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ISOLATION AND CHARACTERISATION OF THE ACTIVE PRINCIPLE FROM THE BARK OF *Aphanamixis polystachya*

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ABSTRACT

The hepatoprotective plants are capable of curing liver ailments. These herbal Plants have the phyto constituents such as phenyl compounds, coumarins, essential oils, terpenoids, monoterpenoids, diterpenoids, triterpenoids, steroids, alkaloids and other nitrogenous compounds. The plant taken for this study is *Aphanamixis polystachya* (Wall) *Parker*. It is called Rohituka in Sanskrit, malampuluvan in Tamil. This plant belongs to Meliaceae family. Its seed and bark are used medicinally. Its habitat is Western Ghats in Thirunelveli district. This plant is used for liver disorders, rheumatoid arthritis, leucorrhoea and anthelmintic ulcers. There has not been sufficient scientific study on hepatoprotective activity of this plant. The scope of the study on bark of *Aphanmixis polystachya* includes phytochemistry. The finding in phytochemistry is that this plant contains flavonoid in major quantities.

Key words: *Aphanmixis polystachya*, Hepatoprotective activity, Flavonoid, 5, 4′-Dihydroxy 6,8-dimethoxy 7-O-rhamnosyl flavone.

INTRODUCTION

An antihepatotoxic plant is used for curing liver ailments. *Aphanamixis polystachya* (Wall) *Parker* is a plant used in a few liver drugs that are produced in India. A scientific study on this plant is necessary in order to promote its medicinal use. Seeds and bark of these plants are used medicinally. The seeds are credited with anthelmintic, laxative and refrigent activities. Also, they are used for curing ulcers and muscular pains. The bark is strong astringent and is used in diseases of the liver and the spleen and for tumours and abdominal complaints. In the preliminary phytochemical analysis flavonoid [1] was found to be present in the ethanolic extract of bark of *Aphanamixis polystachya*.

The tropical plant *Aphanamixis polystachya* J. N. Parker (synonyms: *Amoora rohituka* (Weight) and Arn.; *Aphanamixis rohituka* (Roxb.) (Meliaceae) is a large evergreen and useful timber tree. It has been extensively investigated since the 1960s because of the anticancer, antimicrobial and antifungal, anti-inflammatory, and insecticidal potential, and hepatoprotective properties of the plant extracts. Earlier studies on this plant have disclosed the presence of alkaloids, fatty acids, flavonoids, lignans, steroids, diterpenoids, sesquiterpenoids, triterpenoids and a series of complex tetra nor triterpenoids (limonoids) [2] (including dihydroamoorinin and amoorinin, and its glucosides, aphanamixinin, kihadalactone and polystachin, rohituka , rohituka , androhitukin). Our early work [3,4] on investigating the chemical constituents of the plant seed resulted in the isolation of a known dregeana-1 and a novel limonoid rohituka-15 . Further investigation led to the isolation and identification of 5, 4′-Dihydroxy 6, 8-dimethoxy 7-O-rhamnosyl flavone .

MATERIALS AND METHODS

Bark of the plant *Aphanamixis polystachya* (Wall) *Parker* was collected from Papanasam hills in Tirunelveli district. Ethyl alcohol extracts of leaves and bark were prepared by cold percolation method [5,6,7] Powdered leaves and powdered bark were soaked in ethyl alcohol for 48 hours.

ISOLATION OF COMPOUND

The concentrate of the alcohol extract of

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Aphanamixis polystachya was fractionated using benzene (3 × 300 ml), diethyl ether (3 × 300 ml) and ethyl acetate (4 × 300 ml). The ethyl acetate fraction on concentration yielded a yellow solid, which was non homogenous in TLC and hence was further subjected to separation and purification on column chromatography [8-10].

Column chromatographic analysis

The residue obtained from the ethyl acetate fraction (15g) of *Aphanamixis polystachya* was chromatographed in silica gel column (60-120 mesh, 300 gm, 100 × 5 cm) using gradient elution with the solvents of increasing polarity. Fractions of 100 ml were collected each time and the homogeneity was examined on TLC with suitable solvents. The details of the fractionations and their characteristics are given in Table 1**.**

Fractions 77-90 on concentration yielded a pure yellowish homogeneous solid and were designated as compound I. It gave dark green colouration with neutral ferric chloride and violet colouration with Molish's reagent. The R_f values of compound I in various solvent systems are given in Table 2**.**

Acid hydrolyis of compound I

In order to find out the nature of the glycoside, compound I was subjected to acid hydrolysis. To a solution of the glycoside (10 mg) in hot methanol (10 ml), an equal volume of H_2 SO_4 (7%) was added and the mixture was gently refluxed at 100°C for 2 hours. The excess of alcohol was distilled off *in vacuo* and the resulting aqueous solution was partitioned with ether to separate the ether soluble aglycone and the aqueous sugar.

