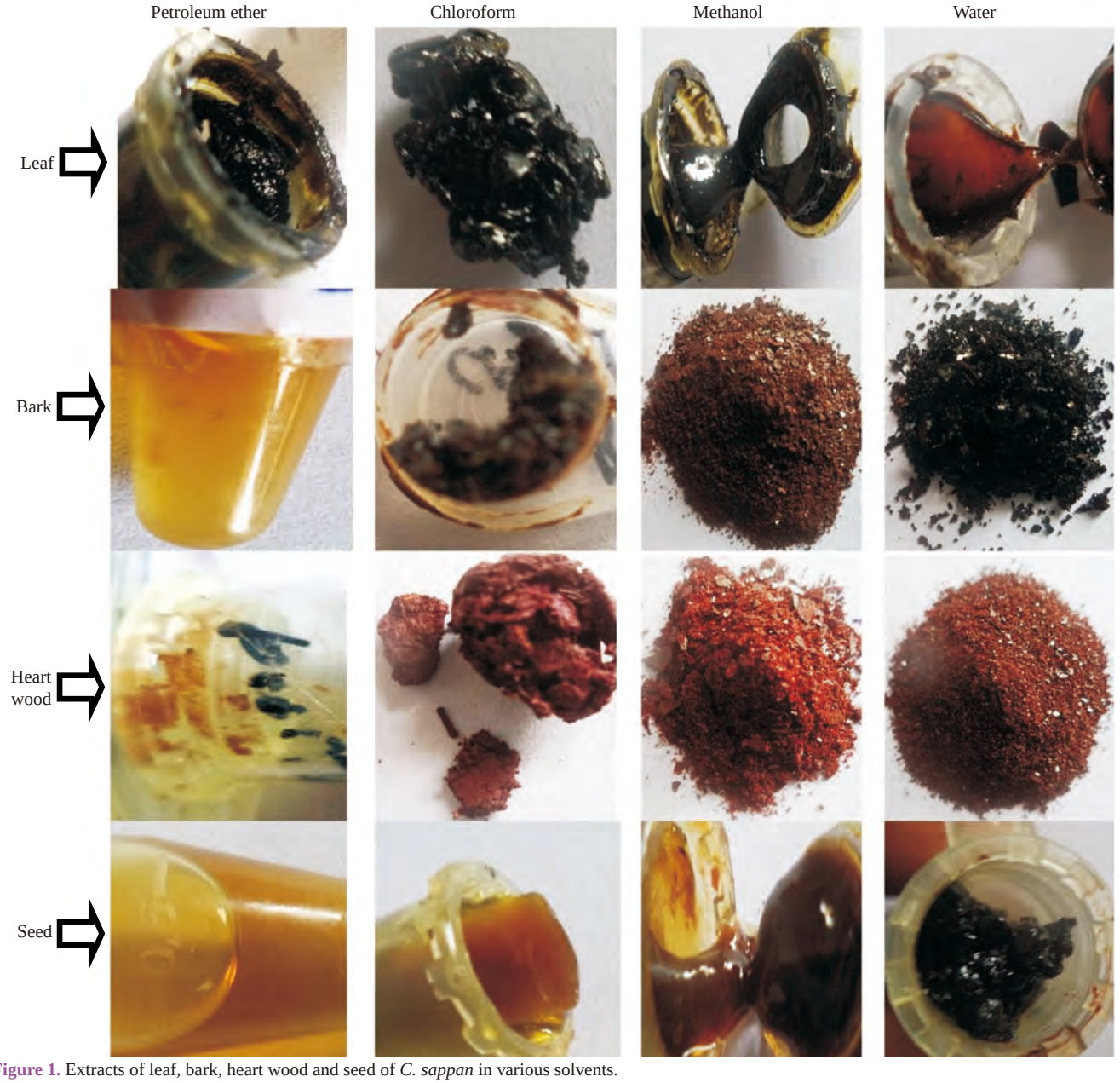

Figure 1. Extracts of leaf, bark, heart wood and seed of *C. sappan* in various solvents.

the flask. After that, the flask containing the extract was distilled and the solvent was collected back. Then the final filtrate evaporated to dryness in a steady air for about 24 h in a previously weighed Petri plate and the final weight obtained and color was observed (Figure 1 and Table 1). The dried extract was sterilized under UV rays and checked for sterility on nutrient agar plate and further used for antibacterial assay.

