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Isolation, structural elucidation of flavonoid constituents from *Lawsonia Inermis Linn*

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ABSTRACT

The aim of the present study was to isolate and characterize the active components present in the root of Lawsonia inermis. The plant was extracted with ethanolic extract. The phytochemical results reveal the presence of carbohydrates, glycosides, tannins, phenolic compounds, flavonoids and terpenes in the plant. The ethanol extract of Lawsonia inermis was undergone coloumn chromatography with different solvent fractions. Two compounds were isolated from ethanolic extract. Compound 1 was eluted with benzene: Chloroform 10:90 v/v and compound 2 was eluted with ethyl acetate: ethanol, 30:70, v/v. The structures of the 2 compounds was characterised by using FT-IR, NMR and Mass spectrometry. Compound 1 was characterised as 24β-ethyl cholest-4-en-3β-ol as $C_{29}H_{50}O$ and compound 2 is 3,7,4',5'-Tetrahydroxy-6-methoxyflavone as $C_{16}H_{12}O_7$.Therefore, further biological investigation need to carry out isolated compounds present in this plant.

Keywords: *Lawsonia inermis*, Isolation, coloumn chromatography, FT-IR, NMR.

INTRODUCTION

This plant is commonly known as Henna or Mhendi and abundantly available in tropical and subtropical areas. Ancient history of India describes its diverse uses and also plays appreciable role in Ayurvedic or natural herbal medicines[1]. It is much branched, deciduous, glabrous, sometime spinescent shrub or small tree with grayish brown bark, attaining a height of 2.4 -5 m. It is cultivated as a hedge plant throughout India, and as a commercial crop in certain states of India for its dye [2]. Leaves are small, opposite, entire margin elliptical to broadly lanceolate, sub-sessile, about 1.5 to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, petiole short and glabrous acute or obtuse apex with tapering base. New branches are green in colour and quadrangular, turn red with age. Young barks are greyish brown, older plants have spine-tipped branchlets. Inflorescence has large pyramid shaped cyme. Flowers are small, numerous, aromatic, white or red coloured with four crumbled petals. Calyx has 0.2 cm tube and 0.3 cm spread lobes. The fruits are small, brown globose capsule, opening irregularly and split into four sections with a permanent style. Seeds have typical, pyramidal, hard and thick seed coat with brownish coloration [3-5].In folk medicines, henna has been used as astringent, anti-hemorrhagic, intestinal antineoplastic, cardio-inhibitory, hypotensive, sedative and also as therapeutic against amoebiasis, headache, jaundice and leprosy [6,7].Leaves are used as Bitter, astringent, acrid, diuretic, emetic, edema, expectorant, anodyne, anti-inflammatory, constipating, depurative, liver tonic, haematinic, styptic, febrifuge, trichogenous, wound, ulcers, strangury, cough, bronchitis, burning sensation, cephalalgia, hemicranias, lumbago, rheumatalgia, inflammations, diarrhoea, dysentery, leprosy, leucoderma, scabies, boils, hepatopathy, splenopathy, anemia, hemorrhages, hemoptysis, fever, ophthalmia, amenorrhoea, falling of hair, greyness of hair, jaundice [8-11].

MATERIALS AND METHODS

Plant material

The dry root of *Lawsonia inermis* (Linn) were collected from Thirunelveli district, Tamil Nadu, India. This root was identified and authenticated by Dr. V. Chelladurai, Research officer-Botany, Central council for research in Ayurveda and siddha, Govt of India, Thirunelveli. The roots were dried under shade, segregrated, pulverized by a mechanical grinder and passed through a 40 mesh sieve.

Extraction

The powdered root material were successively extracted with ethanol by hot continuous percolation method in soxhlet apparatus[(12)] for 24 hours. The extract was concentrated with rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained. The ethanolic extract was stored in screw cap vial at 4 ^{0}C until further use.

Preliminary phytochemical screening

The extract was subjected to preliminary phytochemical screening for the detection of various plant constituents like Alkaloids[13],Carbohydrates[13],Glycosides[13], Phytosterols[14], coumarins[14], Flavonoids[15,16],Tannins and phenolic compounds[17],Proteins and amino acids[13], Saponins[13], Fixed oils[13].