Identification of the sugar

The aqueous layer was treated with $BaCO_3$ to remove excess sulphuric acid and the barium sulphate formed was filtered off using Whatman No. 42 filter paper and the filtrate (sugar portion) was concentrated.

The concentrate was analyzed by Paper chromatography (PC) with various authentic sugar samples on a Whatman No.1 filter paper strip and identified using Aniline hydrogen phthalate spray reagent (prepared by dissolving 9.2 ml of aniline and 16 g of phthalic acid in 490 ml of n-butanol, 490 ml of ether and 20 ml of water). The various solvent systems used for PC and R_f values of the identified sugar in these solvent systems are presented in Table 3.

UV spectral characteristics of compound I

Basic flavonoid structure of compound I and position of attachment of hydroxy and other substituents were conveniently studied by recording UV spectrophotometer (Shimadzu 1601) in MeOH as well as in various Shift reagents. The λ max values are given in Table 4.

^{1}H-NMR Spectral data of compound I

The ^{1}H-NMR spectrum of compound I was recorded using AMX 400 (400 MHz) spectrometer using DMSO-d_6 as the solvent and complete assignment of protons are shown in Table 5**.**

^{13}C-NMR spectral data of compound I

^{13}C-NMR spectrum of compound I was recorded using AMX 400 (100 MHz) spectrometer using DMSO-d_6 as the solvent and the complete assignment of carbon are given in Table 6.

EI-MS study of compound I

EI-MS spectrum (Fenniganmat 8230, 70 eV) of compound I was taken and it gave various fragments at *m/z*: 330, 213 and 118.

Figure 1. 1H-NMR Spectral data of compound I

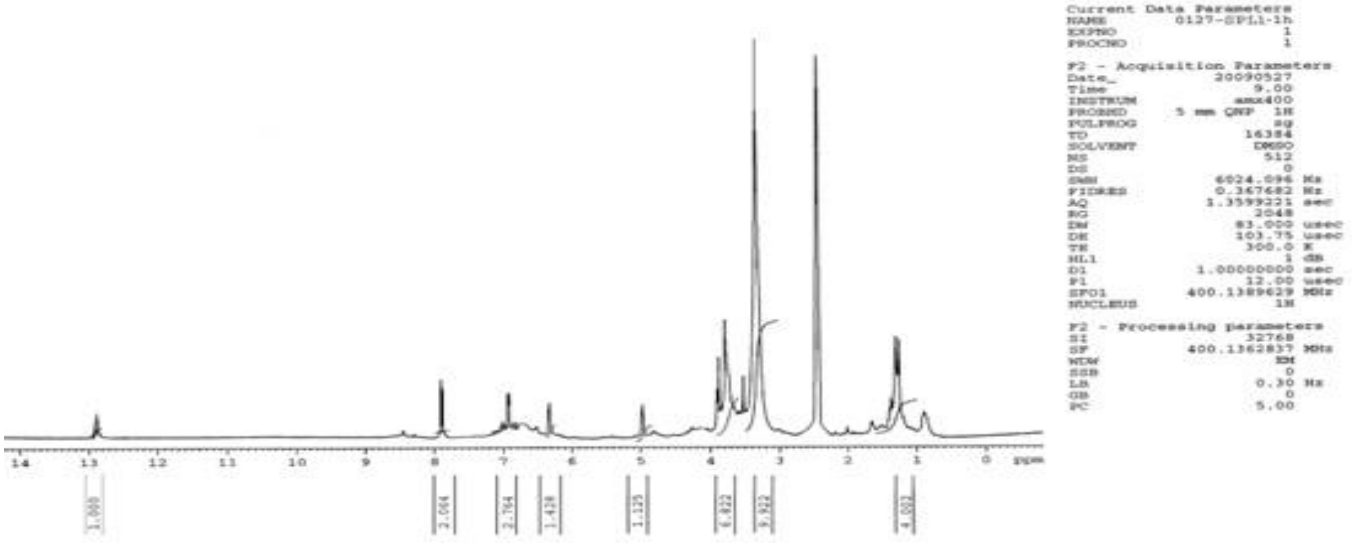

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Figure 2. ^{13}C-NMR spectral data of compound I