Table 1
Yields of extracts of different plant parts in various solvents.

Solvent	Plant part	Total weight	Yield (%)	Characterization
Petroleum ether	Leaf	0.59	2.95	Dark green and viscous
	Bark	0.06	0.30	Brown and viscous
	Heart wood	0.08	0.40	Light brown and shiny crystals
	Seed	0.93	4.65	Yellow viscous gel
Chloroform	Leaf	3.16	15.80	Black and jelly
	Bark	0.21	1.05	Light black and jelly
	Heart wood	0.34	1.70	Brown and crystalline
	Seed	1.48	7.40	Light brown and viscous gel
Methanol	Leaf	3.41	17.05	Dark green and jelly
	Bark	1.33	6.65	Shiny brown powder
	Heart wood	3.52	17.60	Shiny brown powder
	Seed	2.05	10.25	Light brown viscous
Water	Leaf	3.94	19.70	Black and hard jelly
	Bark	2.45	12.25	Light black crystals
	Heart wood	0.79	3.95	Shiny brown crystal powder
	Seed	2.14	10.70	Light brown jelly

2.3. Culture of bacterial strains

The pure cultures of *S. ebony*, *K. pneumonia*, *B. subtilis* and *E. coli* were obtained from Institute of Microbial Technology, Chandigarh, India. All the cultures were revived aseptically in freshly prepared nutrient broth medium and incubated at 37 ˚C.

2.4. Minimum inhibition concentration (MIC)

MICs are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism.

The MIC was determined by agar well diffusion technique. A serial dilution method was used for reconstituting the extracts. In first reconstituting process, 100 mg of extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO) then diluted with sterile distilled water to achieve decreasing concentration range of 1, 2, 5, 10 and 30 mg/mL. One milliliter of each dilution was introduced with micropipette in wells of nutrient agar plates which had been already seeded with standardized (0.6 optical density) of test bacterial cells. All the plates were incubated at 37 ˚C for 24 h. The least concentration of each extract showing a clear inhibition zone was taken as MIC.

2.5. Antibacterial activity assay

Agar well diffusion method was followed to determine the antimicrobial activity[28]. Nutrient agar plates were swabbed (sterile cotton swabs) with 8-h broth culture of respective bacteria. Each of these plates' wells (6 mm diameter and about 2 cm apart) were made of sterile cork borer. Each plant extract of methanol, chloroform, petroleum ether and water was further diluted in DMSO with the concentration of material used of 100 mg/mL. About 50 μL of different concentrations of plant solvent extracts were added with micropipette into wells made by sterile cork borer (6 mm) and allowed to diffuse at room temperature in laminar air flow chamber for 30 min. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37 ˚C for 18–24 h for bacterial pathogens. The diameter of the zone (mm) was measured. The experiment was performed in triplicate and repeated three times for each replicates, and the readings were taken.

3. Results

The response of *S. ebony*, *K. pneumonia*, *E. coli* and *B. subtilis* to the four solvent extracts of leaves varied (Table 2, Figures 2 and 3).

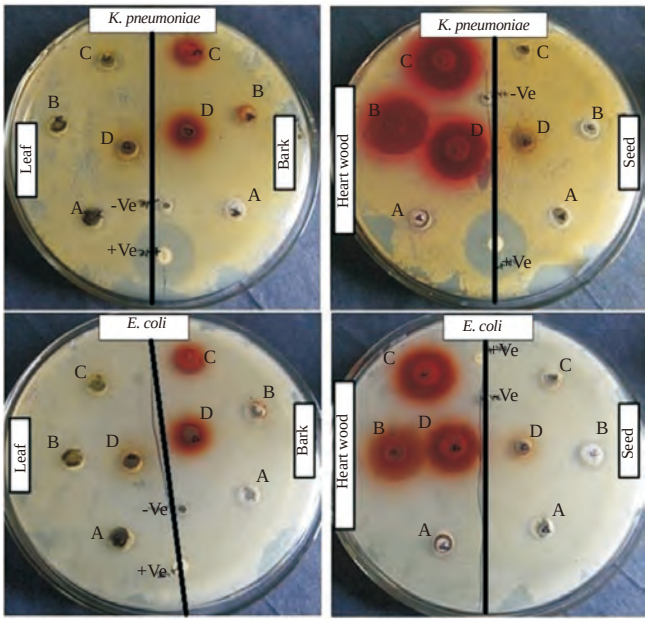

Figure 2. The antimicrobial activity of leaf, bark, heart wood and seed samples of *C. sappan* on *K. pneumoniae* and *E. coli*.
A: Pertoleum ether; B: Chloroform; C: Methanol; D: Water; -Ve: Negative control (DMSO); +Ve: Control (Ampicillin).

