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Research Article

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *MORINGA OLEIFERA* (MORINGAECEAE) PODS

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ABSTRACT

Objective: *Moringa oleifera* Lam. is the most widely cultivated species of the monogeneric family Moringaceae and has an impressive range of medicinal uses with high nutritional value. In this study saponin was isolated from *Moringa oleifera* pods.

Methods: Thin layer chromatography (TLC) was performed using a mobile phase of Chloroform: methanol: H_2O (7:3:1) on silica gel glass plates. High performance liquid chromatography (HPLC) of isolated compound from benzene extract obtained by Successive extraction method was carried out to confirm its nature by analyzing HPLC chromatograms.

Results: Characterization of isolated saponin was done using IR and ^{1}H NMR. Saponin from *M. oleifera* pods was isolated having R_f 0.90. The IR spectrum of isolated compound exhibited the presence for hydroxyl group (-OH), carboxylic acids, alkynes, presence of –C=O (esters) and >C-O (ethers) and the ring involvement or aromatic structure of the compound. ^{1}NMR spectrum of isolated compound revealed presence of protons in the compound.

Conclusion: The isolated compound was then nomenclatured as SM (saponin from Moringa pods) and was further used to determine its biological and pharmacological properties.

Keywords: *Moringa oleifera*, Saponin, Thin layer chromatography, HPLC/IR/NMR

INTRODUCTION

Medicinal plants represent a rich source of cancer drug leads. Saponins are plant glycosides with a triterpene or steroid aglycone. Saponins have been found in many medicinal plants used in folk medicines. In this study, isolation of saponins was conducted from *M. oleifera* pods. *Moringa oleifera* Lam (Moringaceae) is a highly valued plant, distributed in many countries of the tropics and subtropics [1]. It has an impressive range of medicinal uses with high nutritional value. Different parts of this plant contain a profile of important minerals, and are a good source of proteins, vitamins, β-carotene, amino acids and various phenolics. The Moringa plant provides a rich and rare combination of zeatin, quercetin, β-sitosterol, caffeoylquinic acid and kaempferol. Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, possess antitumor, antipyretic, analgesic [2] antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant [3,4], antidiabetic, hepatoprotective [5,6], renoprotective [7,8] antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of medicine. *Moringa oleifera* is rich in compounds containing the simple sugar, rhamnose and a fairly unique group of compounds called glucosinolates and isothiocyanates. The stem bark has been reported to contain two alkaloids, namely moringine and moringinine, Vanillin, β-sitosterol, β-sitostenone, 4-hydroxymellin and octacosanoic acid have been isolated from the stem of *M. oleifera* [9]. Till date saponins from *M. oleifera* have not been reported hence this study was an attempt to isolate saponin component from the pods.

Saponins are natural high-molecular-weight glycosides of triterpene or steroids with a very wide distribution in the plant kingdom [10], as well as in lower marine animals, such as starfish [11]. In the past, saponins were characterized according to their surface-active properties and ability to form persistent foams [12].

Saponins exhibited a range of biological activities [13]. On the other hand, saponins also have beneficial pharmacological effects. They are anticholesterolemic due to the formation of a complex with cholesterol in gastrointestinal tract thus preventing absorption [14]. Other activities include anti-inflammation, anti-parasite and anti-virus [15,16]. Numerous lines of evidence now indicate that saponins can kill tumor cells by triggering tumor cell death via different signaling pathways, by activating death receptors [17], targeting mitochondria [18], and inducing oxidative stress [19].

Saponins, by virtue of their multiple apoptotic actions on cancer cells, may provide a new line of anticancer agents. They are also effective against drug-resistant cancer cells [17]. To date, over hundreds of saponins have been described. However, given the diverse distribution of saponins, it can be conceived that a lot of novel anticancer saponins remain unexploited. A variety of techniques can be used to determine and estimate the presence of such phytochemical compounds, including saponins [20]. Various chromatography methods like High Pressure Liquid Chromatography (HPLC) and Thin layer Chromatography (TLC) are commonly used. Hence the aim of current investigation is to isolate active saponins from *M. oleifera* pods.

MATERIALS AND METHODS

Chemicals

All chemicals used in the study were of analytical reagent grade and of highest quality available and were purchased from reliable firms and institutes

Procurement of experimental plant

The experimental plant *Moringa oleifera* was collected from Krishi Vigyan Kendra, Banasthali University, Banasthali, India, in the month of October 2009. The plant material was taxonomically identified by Botanist of Krishi Vigyan Kendra, Banasthali, Tonk district.

