RESEARCH PAPER | Pharma | Volume : 4 | Issue : 2 | Feb 2014 | ISSN - 2249-555X

Extraction, Isolation and Chemical Structure Elucidation of Isorhamnetin From Leaves and Stems of Argemone Mexicana Linn of Bhopal, Madhya Pradesh, India

KEYWORDS: Argemone mexicana, Isorhamnetin, Flavonoid

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ABSTRACT *PURPOSE: To extract, isolate and identify isorhamnetin from the stems and leaves of Argemone mexicana Linn of Bhopal, Madhya Pradesh, India.*

METHODS: Ethyl acetate soluble fraction of successive methanol extract from the stems and leaves of A. mexicana was subjected to column chromatography and fractionated by benzene, n-butanol, and acetone successively. Eluents having similar Rf values were pooled together. These pools were subjected to flavonoid test and only a single pool passed it which was again subjected to chromatographic separation for purification and crystallized powers were subjected to FT-IR, EI-MS and 1H-NMR for structure identification.

CONCLUSION: From this study it can be concluded that the whole plant (except roots and capsules) can be used as a source of isorhamnetin.

INTRODUCTION

The plant Argemone mexicana is the source of a diverse kind of chemical constituents having mostly abundant alkaloids as berberine[1], protopine[2], sanguinarine[3], nor-sanguinarine[2], angoline[3, 4], chelerytherine[3] etc. Other active constituents which are found are terpenoids {trans-phytol[4], β-amyrin[5]}, steroids {stigma-4-en-3, 6-dione[4], β-sitosterol[6]}, long-chain alcohols {myristic acid[7], palmitic acid[7], stearic acid[7], arachidic acid[7], oleic acid[7], linoleic acid[7], argemonic acid[8] etc.}, fatty acids from seed oil {9-and 11-oxo-octacosanoic and 11-oxotriacontanoic acids[9]}, amino acids {cysteine and phenylalanine}[5], flavonoids {luteolin[10], eriodictyol[10], isorhamnetin-3-O-β-Dglucopyanoside[4, 5, 6], isorhamnetin[6, 11], isorhamnetin-7-O-β-Ddiglucopyanoside[11], isorhamnetin-3,7-O-β-Ddiglucopyranoside, quercetin[12], rutin[12, 13], mexitin[13]}, aromatic acids {5,7-dihydroxy chromone -7-neohesperidoside[14], tannic acid[15], caffeic acid[15], ferulic acid[15], vanillic acid[6]}, miscellaneous compounds {α-tocopherol, adenosine, adenine}[4].

As mentioned earlier, previous studies have reported the presence of isorhamnetin-3-O-β-D-glucopyranoside in the leaves and flowers[4, 5, 11] and isorhamnetin from flowers[6, 11] however in the present study an attempt has been made to isolate isorhamnetin possibly for the first time from the stems and leaves.

EXPERIMENTAL

Collection of plant material

Fresh plants of A. mexicana were collected in the month of May 2010 from the local area of Berasia Tahasil, District Bhopal, Madhya Pradesh, India after collection seeds and flowers of the plant were rejected. The specimens were identified by Dr. Padma Shrivastava, Professor and Head of Post Graduate Department of Botany, Government Post Graduate College BHEL, Bhopal, Madhya Pradesh, India and voucher specimen (PGDB/BHEL/Ph.D/AM24) was deposited there for future reference .

Chemicals

Ethyl acetate, n-hexane, methanol, ethanol, chloroform, diethyl ether, hexane were analytical grade and purchased from Himedia Laboratories (Bhopal, India). Acetonitrile was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MV) (Millipore, USA) was used for all solutions and dilutions. All other reagents were of the highest commercial grade available.

General experimental procedures

UV spectra of the isolated compounds were recorded in methanol over a scanning range of 200-400 nm and λ_{max} of compounds were determined. Spectra were recorded with a Systronics double beam-2203 UV-VIS spectrophotometer. EIMS (electron impact mass spectrum) in positive mode, were recorded on Bruker's aurora M90 (USA) instrument. The isolate was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 375-7500 cm^{-1} in FT-IR spectroscopy (Bruker FT-IR Spectrometer, USA). ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker BioSpin Advance III FT-NMR spectrometer, USA, operating at 700 MHz both for proton and carbon using tetramethylsilane (TMS) as internal standard. The solvents used were methanol and DMSO-d_6. Chemical shifts ware shown in δ values (ppm) with TMS as an internal reference. Paper chromatography was carried out by Whattman 3 MM CHR. For column chromatography silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany) was used. Thin layer chromatography (TLC) was performed using precoated TLC plates (Silica Gel G-60 F254, Merck, Germany).

