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Asian Pacific Journal of Tropical Biomedicine

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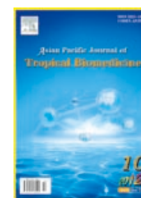

Document heading doi:10.1016/S2221-1691(12)60232-9 © 2012 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

Antibacterial activity of sequentially extracted organic solvent extracts of fruits, flowers and leaves of *Lawsonia inermis* L. from Jaffna

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ARTICLE INFO

Article history:
Received 15 March 2012
Received in revised form 27 April 2012
Accepted 28 May 2012
Available online 28 October 2012

Keywords:
Lawsonia inermis
Antibacterial activity
Sequential extraction
Well diffusion method

ABSTRACT

Objective: To reveal the antibacterial activity of sequentially extracted different cold organic solvent extracts of fruits, flowers and leaves of *Lawsonia inermis* (*L. against)* some pathogenic bacteria. **Methods:** Powders of fruits, flowers and leaves of *L. inermis* were continuously extracted with dichloromethane (DCM), ethyl acetate and ethanol at ambient temperature. The dried extracts were prepared into different concentrations and tested for antibacterial activity by agar well diffusion method, and also the extracts were tested to determine the available phytochemicals. **Results:** Except DCM extract of flower all other test extracts revealed inhibitory effect on all tested bacteria and their inhibitory effect differed significantly (P<0.05). The highest inhibitory effect was showed by ethyl acetate extract of flower against *Staphylococcus aureus (S. aureus)* and *Pseudomonas aeruginosa* (*P. aeruginosa)*, and ethyl acetate extract of fruit on *Escherichia coli (E. coli)* and *Bacillus subtilis* (*B. subtilis)*. The ethyl acetate and ethanol extracts of flower, fruit and leaf expressed inhibition even at 1 mg/ 100 μl against all test bacteria. Among the tested phytochemicals flavonoids were detected in all test extracts except DCM extract of flower. **Conclusions:** The study demonstrated that the ethyl acetate and ethanol extracts of fruit and flower of *L. inermis* are potentially better source of antibacterial agents compared to leaf extracts of respective solvents.

1. Introduction

The development of resistance in microorganisms to antibiotics and emergence of new infectious diseases create urgent need to discover novel, safe and effective antimicrobial compounds[1]. In modern pharmaceutical industries, natural sources and semi synthetic derivatives of natural products play a key role for the production of novel drugs[2]. Plants are found to be an immense source for variety of bioactive molecules with diverse molecular structure and function. These molecules are primarily derived from the secondary metabolism of plants and protect the plants against predation by microorganisms, insects and herbivorous[3]. Since prehistoric period, man has used various plants to cure and prevent a range of ailments, and still it is found to be an effective source in traditional medical practices[4]. Therefore, evaluation of the activities of medicinal plants claimed for possessing antimicrobial property is getting attention nowadays.

Lawsonia inermis L. (*L. inermis)*, commonly called as Maruthondi (Tamil), Henna (English), is a well known medicinal plant in Jaffna Peninsula, Sri Lanka. It belongs to the family Lythraceae. It widely occurs in the drier parts especially near the sea coast of Sri Lanka and India and also in Africa and Arabia[5]. Henna is a glabrous much branched deciduous shrub with lateral branches often ending in spines; leaves simple, opposite, entire, lanceolate, petioles very short or absent; flowers white colored; fruits globose, capsules with numerous seeds[6]. Roots, leaves, flowers and seeds are used in traditional medicinal systems for the treatment of various ailments. Leaves are useful for the treatment of diarrhea, dysentery, leprosy, scabies and boils. Flowers are used in cephalalgia, burning sensation, sardiopathy, anemia, insomnia and fever[6]. Seeds are effective in the treatment of intermittent fever, insanity, dysentery, diarrhea, and amentia[7]. The bark is given in the form of decoction for the enlargement of spleen and obstinate skin disease, and the root is regarded as a specific for leprosy[5].