Successive extraction of *M. oleifera* pods

Successive extraction of plant material was performed using solvents (non-polar to polar) that were pet ether, benzene, chloroform, ethyl acetate and ethanol for 16 h in soxhlet apparatus. The extracts were than concentrated on a rotary evaporator below 50 ºC and were stored in air-tight containers in cold room for further studies.

Chromatographic purification and isolation: Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was carried out to isolate the principle components that were present in most effective extracts of plant. By phytochemical screening of successive extracts it was

confirmed that maximum saponin was present in benzene extract, hence TLC study was carried out for benzene extract. The solvent system was prepared and a TLC study was carried out to select the solvent system capable of showing better resolution. The solvent system for isolation of saponin from benzene extract used was: Chloroform: methanol: H_2O (7:3:1).

Phytochemical analysis of isolated compounds by TLC

Phytochemical screening of isolated compounds by TLC of benzene extract was carried out according to the methods described by Harborne [21], Trease and Evans [22] and Sofowara [23].

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (Waters C 18 column, USA) was performed for sample isolated by TLC. Sample was dissolved in HPLC grade methanol in concentration of about 1-10µg/ml and 20µl of the solution was injected in the column RP-C18 and analyzed by PDA detector. The wavelength range was 250 - 500nm. The mobile phase components acetonitrile: water was used in a gradient form, which varied with change in time. The sampling rate was kept 2 (points/sec), total flow rate was kept 0.70 ml/min, filter time constant was 1.0000 sec and the software installed was Empower 2 software build 2154 SPs. Service pack H DB ID: 908711544.

Compound Characterization

As relatively large molecular weight natural products, saponins require a variety of IR and ^1H NMR in order to characterize an unknown saponin. As the amount of saponin isolated is often small, non-destructive methods such as IR and NMR was preferred.

FTIR: (Fourier transform Infra red) spectroscopy

FTIR (Model - Varian 3600; Range: 12000-100 cm^{-1}) was obtained for Successive benzene extract and for isolated compound. Sample (1-2mg) was crushed with KBr (3-4mg) and pellet was formed with the help of mechanical pressure formed pellet was observed at the different coming wavelengths in FTIR instrument.

Nuclear Magnetic Resonance (^1H NMR)

Nuclear Magnetic Resonance (DRX-300Mega Hz Bruker, Switzerland) was obtained for the isolated compound. Sample was dissolved in respective dutrirated solvents ($CDCl_3$) and about 600µl was poured in NMR tube and observed on the applied magnetic field.

RESULTS

Chromatographic purification: Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) of all Successive extracts of *Moringa oleifera* pods obtained by Successive extraction methods was carried out to confirm its nature by analyzing TLC chromatograms and to isolate active saponin ingredients from the extracts.

TLC of benzene extract of *Moringa oleifera* pods revealed the presence of 8 compounds (corresponding to 8 spots) having an R_f values of 0.30, 0.47, 0.62, 0.75 0.87, 0.90, 0.95 and 0.98 respectively when a solvent phase of chloroform: methanol: H_2O (7:3:1) was used (table 1). Compounds having R_f of 0.90 and 0.87 were most prominent and showed clear spots (green spots).

From the above results, it can be seen that compounds having same RF values are of same nature. The R_{fs} of these compounds are 0.90 (IS_1), 0.87 (IS_2), 0.75 (IS_3), 0.47 (IS_4) and 0.30 (IS_5) respectively. Further more from all these isolated saponins (IS_1-IS_5). Benzene extract of *Moringa oleifera* pods showed most prominent spots having R_f of IS_1 and IS_2. Hence, this particular extract was selected for further identification and purification which includes collection of these two spots (IS_1 and IS_2) in large amounts by TLC. For collecting IS_1 and IS_2 in large quantities, the spots were scratched from silica plates, placed in centrifuge tubes with respective solvent (benzene). They are then centrifuged at 4 ºC for 15 min (15000 rpm). The supernatant was discarded as these compounds were absorbed by silica. This was then centrifuged taking methanol as solvent. The supernatant was then vacuum dried to obtain pure IS_1 and IS_2. Percentage yield of compounds isolated from benzene extract of *Moringa oleifera* pods is depicted in table 2.

Table 1: R_f values of compounds isolated from benzene extract of *Moringa oleifera* pods.

S. No.	Extract	Solvent phase	Solvent run (cm)	Peaks obtained (cm)	Rf values	Colors of peaks
1	**Benzene**	Chloroform:methanol: H_2O (7:3:1)	6.3	(1) 1.9	0.30	yellow
				(2) 2.9	0.47	yellow
				(3) 3.9	0.62	brown
				(4) 4.7	0.75	brown
				(5) 5.5	0.87	green
				(6) 5.7	0.90	green
				(7) 6.0	0.95	green
				(8) 6.2	0.98	green

Table 2: Percentage yield of compounds isolated from 50g benzene extract of *Moringa oleifera* pods.