Preparation of extracts

Plant samples were shade dried for 15 days and then pulverized to fine powders using mortar and pestle. 1000g of powder samples were extracted exhaustively with petroleum ether (70^{0}C) and then with methanol (90 %) (300 × 5) in a soxhlet apparatus. Methanol extract was then concentrated by distilling off the solvent and evaporated to dryness. The residue (204.1 g) was suspended in water (300 ml), extracted successively with chloroform, ethyl acetate and acetone (300 ml × 3 each) and resulting solutions were concentrated by distilling off the solvent using hot air oven to yield 31.3, 17.8

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and 23.2 g of dry sub-extracts respectively and stored at 4^0C for further analysis.

Phytochemical Screening for flavonoids
Sodium hydroxide test, lead ethanoate test and Shinoda's test were performed on all the prepared extracts for the presence of flavonoids with suitable solvents according to the standard procedures[16].

Chromatographic characterization
Paper chromatography
Paper chromatography was carried out as per standard procedure. Different solvent extracts of the plant samples were applied on chromatography paper and chromatogram was developed in seven different solvents.

Column chromatography
15g of ethyl acetate extract fraction of the plant sample was adsorbed onto sufficient quantities of silica gel by triturating in a mortar under hood and left for about 9 hours to dry. Meanwhile, silica gel was suspended in water and stirred gently until the formation of a slurry. This slurry was then poured carefully into previously cleaned, uprightly fitted; glass-wool sealed open glass column (2.5 cm inner diameter and 75 cm length) till it is about three-fourths filled, with tapping the walls with a cushioned rod in order to avoid air-bubbles. Solvent system having same proportion of n-butanol, acetic, acid, water was poured continuously into the column and allowed to drain until the silica particles pack. The quantity collected was poured back into the column. The silica gel adsorbed extract sample was sprinkled over the surface of the supernatant in the column using a fine spatula and allowed to settle on the surface of the stationary phase. A small quantity of sand sprinkled on the supernatant after adding the sample to prevent dissolution of the extracts into the mobile phase. Three mobile phases (benzene, n-butanol, and acetone) were successively passed through the column under the force of gravity and the tap at the bottom of the column was allowed to adjust the flow. Several fractions were eluted depending on the visible changes in the colorful bands running out of the column and collected in dry glass bottles.

TLC
1 cm was measured from the base of the TLC plate, marked with a pencil and labeled. Capillary tube was used to spot the plates with the column eluents. Small quantities of the eluents collected with capillary tube by dipping it in the solution. They were then used to spot the plates and put in a lidded tank containing the solvent system, Benzene: Acetone (9:1). The level of solvent system in the tank was about 1 cm beneath the origin. The solvent travelled up the plate by capillary action till it reached the solvent front, marked by a straight line across. The lid was lifted off and the plates ware dried before they were visualized by spraying with 1% ethanolic solution of aluminum chloride under UV light (365 nm). The retention factors were calculated by making use of the distance moved by the solvent and the distance moved by the component as follows.

RF = Distance travelled by the component/Distance travelled by the solvent front

Results
The preliminary phytochemical screening of successively extracted fractions of methanolic extract of leave and stem parts of A. maxicana showed the presence of flavonoids only in the ethyl acetate fraction as a pink coloration, buff-colored precipitate and a colorless solution appeared in Shinoda's test, Lead ethanoate test and Sodium hydroxide test respectively and only this fraction was used for further characterization study.

Prior to the chromatographic separation, solvents with different polarities such as cyclohexane, benzene, chloroform, n-butanol, acetone, methanol and water were chosen to determine the appropriate solvent for separation of compounds by paper chromatography for the selected extract. Among these seven solvents, better separation of compounds with clear and distinct spots was achieved with benzene, n-butanol and acetone against the other four solvents which gave unclear spots with varying Rf values. Subsequently the extract was fractionated by column chromatography with benzene, n-butanol, and acetone successively. A total of 26, 33 and 4 fractions were eluted by benzene, n-butanol and acetone respectively. The column fractions were tested with TLC chromatogram and the Rf values were determined. Eluents showing similar pattern of TLC separation with respect to their Rf values were considered as one fraction alike and were pooled together. A total of 14 pools were made, from which a single pool was selected for further studies as the eluents in this pool showed positive inferences when subjected to the flavonoid test i.e., they shown yellow fluorescence in UV light (365 nm) after spraying with a 1% ethanolic solution of aluminum chloride. This fraction was again chromatographed under the same conditions and finally purified (70 ml) by crystallization to yield 27.56 mg of yellow amorphous powder

The chemical structure identification was carried out by FT-IR, EI-MS and ^{1}H-NMR as follows.