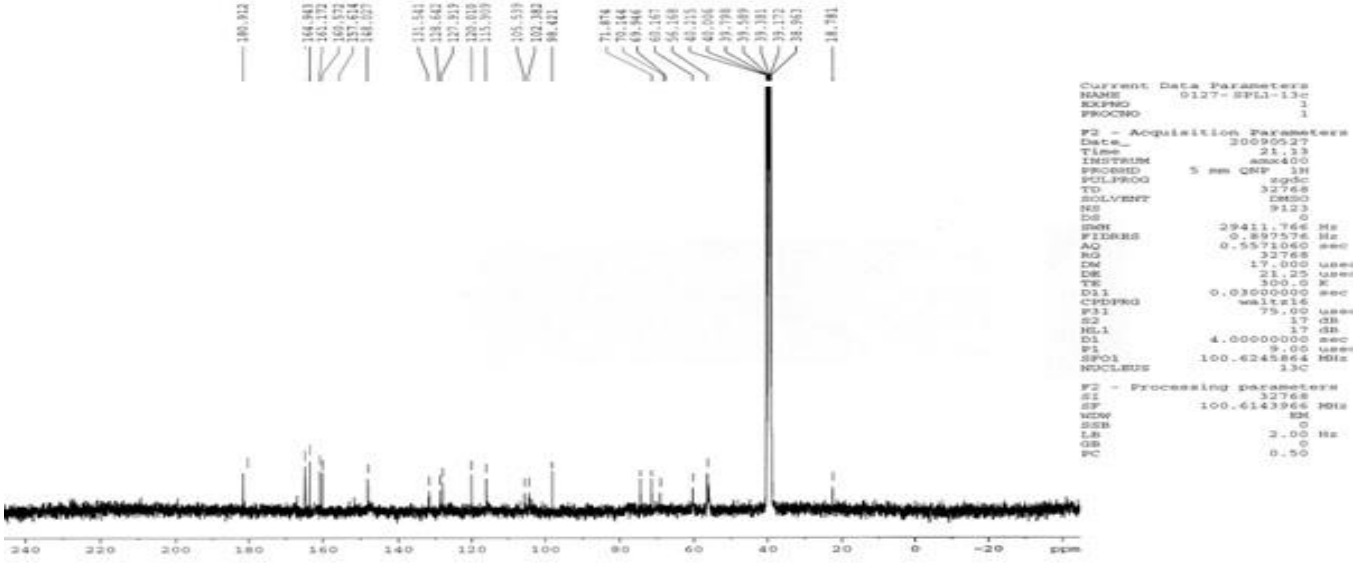

Figure 3. EI-MS study of compound I

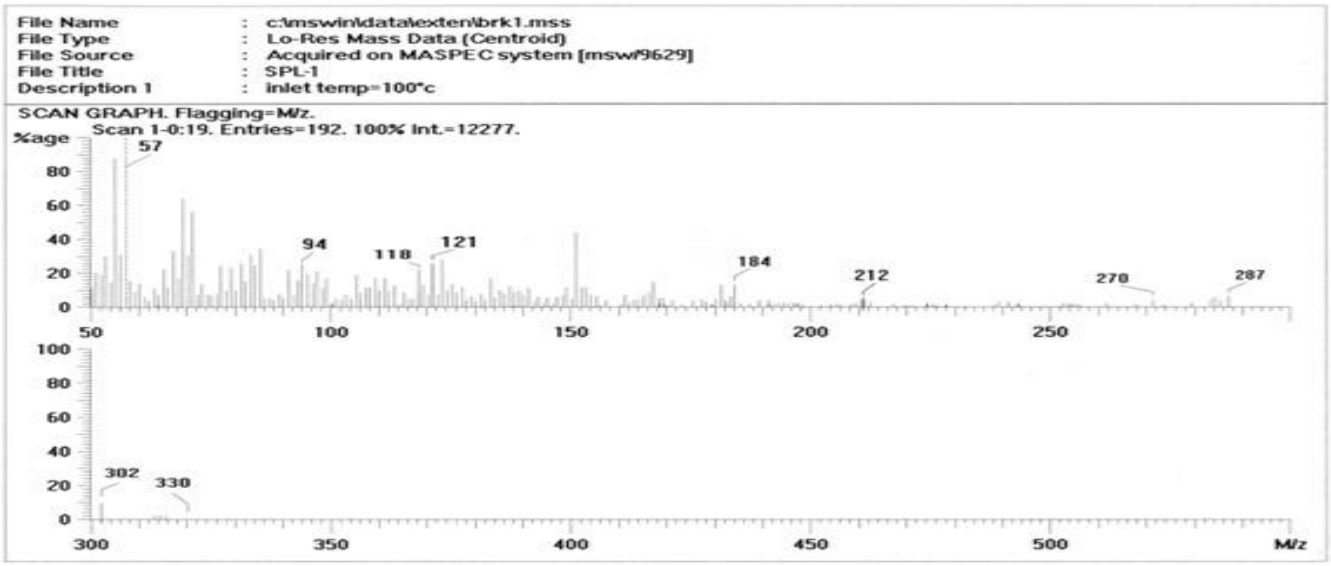

Figure 4. 5, 4′-Dihydroxy 6,8-dimethoxy 7-O-rhamnosyl flavone

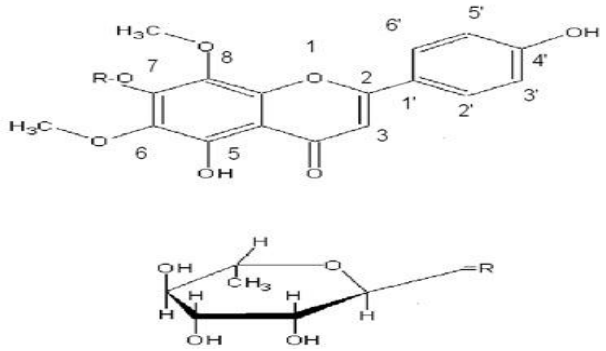

Figure 5. EI-MS Fragmentation

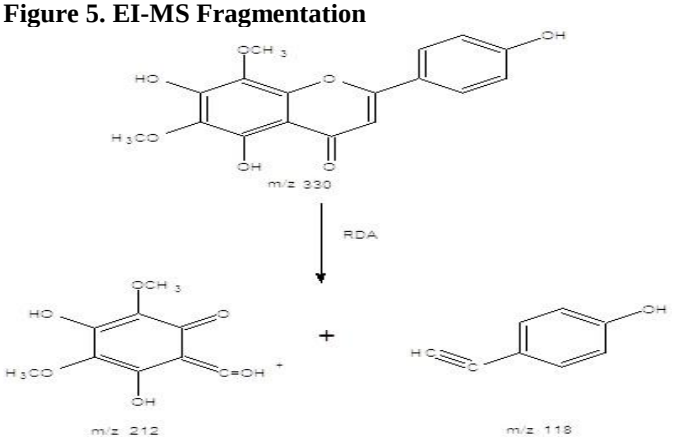

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Table 1. Chromatographic Fractions of Ethyl Acetate Concentrate of *Aphanamixis polystachya*

Fractions collected	% Eluent composition	*Remarks*
1-5	100 Petroleum ether	Yellow waxy substance
6-10	90/10 Pet. Ether/benzene	Yellow waxy substance
11-48	80/20 to 10/90 Pet. Ether/benzene	Pale yellow solid
49-64	100 benzene	Pale yellow solid
65-76	90/5 benzene /EtOAc	Yellow solid
77-90	80/20 benzene /EtOAc	Yellow solid
91-104	70/30 benzene /EtOAc	Yellow brown solid

Table 2. $R_f \times 100$ Values of Compound in Paper Chromatography