There are number of studies have been conducted to prove the bioactivity of *L. inermis*; free radical scavenging and reducing power[8], anti ulcer potential[9], hypoglycemic

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Foundation Project: Supported by Ministry of Higher Education, Sri Lanka for providing the financial assistance for the work as payment of research allowance in accordance with circular no 1/2011.

and antihyperglycemic effect[10], anthelmintic activity[11], protein glycation inhibitory activity[12], osteoclastogenesis inhibition[13] and antibacterial activity[2, 14-17] . However, in antibacterial studies of *L. inermis* there are some gaps to fill. Most of the previous studies were based on the leaf extracts of *L. inermis*, but in traditional medicine the fruit and flowers are also used for the treatment. In addition to that in most of the antibacterial studies single solvent extraction or hot (Soxhlet) sequential extraction method was used to get extracts. Therefore, the present study was carried out to evaluate the in vitro antibacterial activity of sequentially extracted different cold organic solvent extracts of fruit, flower and leaf of *L. inermis*, present in Jaffna, against some selected pathogenic bacteria, and to elucidate the available phytochemicals in the test extracts.

2. Materials and methods

2.1. Collection and identification of plant materials

Fresh and healthy fruits, flowers and leaves of Lawsonia inermis were collected from the botanical garden of the Department of Botany, University of Jaffna, Sri Lanka, and the taxonomic identities of this plant were authenticated by a taxonomist in the Department of Botany, University of Jaffna, Sri Lanka. The voucher specimen was deposited in the same department. The collected plant parts were thoroughly washed under running tap water, dried in shade and then triturated into fine powders by using an electric grinder. These powders were stored in air sealed brown bottles at ambient temperature.

2.2. Preparation of plant extracts

Each powdered plant part of *L. inermis* was successively extracted with different organic solvents in the increasing polarity order[18]. 100 g of each powder was macerated separately in 300 mL Dichloromethane (DCM) with intermittent shaking for three days. Then they were first filtered with muslin cloth and then through Whatman no1 filter paper. The residue was further extracted two times by using the same fresh solvent and all the filtrates were pooled together. The resulting residue was air dried and further extracted with ethyl acetate and followed by ethanol similar to the procedure carried out for the DCM extraction. Finally from each filtrate the solvent was removed using rotary evaporator under reduced pressure and low temperature. The yield of each extract was weighed and stored at 4 ℃ until used.

2.3. Test Bacteria

Four bacterial isolates namely *Staphylococcus aureus* (*S.aureus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were obtained from the culture collection of Department of Microbiology, Faculty of Medicine, University of Jaffna, Sri Lanka. The above bacteria were maintained on nutrient agar slants at 4 ℃ until used for the study.

2.4. Antibacterial assay

The *in vitro* antibacterial activity of the crude extracts of different parts of *L. inermis* was determined by agar well diffusion method[19]. The test bacteria were cultured in nutrient broth at 37 ℃ for 18 hours. Autoclaved Mueller Hinton agar (MHA) medium was cold down to 40 ℃, and then 1 mL of above bacterial suspension (10^6 cfu/mL) was mixed with 15 mL of this medium, poured into a sterile Petri dish and allowed to set. Test extracts were prepared by dissolving 60 mg of each extract in mixture of 100 μL dimethyl sulfoxide (DMSO) and acetone (1:1 V/V). Wells were made on solidified medium using a sterile cork borer (8 mm) and filled with 100 μL of each extract. Streptomycin (50 μg / 100 μL) and 100 μL of mixture of DMSO and acetone were used as standard and control respectively. Culture plates were incubated at 37 ℃ for 24 hours and the antibacterial activity was determined by measuring the diameter of inhibition zone around the well. Each experiment was repeated thrice.

2.5. Determination of lowest inhibitive concentration

The lowest inhibitive concentration of the test extracts were determined by agar well diffusion method, as described above, with different concentrations of test extracts ranging from 1 mg/100 μL to 40 mg/100 μL. The test culture plates were incubated at 37 0C for 24 hours and the antibacterial activity was determined by measuring the diameter of inhibition zone around the well. Each experiment was repeated thrice.