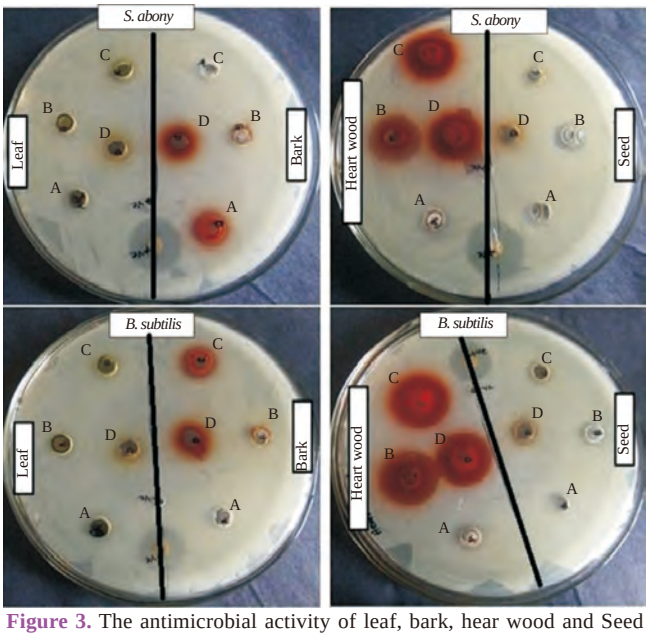

Figure 3. The antimicrobial activity of leaf, bark, hear wood and Seed samples of *C. sappan* on *S. ebony* and *B. subtilis*.
A: Petroleum ether; B: Chloroform; C: Methanol; D: Water; -Ve: Negative control (DMSO); +Ve: Control (Tetracyclin).

Table 2
The antibacterial activity of different parts of *C. sappan* extracts against the pathogenic bacterial strains. mm.

Plant part	Solvent	Zone of inhibition			
		S. ebony	*K. pneumonaie*	*E. coli*	*B. subtilis*
	Control	22T	28A	13A	20T
Leaf	Petroleum ether	8.500 ± 0.500	10.833 ± 0.440	0.000 ± 0.000	0.000 ± 0.000
	Chloroform	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
	Methanol	16.000 ± 0.290	13.000 ± 0.290	0.000 ± 0.000	0.000 ± 0.000
	Water	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Bark	Petroleum ether	0.000 ± 0.000	12.833 ± 0.600	0.000 ± 0.000	0.000 ± 0.000
	Chloroform	0.000 ± 0.000	13.166 ± 0.930	0.000 ± 0.000	0.000 ± 0.000
	Methanol	16.000 ± 0.580	17.500 ± 0.760	14.833 ± 0.600	16.500 ± 0.580
	Water	15.666 ± 0.600	0.000 ± 0.000	0.000 ± 0.000	12.000 ± 0.290
Heart wood	Petroleum ether	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	12.666 ± 0.170
	Chloroform	24.500 ± 0.500	28.166 ± 0.170	26.166 ± 0.170	27.166 ± 0.170
	Methanol	26.000 ± 0.000	30.333 ± 0.330	26.666 ± 0.440	27.333 ± 0.440
	Water	24.167 ± 0.730	28.166 ± 0.730	24.000 ± 0.290	26.166 ± 0.440
Seed	Petroleum ether	9.333 ± 0.330	0.000 ± 0.000	0.000 ± 0.000	11.500 ± 0.760
	Chloroform	16.000 ± 0.580	14.000 ± 0.580	15.500 ± 0.290	11.833 ± 0.600
	Methanol	9.666 ± 0.670	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
	Water	9.000 ± 0.580	13.833 ± 0.600	0.000 ± 0.000	12.166 ± 0.170

The data are expressed as mean ± SE; Each value is a mean of triplicate; Test concentration of extracts is 5 mg/mL (50 μL/well), and concentration of the standard antibiotic discs of ampicillin and tetracyclin is 30 mg/disc; A: Ampicillin; T: Tetracyclin.