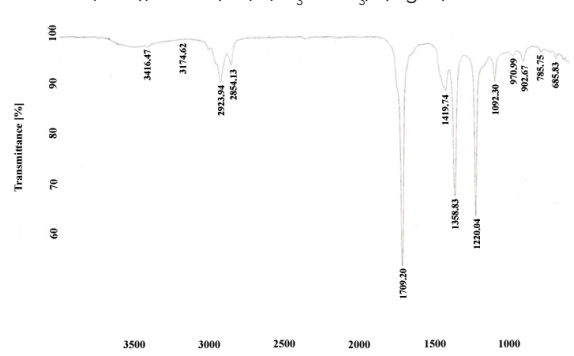
M.P.: 307°C.

UV (MeOH) λ_{max}: 272 and 334 nm.

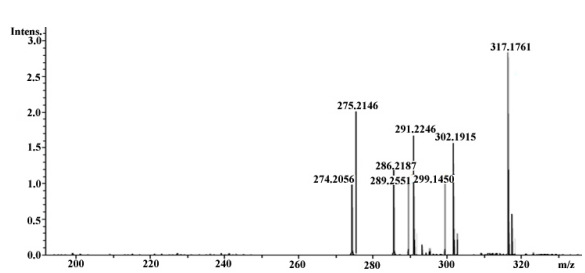
IR (KBr) ν_{max} cm^{-1}: 3416.47, 3174.62, 2923.94, 2854.13, 1709.20, 1419.74, 1358.83, 1220.04, 1092.30, 970.99, 902.67, 785.75. (Fig. 1)

EI/MS m/z: 316 [M^+], 316, 301, 287, 245, 153, 142, 128, 108, 69. (Fig. 2)

^{1}H NMR (500 Hz, DMSO-d_6) δ: 12.472 (1H, s, C_5-OH), 10.774 (1H, s, OH), 9.781 (1H, s, OH), 9.435 (1H, s, OH), 7.780 (1H, d, J=1.5 Hz, H-2′), 7.774 (1H, dd, J= 1.5 Hz, H-6′), 6.951 (1H, d, J=8.5 Hz, H-5′), 6.498 (1H, d, J=2.0 Hz, H-8), 6.211 (1H, d, J=2.0 Hz, H-6), 3.184 (3H, s, C_3-OCH_3). (Fig. 3)



(Fig. 1: FTIR spectrum of flavonoid compound)



(Fig. 2: EI-MS of flavonoid compound)

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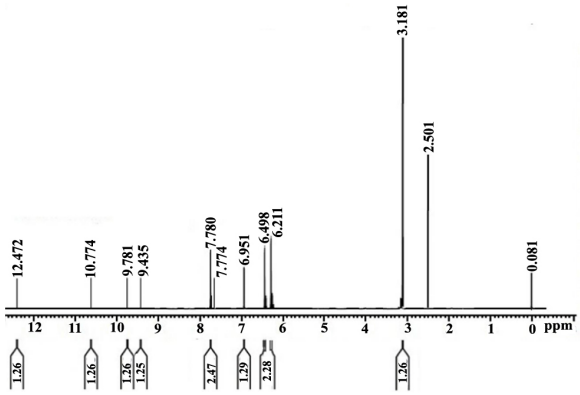


(Fig. 3: ^{1}H NMR (500 MHz) spectrum of compound in DMSO-d_6)

Discussion

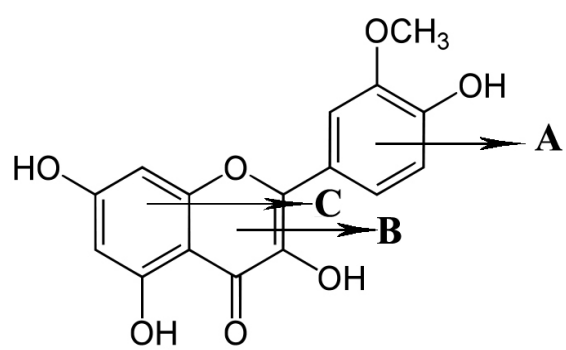
Different non polar and polar solvents such as cyclohexane, benzene, chloroform, n-butanol, acetone, methanol and water in different combinations were used to carry out TLC. Except Benzene: Acetone (9:1), no other solvent system gave successful results in chromatographic separation of the components. The successful separation of active constituents by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient for each target compound[17]. TLC chromatogram of a specific pool of column chromatography eluted plant extracts showed the presence of flavonids because they have shown yellow fluorescence in UV light at 365 nm after spraying with 1% ethanolic solution of aluminum chloride. There after the re-chromatogarphed and crystallized flavonoid fraction was used for the following studies for structure elucidation.