Compound I	Solvent System						
	15% HOAc	30% HOAc	50% HOAc	60% HOAc	BAW*	Forestal[#]	PhOH[+]
	11.26	31.34	78.76	83.60	20.83	93.84	34.42

*BAW – n- butanol: acetic acid: water (4:1:5); [#]Forestal- Acetic acid: Conc HCl: H_2O (30:3:10); [+]PhOH- Phenol saturated with water (3:1)

Table 3. $R_f \times 100$ Values of Sugar of Compound

Sugar	Developing solvents			
	BAW*	**PhOH[+]**	**Forestal[#]**	**EtOAc:Pyridine: H_2O 10:4:3**
Sugar from compound I	38	55	58	54
Authentic Rhamnose	37	55	59	55

* BAW – n- butanol: acetic acid: water (4:1:5); [+]PhOH- Phenol saturated with water (3:1); [#]Forestal- Acetic acid: Conc HCl: H_2O (30:3:10)

Table 4. λ_{max} Values of Compound I

Solvent / Shift Reagents	λ_{max} (nm)
MeOH	273, 329
+NaOMe	273, 340, 389
+NaOAc	273, 313
+NaOAc +H_3BO_3	272, 329
+$AlCl_3$	270, 348
+$AlCl_3$ + HCl	270, 300, 315, 347