TLC characterization of ethanolic extract of *Lawsonia inermis*

This principle of separation is either partition or adsorption. The constituent which is having more affinity for mobile phase moves with it, while the constituent which is having more affinity for stationery phase gets adsorbed on it. This way various compounds appear as a band on the TLC plate, having different R_f values. The ethanolic extract of *Lawsonia inermis* was subjected to thin layer and high performance thin layer chromatographic studies for the separation and identification of their components.

Preparation of plates

100g of silica gel G was weighed and made into a homogenous suspension with 200 mL of distilled water to form slurry. The slurry was poured into a TLC applicator, which was adjusted to 0.25mm thicknesson flat glass plate of different dimensions(10X2, 10X5, 30X5, 20X10cm etc). The coated plates were allowed to dry in air, followed by heating at 100-105^{0}C for 1hour, cooled and protected from moisture. Before using, the plates were activated at 110^{0}C for 10 minutes.

Identification method

The ethanolic extract of *Lawsonia inermis* was dissolved in ethanol separately and spotted using a capillary tube on TLC plates 2 cm above from the bottom of the plate. The selection of solvent systems were based on increasing the order of polarity. The different spots developed in each system were detected by means of iodine staining.

Isolation of ethanolic extract of Lawsonia inermis by using column chromatography

The 20gms of ethanolic extract of *Lawsonia inermis* was admixed with 20gms silica gel (60/120 meshes) to get uniform mixing. 200gms of silica gel (70/325 meshes) was taken in a suitable column and packed very carefully without air bubbles using petroleum ether as filling solvent. The column was kept aside for 1 hour and allowed for close packing. Admixture was then added at the top of the stationary phase and started separation of compounds by the eluting with various solvent mixtures with increasing order of polarity. All the column fractions were collected separately and concentrated under reduced pressure. Finally the column was washed with hexane, ethyl acetate and ethanol.

Characterization of isolated Compounds

FT-IR

IR spectra of the compounds isolated from the ethanolic extract of *Lawsonia inermis* were recorded using a Nicolet 170SX. The spectral resolution for the Nicolet 170SX was 0.25cm^{-1}, and the spectral data were stored in the database at intervals of 0.5 cm-1 at 4000-2000 cm-1, and of 0.25 cm-1 at 2000-400 cm-1. The solid samples were measured by using KBr disc methods.

^{1}H NMR

1H NMR spectra of the compounds isolated from the ethanolic extract of *Lawsonia inermis* were recorded using a JEOL AL-400 (399.65 MHz). The measuring conditions for the most of the spectra were as follows: flip angle of 22.5-30.0 degrees, pulse repetition time of 30s. The long pulse repetition time and small flip angle was used to ensure precise relative intensities. The 1H NMR chemical shifts were referred to TMS in organic solvents.

^{13}C NMR

13C NMR spectra of the compounds isolated from the ethanolic extract of *Lawsonia inermis* were recorded with a Bruker AC-200 (50.323 MHz). The measuring conditions for the most of the spectra were as follows: a pulse flips angle of 22.45-45 degrees, a pulse repetition time of 4-7 seconds, and a resolution of 0.025-0.045 ppm. The spectra whose spectral codes started with "CDS" were reconstructed from peak positions, intensities, and line widths by assuming all resonance peaks were Lorenz lines. The chemical shift was referred to a TMS for all solvents.

Mass Spectrum

Mass spectra of the compounds isolated from the ethanolic extract of *Lawsonia inermis* was recorded with JEOL JMS-700 by the electron impact method where an electron is accelerating voltage 75eV and an ion accelerating voltage of 8-10nV. The reservoir inlet systems were used. The dynamic range for the peak intensities were 3 digits and the accuracy of the mass number was 0.5.

RESULTS

The ethanolic extract of *Lawsonia inermis* (Linn.) was subjected to screening for its phytochemical constituents. The phytochemical screening results are shown in Table 1. The ethanolic extract contains carbohydrates, glycosides, phenolic compounds, tannins, Terpenes and flavonoids.

Table 1- Phytochemical analysis of ethanolic extract of root of *Lawsonia inermis* (Linn.)

S.NO	TEST	ETHANOLIC EXTRACT
1.	ALKALOIDS	----
2.	CARBOHYDRATES	+
3.	GLYCOSIDES	+
4.	PHYTOSTEROLS	---
5.	FIXED OILS AND FATS	---
6.	SAPONINS	----
7.	TANNINS AND PHENOLIC COMPOUNDS	+
8.	PROTEINS AND AMINOACIDS	----
9.	FLAVONOIDS	+
10	LIGNIN	---
11.	TERPENES	+

Positive + , *Negative* -

The ethanolic extract of *Lawsonia inermis* was subjected to the TLC chromatographic profile and column chromatographic separation. The ethanolic extract of *Lawsonia inermis* dissolved in their mother solvent was taken in a capillary tube and spotted on TLC plates 2cm above its bottom. Most of the sample for application were between 0.1 – 1%. The applied spots were of equal size as far as possible and diameter ranging from 2-3mm. The solvent system for ethanolic extract was developed by trial and error method using various solvents which were differing in polarities.