The spectral analysis of the flavonoid fraction shown in Fig. 1 was carried out by comparing with previous published literatures[18- 20] following remarks were manifested. The absorbance bands at 3416.47, 3174.62 and at 2923.94, 2854.13 cm^{-1} possibly due to the presence of –OH stretch of phenols or alcohols, C–H stretching vibration of alkenes and alkanes respectively. The strong stretching band at 1709.20 cm^{-1} indicates the presence of carbonyl group (C=O). The most characteristic C=C stretching at 1419.74 cm^{-1} confirms the compound to be an aromatic one, also other inferences in favor of the aromatic structure we had drawn from the band at 1358.83, 902.67 and 785.75 cm^{-1} which were represented as C–H deformation in methyl moiety of aromatic nucleus, C–H deformation in aromatic ring and for the presence of a di-substituted (meta) benzene ring respectively. A strong broad band appeared at 1220.04 cm^{-1} represents the characteristic band for C–O stretch of phenol which is slightly deviated from its normal frequency at 1230 cm^{-1} may be due to split. A band at 1092.30 cm^{-1} can be interpreted for alkyl C–O stretch of ethers. Mild bands at 970.99 and 685.83 cm^{-1} can be identified for C–H out of plane deformation in alkene.

In the mass spectra (Table 1.) the peak results are in M+1 form, the peaks will be as the followings- molecular ion peak (316) will be at 317 of C(16)H(12)0(7) and the two main fragment ion peak (301) will be at 302 of C(15)H(9)0(7) and (285) will be at 286 of C(15)H(9)0(6). The molecular formula obtained was $C_{15}H_{10}O_6$ from the [M+] ion at m/z 286[21]. The MS fragmentation pattern clearly indicated that one methoxy and hydroxyl groups were attached to the ring-A, (Fig. 4) while the remaining hydroxyl groups were linked with the ring-B and C at C-3, 5, 7 position. The mass fragmentation pattern also confirmed that the C-4 position was blocked by a carbonyl group. In view of these spectral data, the compound was identified as 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one (Fig. 4). Compound was identified as isorhamnetin, flavonol, by comparison of spectroscopic data with previously published literature[22].

Table 1: Interpretation from EI-MS of flavonoid compound

S. No.	Peak/ M.W.	Name	Remark	M+1 peaks
1.	26	C(2)H(2)		26.0220
2.	28	Carbonmo-nooxide		28.1722
3.	42	CH(2)=C=O		42
4.	273	C(14)H(9)0(6)	A,B and C excluding methoxy group with carbon at A	274.2056
5.	274	C(14) H(10)0(6)	A,B and C excluding hydroxyl group with carbon and CH at C	275.2146
6.	285	C(15)H(9)0(6)	At A,B and C excluding methoxy at C nucleus	286.2187
7.	288	C(15) H(12)0(6)	At A,B and C excluding CO at C	289.2551
8.	290	C(14)H(10) O(7)	At A,B and open C nucleus without C(2)H(2)	291.2246
9.	299	C(16) H(11)0(6)	At A,B and C excluding para hydroxy at C nucleus	291.2246
10.	299	C(16) H(11)0(6)	At A,B and C excluding hydroxy at B nucleus	299.1450
11.	299	C(16) H(11)0(6)	At A,B and C excluding ortho hydroxy at C nucleus	299.2501
12.	299	C(16) H(11)0(6)	At A,B and C excluding hydroxy at A	302.1915
13.	301	C(15)H(9)0(7)	At A,B and C excluding para hydroxy at C	302.1915
14.	316	C(16)H(12) O(7)	Molecular Ion	317.1761



(Fig. 4: Isorhamnetin- 3,5,7- trihydroxy -2- (4-hydroxy-3-methoxyphenyl) chromen-4-one)

This structure was further confirmed by ^{1}H-NMR spectral studies. The ^{1}H-NMR spectrum of the compound (Fig. 3) showed a total of 12 signals for 15 carbons. The signals observed were allocated as following. The ^{1}H-NMR spectrum shows, the aromatic region exhibited at δ 7.780 (1H, d, J=1.5 Hz, H-2′), 7.774 (1H, dd, J= 1.5 Hz, H-6′) and 6.951 (1H, d, J=8.5 Hz, H-5′) due to a 3′, 4′ disubstitution of ring B and a typical meta-coupled pattern for H-6 and H-8 protons (δ 6.211 and 6.498, d, J = 2.0 Hz). The presence of the methoxy group at 3 was supported by δ 3.184 signals[23]. All these spectral data were in good concurrence with those reported in several literatures[23- 26] for Isorhamnetin, so the structure of the compound was identified as isorhamnetin.

Conclusion

We have successfully isolated Isorhamnetin from the stems and leaves of Argemone mexicana L., which was previously

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been reported from the flowers[6, 11]. Therefore, from this study it can be concluded that the plant as a whole (except roots and capsules) can be used as a source of isorhamnetin which has several bio-active characteristics, out of which antiviral, antitumor, apoptosis inducer and antioxidant activities are predominant.

Acknowledgement
The authors are very much thankful to Mr. Aashish Pathak, Vice-President, Sapience Bioanalytical Research Laboratory, Bhopal for facilities and encouragement.

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