Table 5. ^{1}H-NMR Data of Compound I in DMSO-d_6

δ H (ppm)	Signal Assignment
12.95	1 H, s, 5-OH
7.89	2H, d, (J=8.7 Hz), H-2′ 6′
6.93	2H, d, (J=8.7 Hz), H-3′ 5′
6.38	1H, s, H-3
5.15	1H, d, (J=2 Hz), H-1 of rhamnose
3.9	3H, s, OCH_3 group
3.8	3H, s, OCH_3 group
3.0 - 3.75	Sugar protons
1.2	3H, d, J=6 Hz, H-6″

Table 6. ^{13}C-NMR Data of Compound I in DMSO - d_6

C	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
ppm	164.9	102.3	180.9	157.6	131.5	161.1	128.6	148.0	105.5

C	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
ppm	120.0	127.9	115.9	160.5	115.9	127.9

C	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″	OCH_3	OCH_3
ppm	98.4	70.1	69.9	71.8	69.9	18.7	60.1	56.1

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RESULTS & DISCUSSION

Characterization of Compound I

The compound **I** was crystallized from alcohol as pale yellow amorphous powder. It showed an intense UV maxima at 273 nm (band II) and 329 nm (band I) indicating the flavone nature of it. Band I underwent a significant bathochromic shift of +60 nm on addition of NaOMe which suggested the presence of free 4′-OH group in ring B.

Absence of characteristic bathochromic shift (5-20 nm) on addition of NaOAc suggested that C-7 was not free. Absence of characteristic bathochromic shift on addition of $NaOAc/H_3BO_3$ indicated the absence of O-dihydroxy substituent in ring B. A consistent bathochromic shift of band II (14 nm) with $AlCl_3$/ HCl indicated the presence of hydroxyl substituent at C-5 along with oxygen at C-6 position [12-15].

In the ^{1}H-NMR spectrum of compound I showed a pair of doublets in the aromatic region at δ 7.89 ppm and δ 6.93 ppm each integrating two protons indicated the presence of two A_2 B_2 pattern due to protons a C-3′, C-5′and C-2′, C-6′ respectively of ring B of flavone. This was supported by the UV shift experiments and ^{13}C-NMR values. Violet colouration of compound I with Molisch's reagent indicated the presence of glycoside moiety [16,17]

The position of glycosylation at C-7 as indicated by UV studies was confirmed by the presence of anomeric proton signal displayed at 5.15 ppm [For C-3 anomeric proton appears at δ 5.8 ppm].

Rhamnosyl nature of the sugar [18] and its attachment to C-7 carbon was confirmed by acid hydrolysis and ^{1}H-NMR studies.

The ^{1}H-NMR spectrum also showed one singlet at δ 6.2 ppm corresponding to C-3 proton of the flavone skeleton, which is also, supported by the ^{13}C-NMR signals at δ 164. 9 (C-2), 102.3 (C-3) and a quaternary signal at 180.9 (C-4).

Absence of other characteristic signals in the aromatic region of the ^{1}H-NMR spectrum suggested that all the carbon atoms of ring A are substituted. The 5-hydroxy and C-6, C-7 and C-8 substitution of ring A is further supported by the ^{13}C-NMR values at δ 157.6 (C-5), 161.1(C-7), 131.5(C-6) and 128.6 ppm (C-8).

The absence of signals for H-6 and H-8 in ^{1}H-NMR, the downfield shift of C-6 and C-8 in ^{13}C-NMR and the appearance of two methoxyl signals at δ 60.1 and δ 56.1ppm suggested the possibility of substitution of C-6 and C-8 by methoxyl groups.

EI-MS of the compound exhibited M^+ *m/z* 330 and fragment ion at *m/z* 212 and *m/z* 118 consistent with retero-Diel's Alder fragmentation and a fragment ion at *m/z* 118 confirmed the presence of C-4′ hydroxyl group in ring B.

Thus based on the R_f values, UV, ^{1}H-NMR, ^{13}C-NMR and EIMS spectral studies the structure of compound I has identified as 5, 4′-dihydroxy 6,8-dimethoxy flavone. This compound is named as Aphanamixine.

CONCLUSION

5, 4′-Dihydroxy 6,8-dimethoxy 7-O-rhamnosyl flavone i.e., Aphanamixine was taken for yet another study to confirm the hepatoprotective effect. The results were positive. The further scope of this study is to formulate a herbal drug that can cure liver ailments.

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