Table 2 - TLC profiles of ethanolic extract of *Lawsonia inermis*

S.NO	SOLVENT SYSTEM	NO OF SPOT	Rf Value
1.	Benzene:choloroform (60:40)	2	0.30 , 0.50
2.	Benzene:choloroform(10:90)	2	0.50, 0.60
3.	Ethyl acetate:Methanol(80:20)	2	0.40 , 0.30
4.	Ethyl acetate:Methanol(30:70)	2	0.50 , 0.40

The ethanolic extract of *Lawsonia inermis* was subjected to column chromatographic separation using normal phase silica gel column. The dark brown solid (20 g ethanolic extract of *Lawsonia inermis*) was adsorbed on silica gel (20 g) and transferred to a column of silica gel (200g equilibrated with benzene). Two compounds were isolated in column chromatography using different solvents. Obviously, the compound 1 (140 mg) was eluted with benzene:

Choloroform 60:40, v/v, compound 2 (220mg) was eluted with ethyl acetate: ethanol, 80:20 v/v. This active fraction was used to identify the chemical tests showed the presence of phytosterols, flavonoids and amino acid as active compounds. The actual compounds were isolated from column chromatography as mentioned in the experimental section. The spectra (IR, 1H & 13CNMR and Mass) of these compounds as mentioned in the experimental section.

DISCUSSION

Characterization of compound 1

The spectral data IR, ^{1}HNMR & 13CNMR and Mass of the compound 1 are good in agreement with the structure proposed for the compound .The melting point of compound 1 was found as 140^{0}c.The IR spectrum of the compound of flavonoid is analyzed from the IR data. The presence of –OH group known from the absorption at the range3324 cm^{-1}.A strong band at the range 1684cm^{-1} is due to the presence of –C=O group. The presence of –C=O group. The presence of –C-O-C- indicates in the absorption at 1017cm^{-1}. In ^{1}HNMR and ^{13}CNMR spectral data of the compounds of pytosterols are analysed. Based on the^{1}HNMR chemical shift values and ^{13}CNMR chemical shift values of the sterol compound was found to be 24β-ethyl cholest-4-en-3β-ol. The mass spectrum of the isolated compound is presented in the fig. the m/z value of the isolated compound of the molecular ion is found as 415(M^{+}) which includes the isotopes of corresponding atoms. On the basis of spectral data (IR, ^{1}H & ^{13}CNMR and Mass), the isolated compound 1 was found as 24β-ethyl cholest-4-en-3β-ol and the Molecular Formula deduced as $C_{29}H_{50}O$.