2.6. Qualitative phytochemical screening

The qualitative phytochemical analysis for the presence of tannins, saponins, flavonoids, steroids and alkaloids was carried out to all test extracts of fruit, flower and leaf of *L. inermis* using standard procedures give by Trease and Evans[20].

2.6. Statistical analysis

The mean value and standard deviation of three replicates were calculated and the data were subjected to examine by analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$) by using a software, SPSS 13.0 for Windows version.

3. Results

The results of the sequential extraction revealed the production of higher percentage of yield by the solvent ethanol on flower and leaf, and the solvent DCM on fruit (Table 1).

Table 1
The percentage of yield in the sequential extraction of different parts of *L. inermis*.

Solvent	Plant part	yield (%)
DCM	Flower	2.16
	Fruit	3.89
	Leaf	1.15
Ethyl acetate	Flower	2.05
	Fruit	1.56
	Leaf	1.83
Ethanol	Flower	9.43
	Fruit	1.91
	Leaf	8.13

DCM- Dichloromethane

Table 2
The inhibitory effect of *L. inermis* at 60 mg/100 μL concentration on bacterial pathogens.

Plant parts	Extracts	Diameter of inhibition zone (mm)*			
		E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
Flower	DCM	11.1±0.3[e]	–	–	16.3±0.5[e]
	EA	21.8±0.5[b]	26.4±0.3[a]	24.3±0.5[b]	26.7±0.3[a]
	EtOH	19.2±0.7[c]	24.7±0.2[b]	20.5±0.2[d]	25.2±0.4[b]
Fruit	DCM	11.7±0.8[d]	12.2±0.8[e]	11.2±0.9[f]	11.9±0.6[f]
	EA	26.2±0.6[a]	23.8±0.3[c]	26.4±0.7[a]	24.2±0.2[c]
	EtOH	19.2±0.4[c]	25.3±0.4[b]	20.7±0.3[d]	25.3±0.2[b]
Leaf	DCM	10.3±0.3[f]	9.8±0.4[f]	11.2± 0.2[f]	10.4±0.5[g]
	EA	12.1±0.5[d]	12.2±0.4[e]	16.7±0.7[e]	19.2±0.6[d]
	EtOH	18.7±0.6[c]	22.3±0.6[d]	22.4±0.6[c]	24.8±0.3[bc]
Streptomycin		13.1±0.9	26.0±0.8	27.9±0.3	16.2±0.8
Control (DMSO: Acetone)		–	–	–	–

*Diameter of inhibition zones includes the diameter of well (8 mm); Values are represented as mean±SD; Values with different superscript in the same column differ significantly (P<0.05); DCM – Dichloromethane; EA –Ethyl acetate; EtOH – Ethanol.

Table 3
The inhibitory effect of *L. inermis* at different solvent concentrations on bacterial pathogens.