The leaf extracts in methanol showed the maximum antibacterial activity against *S. ebony* as seen by inhibition zone [(16.000 ± 0.290) mm] and *K. pneumoniae* [(13.000 ± 0.290) mm]. This was followed by petroleum ether [(10.833 ± 0.440) mm]. The antibacterial activity was absent in leaves extracted in chloroform and water against *S. ebony* and *K. pneumoniae*. The leaf extracts in all solvents did not show any antibacterial activity against *E. coli* and *B. subtilis* strains.

The bark extracts in methanol showed the maximum antibacterial activity against all the tested strains. The maximum inhibitory zone against *K. pneumoniae* was (17.500 ± 0.760) mm, followed by *B. subtilis* (16.500 ± 0.580) mm, *S. ebony* (16.000 ± 0.580) mm and *E. coli* (14.833 ± 0.600) mm, and this was followed by water extract against *S. ebony* [(15.666 ± 0.600) mm] and *B. subtilis* [(12.000 ± 0.290) mm]. The petroleum ether extract of bark showed good response against *K. pneumoniae* [(12.833 ± 0.600) mm] and no antibacterial response against *S. ebony*, *E. coli* and *B. subtilis*. Similarly, the chloroform extract of the bark showed good antibacterial response against *K. pneumoniae* [(13.166 ± 0.930) mm] and none against *S. ebony*, *E. coli* and *B. subtilis*. Among all the bark extracts in different solvents the petroleum ether, chloroform and methanol showed good antibacterial activity.

The heart wood extracts in chloroform, methanol and water overall showed a very good antibacterial activity against *S. ebony*, *K. pneumoniae*, *E. coli* and *B. subtilis*, while the petroleum ether showed antibacterial activity aginst *B. subtilis* only (12.666 ± 0.170) mm). The methanol extract of heart wood showed the strongest activity against *K. pneumoniae* as seen by the inhibition zone (30.333 ± 0.330) mm followed by water extract (28.166 ± 0.730) mm and chloroform extract (28.166 ± 0.170) mm. This was followed by *B. subtilis* (27.333 ± 0.440) mm in methanol, chloroform (27.166 ± 0.170 mm, water (26.166 ± 0.440) mm and the least in petroleum ether (12.660 ± 0.170) mm.

The seed extracts showed good responses against *S. ebony* in all the solvents (petroleum ether, chloroform, methanol and water). The maximum inhibition zones for *S. ebony* was observed in chloroform [(16.000 ± 0.580) mm], followed by methanol [(9.666 ± 0.670) mm], petroleum ether [(9.333 ± 0.330) mm] and water [(9.000 ± 0.580) mm]. The next best response for inhibition activity was seen against *B. subtilis* where the inhibition zone was maximum in water extract [(12.166 ± 0.170) mm], followed by in chloroform [(11.833 ± 0.600) mm] and petroleum ether [(11.500 ± 0.760) mm], but no antibacterial activity was seen in the methanol extract. The maximum antibacterial response of the seed extract was against *K. pneumoniae* was observed in chloroform extract [(14.000 ± 0.580) mm], followed by water extract [(13.833 ± 0.600) mm]. No response was observed in petroleum ether and methanol extracts.

4. Discussion

The search for antimicrobials from natural sources has received many attentions and efforts having been put in to identify compounds which can act as suitable antimicrobials agent to replace those synthetic ones. Here we present a comparative study of antimicrobial activity of different solvent extracts of seed, leaves, bark and heart wood against *S. ebony*, *K. pneumoniae*, *E. coli* and *B. subtilis*. Our preliminary investigation showed that all solvents selected (methanol, chloroform, petroleum ether and aqueous extracts) for *C. sappan* were active against the tested strains (Figures 2 and 3). Susceptibility of each plant extract was tested by serial micro-dilution method and agar well diffusion method.