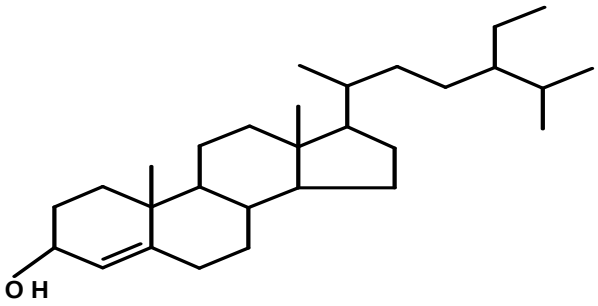

Fig1: 24β-ethyl cholest-4-en-3β-ol structure of compound 1

H signals appearing in the spectra of the isolated compound due to protons attached to carbon atoms can be assigned as follows. Signal at 5.40 produced by the aliphatic proton attached to the carbon atom at 24. Signal appeared at 5.19 due to the OH group proton on carbon atom at 3. Signal represents at 5.26-5.25(4),5.13-5.12(6),4.57-4.56(7),4.60-4.59(22),4.62-4.61(23),3.24-3.19(8,9,14,17 and 24) can be assigned to the CH group proton on carbon atom . Signal appeared between 1.12-0.85 can be attributed to the quaternary-CH3 proton attached to carbon atom 18, 19 & 20. The NMR signal appeared at 1.5 assigned as isopropyl group of the compound attached to carbon atom at 25, 26 and 27 positions. The ^{13}C-NMR spectrum peaks at δ 38(C-1),37(C-2),55(C-3).38,36(C-4),109(C-5),107(C-6),35(C-7),34(C-8),48(C-9),23(C-10),28(C-11),29(C-12),40(C-13),42(C-14),27(C-15),26(C-16),46(C-17),17(C-18),18(C-19),19(C-20),25(C-21),55(C-22),76(C-23), 79,24 (C-24),24 (C-25),21(C-26) and 22 (C-27) corresponds to C-H saturated and unsaturated alkanes. Signals at δ154, 50 correspond to the CH2 and CH3 functional of the compound.

Characterization of compound 2

The spectral data IR, ^{1}HNMR & 13CNMR and Mass of the compound 1 are good in agreement with the structure proposed for the compound. The melting point of the compound 2 is 235^{0}C.

The IR spectrum of the compound of flavonoid is analyzed from the IR data. The presence of –OH group known from the absorption at the range 3330-3463cm^{-1}.A strong band at the range 1655-1617cm^{-1} is due to the presence of –C=O group. The presence of –C-O-C- indicates in the absorption at 1060cm^{-1}. The ^{1}HNMR & ^{13}CNMR spectral data of the compounds of flavonoid are analyzed. Based on the ^{1}HNMR chemical shift values and ^{13}CNMR chemical shift values of the flavonoid compound was found to be 3, 7, 4', 5'-Tetrahydroxy-6-methoxyflavone (Fig 2). The mass spectrum of the isolated compound is presented in the fig. the m/z value of the isolated compound of the molecular ion is found as 316 which includes the isotopes of corresponding atoms. On the basis of spectral data (IR, ^{1}H & ^{13}CNMR and Mass), the isolated compound 2 was found as 3, 7, 4', 5'-Tetrahydroxy-6-methoxyflavone and the Molecular Formula deduced as $C_{16}H_{12}O_{7}$.This is first report of occurrence of this compound in nature as well as flavonoids in this plant.

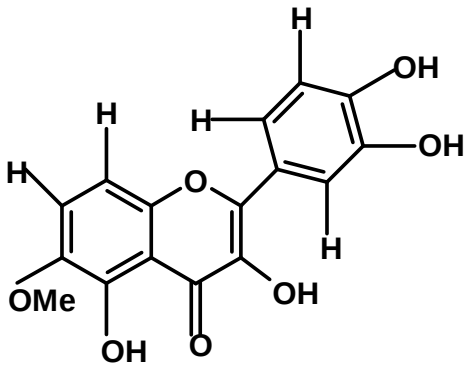

FIG 2: **3, 7, 4', 5'-Tetrahydroxy-6-methoxyflavone**

The ^{1}HNMR chemical shift value at δ, 13.05ppm indicates the presence of phenolic -OH group of the compound. The singlet at 10.72 ppm were due to proton functional of compound and the chemical shift as singlets at δ,9.60 and 8.33 due to presence of -OH at 5' and 4' position respectively. The chemical shifts at δ, 6.83-6.81 and 6.50 ppm were due to the presence of proton at 7 and 8 position of the compound. The chemical shift as singlet at δ, 3.75ppm was due to the presence of methoxy group at C6. The ^{13}C-NMR spectrum peaks at δ 180(C-4), 157.59(C-2), 154(C-3), 152(C-6), 131 C-5,130 (C-7), 121 (C-8), 157.39 (C-9) and 153 (C-10) corresponds to C-H saturated and unsaturated alkanes. Signals at δ, 104(C-2'), 115(C-5'), 121.23(C-6'), 121.78(C-4') corresponds to the aromatic nature of the compound. The chemical shift as singlet at δ, 59ppm was due to the presence of methoxy group at C6.Based on the above results , two compounds were isolated from ethanolic extract of *Lawsonia inermis*

CONCLUSION

Thus the present study reveals that the compound 1 was characterized as 24β-ethyl cholest-4-en-3β-ol and the Molecular Formula deduced as $C_{29}H_{50}O$ and compound 2 was characterized as 3, 7, 4', 5'-Tetrahydroxy-6-methoxyflavone and the Molecular Formula deduced as $C_{16}H_{12}O_7$ and this is the first report of occurrence of this compound in nature as well as flavonoids in this plant. Therefore in future biological investigations are needed for these isolated compounds.

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