Plant parts	Extracts	Test concentration (mg/100 μL)	Diameter of inhibition zone (mm)*			
			E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
Flower	DCM	1	8.4±0.2	–	–	9.3±0.2
		10	9.1±0.1	–	–	14.1±0.1
		20	9.5±0.2	–	–	14.1±0.3
		40	10.9±0.1	–	–	15.1±0.3
	EA	1	8.8±0.3	9.8±0.2	10.2±0.3	10.9±0.2
		10	12.1±0.1	21.1±0.1	21.1±0.3	24.9±0.2
		20	17.4±0.3	23.6± 0.2	22.1±0.1	25.4±0.2
		40	21.3±0.2	26.0±0.1	23.0±0.1	26.1±0.3
	EtOH	1	9.2±0.2	11.1±0.2	9.5±0.2	10.0±0.3
		10	15.1±0.1	22.1±0.1	17.0±0.1	19.1±0.1
		20	16.4±0.3	23.4±0.3	18.4±0.2	21.2±0.2
		40	18.4±0.3	24.6±0.2	20.8±0.3	24.3±0.3
Fruit	DCM	1	–	–	–	–
		10	9.0±0.1	9.1±0.1	9.0±0.1	10.0±0.1
		20	10.2±0.1	9.8±0.2	9.7±0.2	10.7±0.2
		40	11.3±0.2	11.8±0.2	10.8±0.3	11.4±0.3
	EA	1	10.3±0.2	9.7±0.3	10.6±0.2	11.2±0.4
		10	22.0±0.6	17.1±0.3	20.0±0.1	21.0±0.1
		20	22.3±0.2	19.2±0.2	22.4±0.2	22.1±0.2
		40	25.5±0.2	23.3±0.2	25.9±0.3	23.8±0.3
	EtOH	1	9.2±0.2	10.1±0.2	9.2±0.4	9.9±0.2
		10	13.0±0.3	21.2±0.2	17.0±0.1	20.0±0.3
		20	16.3±0.1	22.3±0.2	17.2±0.2	21.2±0.3
		40	18.7±0.4	24.2±0.1	19.3±0.3	24.1±0.2
Leaf	DCM	1	–	–	–	–
		10	–	–	–	–
		20	–	–	10.1±0.2	–
		40	9.1±0.1	9.4±0.1	10.9±0.4	9.8±0.2
	EA	1	9.2±0.1	9.3±0.3	10.1±0.1	10.5±0.4
		10	10.1±0.1	10.1±0.1	15.0±0.2	16.1±0.1
		20	10.8±0.2	10.7±0.4	15.5±0.3	17.3±0.4
		40	11.4±0.2	12.0±0.2	16.7±0.2	18.5±0.2
	EtOH	1	9.2±0.1	10.2±0.2	10.7±0.3	11.2±0.3
		10	12.0±0.3	17.0±0.1	16.1±0.1	20.0±0.1
		20	14.2±0.3	19.2±0.2	17.4±0.2	21.2±0.3
		40	18.1±0.2	21.1±0.2	20.4±0.5	24.1±0.3

*Diameter of inhibition zones includes the diameter of well (8 mm); Values are represent as mean±SD; DCM – Dichloromethane; EA– Ethyl acetate; EtOH – Ethanol.

At 60 mg/ 100 μL, except DCM extract of flower all other test extracts had inhibition on all the test bacteria, the inhibitory effect differed significantly ($P<0.05$) and the diameter of zone of inhibition ranged from (9.8 ± 0.4) mm to (26.7 ± 0.3) mm (Table 2). The highest inhibitory effect was showed by ethyl acetate extract of flower on *S. aureus* and *P. aeruginosa*, and ethyl acetate extract of fruit on *E. coli* and *B. subtilis*.

Among the tested extracts, ethyl acetate and ethanol extracts of fruit, flower and leaf exhibited higher inhibition against all test bacteria compared to DCM extracts of respective plant parts. Furthermore, the results produced by the ethyl acetate extract of flower and fruit were found to be higher than that produced by the same solvent extract of leaf against all test bacteria (Table 2).

Among the leaf extracts, ethanol extract of leaf had higher inhibition on all test bacteria compared to ethyl acetate and DCM extracts. In the case of the fruit extracts, ethyl acetate extract had highest inhibition on *E. coli* and *B. subtilis*, and the ethanol extract on *P. aeruginosa* and *S. aureus*. In flower, the ethyl acetate extract had inhibition on all test bacteria compared to ethanol. DCM extract of flower failed to inhibit the growth of *P. aeruginosa* and *B. subtilis*. There was no inhibition produced by the solvent mixture of DMSO and acetone. Standard antibiotic streptomycin revealed inhibition on all test bacteria and the highest and lowest inhibitions were respectively on *B. subtilis* and *E. coli* (Table 2).

In dose response study, the inhibitory effect of all the test extracts varied in dose dependent manner (Table 3). The ethyl acetate and ethanol extracts of flower, fruit and leaf had inhibition even at 1 mg/100 μL against all test bacteria. The DCM extracts of these plant parts had no activity at the lowest test concentration but as the concentration increased the antibacterial activity was recorded.