The methanolic extracts of *C. sappan* showed significant antimicrobial activity against all tested microorganisms used (Table 2). Though the mechanism of the action of these plant constituents is not yet fully known, it is clear that the effectiveness of the extracts largely depends on the type of solvent used. The methanolic and aqueous extracts showed strong antimicrobial activity as compared to petroleum ether extracts. This observation clearly indicates the existence of non-polar residues in the extracts which have higher bactericidal ability as they were reported earlier[23-26]. Aqueous extracts of plant parts of *C. sappan* showed significant antimicrobial activity, which was similarly reported earlier in bark extracts only[23]. Furthermore, petroleum ether extract from leaves of *C. sappan* had been reported to have the minimum antimicrobial activity against tested pathogenic bacteria used for assay. However, there were no any antimicrobial assay reports on seed extracts of

C. sappan, and in case of leaves, bark and heart wood few reported in aqueous extracts has been observed[23-26], but none in organic solvents such as petroleum ether, methanol and chloroform.

In the present study, the MIC value of the active plant extracts suggested that the plant extracts were more bacteriostatic at lower concentrations and bactericidal at higher concentrations. Among the four tested bacterial strains, *S. ebony* was very sensitive to plant extracts in all solvents tried, followed by *K. pneumoniae*, *B. subtilis* and *E. coli*, the later one was more resistant when compared with the three other organisms. The presence of high amount of secondary metabolites in heart wood of *C. sappan* has a strong inhibiting effect on bacterial growth as seen by the inhibition zone. It has been reported that the aqueous extracts of heart wood of *C. sappan* has also shown antibacterial activity against some oral pathogens[26].

The present comparative study has shown that the heart wood extracts in organic solvents showed the maximum antibacterial activity against the tested strains of human pathogen. This work can be further used as a base for identification, isolation and purification of these phyto constituents, which can be further used as a potential source for new drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Dubey R, Dubey K, Sridhar C, Jayaveera KN. Human vaginal pathogen inhibition studies on aqueous, methanolic and saponins extracts of stem barks of *Ziziphus mauritiana*. *Int J Pharm Sci Res* 2011; **2**(3): 659-63.

[2] Lalitha P, Arathi KA, Sripathi SK, Hemalatha S, Jayanthi P. Antimicrobial activity and phytochemical screening of an ornamental foliage plant, *Pothos aurea* (Linden ex Andre). *An Int J Chem* 2010; **1**(2): 63-71.

[3] Preethi R, Devanathan VV, Loganathan M. Antimicrobial and antioxidant efficacy of some medicinal plants against food borne pathogens. *Adv Biol Res* 2010; **4**(2): 122-5.

[4] Kelmanson JE, Jäger AK, van Staden J. Zulu medicinal plants with antibacterial activity. *J Ethanopharmacol* 2000; **69**(3): 241-6.

[5] Ahmad I, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multiple drug resistant human pathogens. *J Ethanopharmacol* 2001; **74**(2): 113-23.

[6] Sarumathy K, Vijay T, Palani S, Sakthivel K, Dhana Rajan MS. Antioxidant and hepatoprotective effects of *Caesalpinia sappan* against acetaminophen-induced hepatotoxicity in rats. *Int J Pharmacol Ther* 2011; (1): 19-31.

[7] Kim YM, Kim SG, Khil LY, Moon CK. Brazilin stimulates the glucose transport in 3T3-L1 cells. *Planta Med* 1995; **61**(4): 297-301.

[8] Dong SK, Nam-In B, Sei RO, Keun YJ, Im SL, Hyeong-Kyu L. NMR assignment of brazilein. *Phytochemistry* 1997; **46**(1): 177-8.

[9] Namikoshi M, Saitoh T. Homoisoflavonoids and related compounds IV. Absolute configuration of homoisoflavonoids from *Caesalpinia sappan* L. *Chem Pharm Bull* 1987; **35**: 3597-602.

[10] Namikoshi M, Nakata H, Yamada H, Nagai M, Saitoh T. Homoisoflavanoids and related compounds: II. Isolation and absolute configurations of 3,4-dihydroylated homoisoflavans and brazilins from *Caesalpinia sappan* L. *Chem Pharm Bull* 1987; **35**: 2761-73.

[11] Namikoshi M, Nakata H, Saitoh T. Homoisoflavonoids and related compounds V. A novel dibenzoxocin derivative from *Caesalpinia sappan* L. *Chem Pharm Bull* 1987; **35**(9): 3615-9.