Table 4

Phytochemical constituents of different solvent extracts of flower, fruit and leaf of *L. inermis*.

Phytochemical constituents	Leaf			Fruit			Flower		
	DCM	EA	EtOH	DCM	EA	EtOH	DCM	EA	EtOH
Tannins	−	−	+	−	+	+	−	−	+
Saponins	−	−	−	−	−	+	−	−	+
Flavonoids	+	+	+	+	+	+	−	+	+
Steroids	−	−	−	−	−	−	−	−	−
Alkaloids	−	+	−	−	−	−	−	−	−

+: Present; −: Absent; DCM - Dichloromethane; EA – Ethyl acetate; EtOH – Ethanol.

The results for the qualitative phytochemical analysis revealed that among the tested five phytochemicals at least two of them were present in leaf, fruit and flower of *L. inermis*. Flavonoids were detected in all test extracts of *L. inermis* except DCM extract of flower. Tannins were present in ethanol extracts of leaf, fruit and flower and ethyl acetate extract of fruit. Alkaloids and saponins were scarcely present, saponins present in ethanol extract of fruit and flower, and the alkaloids present only in ethyl acetate extract of leaf. None of the test extracts showed positive results for steroid tests (Table 4).

4. Discussion

The present study clearly demonstrated the antibacterial activity of sequentially extracted cold DCM, ethyl acetate and ethanol extracts of leaves, flower and fruit of *L. inermis*. This finding further enriches the pharmaceutical value of *L. inermis*, and also it confirms the validity of traditional use of *L. inermis*. Most of the previous studies were done on leaf extracts, however, the present study proved the bioactivity of fruit and flower of *L. inermis* by providing higher activity than leaf extracts.

Generally, the antimicrobial activities of plant extracts dependent on various factors; the environmental and climate conditions under which the plant grew, the solvent that used for the extraction, the choice of extraction method, test concentration, the method of determination of antimicrobial activity and the test microorganisms[3, 21, 22]. In this study, the plant materials were sequentially extracted with different organic solvents in increasing polarity order. The sequential extraction method ensures the extraction of active compounds from plant material according to their polarity, and also reduces the antagonistic effect of compounds in the extract.

Sukanya *et al* reported the absence of inhibitory effect of sequentially extracted hot (Soxhlet) ethanol, ethyl acetate and chloroform extracts of leaf against *S. aureus* and *E. coli* in disc diffusion method[2]. However, the present study demonstrated the inhibitory effect of cold sequential extracts of leaf against all test bacteria. The variation in the results of previous and present study may be due to the variation in extraction method or method of antibacterial activity or by both. During the Soxhlet extraction, due to the high temperature treatment some of the active biomolecules might escape from the extract[23]. It has been also reported that the agar well diffusion method is better than disc diffusion method, because, the free hydroxyl groups present in the disc may prevent the diffusion of cationic polar molecules[21]. Therefore, the results of the present study further support the usage of agar well diffusion method and cold sequential extraction method.

The ethyl acetate and ethanol extracts of all three test parts of *L. inermis* expressed tremendous activity against both Gram negative (*E. coli* and *P. aeruginosa*) and gram positive (*S. aureus* and *B. subtilis*) bacteria. This shows the presence of active ingredient with broad spectrum of antimicrobial activity. The bioactivity of plant extracts depends on the type and quantity of phytochemicals present in the extract. The phytochemicals show various modes of antibacterial activities[3]. Therefore, further investigations are needed

as far as the chemical constitutes of this plant extracts concerned. Additional deep research is necessary to isolate and characterize their active compounds for pharmacology testing.

In conclusion, the present study clearly demonstrated the antibacterial effect of cold sequentially extracted DCM, ethyl acetate and ethanol extracts of flower, fruit and leaves of *L. inermis* present in Jaffna peninsula, Sri Lanka. Particularly the ethyl acetate and ethanol extracts of fruit and flower extracts are more effective for the in vitro control of pathogenic bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to Ministry of Higher Education, Sri Lanka for providing the financial assistance for the work as payment of research allowance in accordance with circular no 1/2011.

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