[12] Fuke C, Yamahara J, Shimokawa T, Kinjo JE, Tomimatsu T, Nohara T. Two aromatic compounds related to brazilin from *Caesalpinia sappan* L. *Phytochemistry* 1985; **24**(10): 2403-6.

[13] Zeng KW, Liao LX, Zhao MB, Song FJ, Yu Q, Jiang Y, et al. Protosappanin B protects PC12 cells against oxygen–glucose deprivation-induced neuronal death by maintaining mitochondrial homeostasis via induction of ubiquitin-dependent p53 protein degradation. *Eur J Pharmacol* 2015; **751**: 13-23.

[14] Wang Z, Sun JB, Qu W, Guan FQ, Li LZ, Liang JY. Caesappin A and B, two novel protosappanins from *Caesalpinia sappan* L. *Fitoterapia* 2014; **92**: 280-4.

[15] Miyahara K, Kawasaki T, Kinjyo JE, Shimokawa T, Yamahara J, Yamasaki M, et al. The X-ray analysis of caesalpin J from sappan lignum. *Chem Pharm Bull* 1986; **34**(10): 4166-9.

[16] Nirmal NP, Rajput MS, Prasad RG, Ahmad M. Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: a review. *Asian Pac J Trop Med* 2015; **8**(6): 421-30.

[17] You EJ, Khil LY, Kwak WJ, Won HS, Chae SH, Lee BH, et al. Effects of brazilin on the production of fructose-2,6-bisphosphate in rat hepatocytes. *J Ethnopharmacol* 2005; **102**(1): 53-7.

[18] Oh SR, Kim DS, Lee IS, Jung KY, Lee JJ, Lee HK. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. *Planta Med* 1998; **64**(5): 456-8.

[19] Baek NI, Jeon SG, Ahn EM, Hahn JT, Bahn JH, Cho SW, et al. Anticonvulsant compounds from the wood of *Caesalpinia sappan* L. *Arch Pharm Res* 2000; **23**(4): 344-8.

[20] Badami S, Moorkoth S, Rai SR, Kannan E, Bhojraj S. Antioxidant activity of *Caesalpinia sappan* heartwood. *Biol Pharm Bull* 2003; **26**(11): 1534-7.

[21] Wetwitayaklung P, Phaechamud T, Keokitichai S. The antioxidant activity of *Caesalpinia sappan* L. heartwood in various ages. *Naresuan Univ J* 2005; **13**(2): 43-5.

[22] Saenjum C, Chaiyavat C, Kadchumsang S, Chansakaow S, Suttajit M. Antioxidant activity and protective effects on DNA damage of *Caesalpinia sappan* L. extract. *J Med Plant Res* 2010; **4**: 1594-608.

[23] Mohan G, Anand SP, Doss A. Efficacy of aqueous and methanol extracts of *Caesalpinia sappan* L. and *Mimosa pudica* L. for their potential antimicrobial activity. *South Asian J Biol Sci* 2011; **1**: 48-57.

[24] Srinivasan R, Selvam GG, Karthik S, Mathivanan K, Baskaran R, Karthikeyan M, et al. *In vitro* antimicrobial activity of *Caesalpinia sappan*. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 1): S136-9.

[25] Saravanakumar S, Chandra JH. Screening of antimicrobial activity and phytochemical analysis of *Ceasalpinia sappan* L. *J Chem Pharm Res* 2013; **5**(2): 171-5.

[26] Keramat H, Moaddabi A, Ranjbari A. *In vitro* antimicrobial effects of aqueous extracts of *Caesalpinia sappan* Linn. derivatives against oral pathogens. *Indian J Sci Res* 2014; **7**(1): 342-7.

[27] Redfern J, Kinninmonth M, Burdass D, Verran J. Using Soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. *J Microbiol Biol Educ* 2014; **15**(1): 45-6.

[28] Gunasekaran T, Desta ED, Seifu MF, Gelaw BK, G/Mariam ET, Ahmed M. Comparative efficacy evaluation of seven commercial injectable ceftriaxone purchased at Ambo, Oromia Regional State, Ethiopia, using agar well plate diffusion method. *J Pharm Health Serv Res* 2015; **6**(2): 97-101.