Isolated compounds	Rf Value	Yield of isolated compounds (g)	% yield of isolated compounds
IS_1	0.90	0.653	1.3
IS_2	0.87	0.487	0.98

Phytochemical screening of IS_1 and IS_2

The results of various qualitative tests performed in the laboratory for analysis of phytochemicals in compound (saponin) isolated from benzene extract of *Moringa oleifera* pods (IS_1 and IS_2) are outlined in table 3. The phytochemical screening of IS_1 and IS_2 confirmed the presence of saponins and negative for other phytochemicals.

Table 3: Analysis of phytochemicals in compound (saponin) isolated from benzene extract of *Moringa oleifera* pods.

Name of extracts	Name of test			
	Saponin	Steroids	Terpenoids	Cardiac glycosides
IS_1	+++	-	-	-
IS_2	+++	-	-	-

Abbreviations: IS_1-IS_2: Compounds (saponins) isolated from benzene extract

High Performance Liquid Chromatography (HPLC)

HPLC of isolated compound from benzene extract obtained by Successive extraction methods was carried out to confirm its nature by analyzing HPLC chromatograms. From TLC analysis it has been found that benzene extract contained maximum saponin content as proved by the spot analysis. Hence we have chosen spot no 1 i.e. IS_1 (R_f 0.90) nomenclatured as SM (saponin from Moringa pods) out of all the spots (8) isolated from benzene extract because this spot contain maximum saponin content as proved by phytochemical screening and the yield of the compound is more than IS_2 (table 2).

The HPLC profile of Successive benzene extract of *Moringa oleifera* pods along with its isolated saponin SM was detected at a wavelength range of 200-400nm. The sharpness of peaks, its retention time (Rt min), height and percent area were recorded as shown in fig 1 and 2.

The HPLC chromatogram of benzene extract has shown 12 peaks (fig 1). However, only 4 peaks were prominent with significant height and percent area (> 10%). One of the most prominent peak with 17.07 percent area and 61995 height is observed at the retention time 14.981 (Rt min), which is somewhat similar to that observed in case of isolated compound SM (15.201 Rt min). The other prominent peaks were recorded with retention time 3.215, 10.020 and 12.780 (Rt min) respectively.

However in the HPLC chromatogram of SM, only one prominent peak was visible with 70.11 % percent area and 30259 height, whose Rt was found to be 15.201 (min). In the chromatogram of SM apart from this peak, few inconspicuous peaks were also detected having percent area >10 %, which may be attributed to the presence of certain impurities in very small concentration along with isolated compound (fig 2)

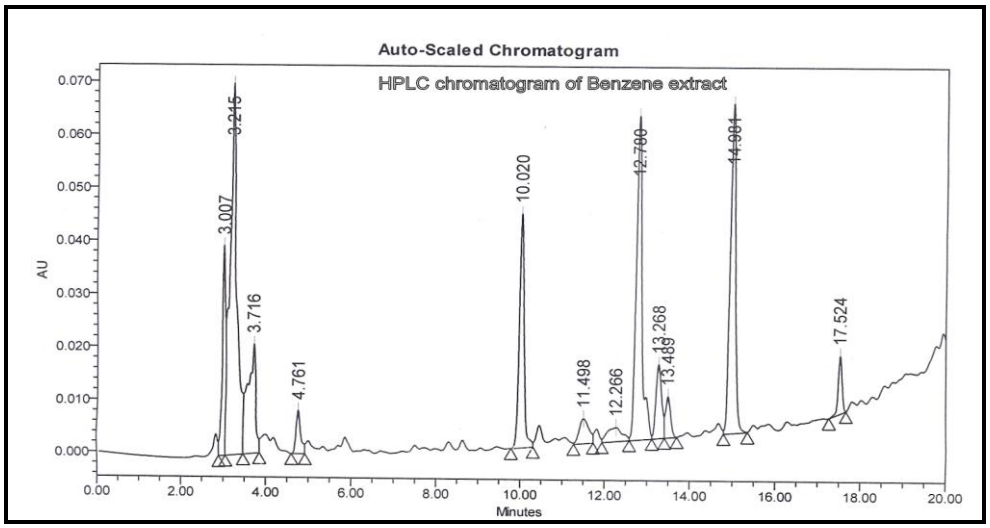

Fig. 1: HPLC chromatogram of Successive benzene extract of *M. oleifera* pods

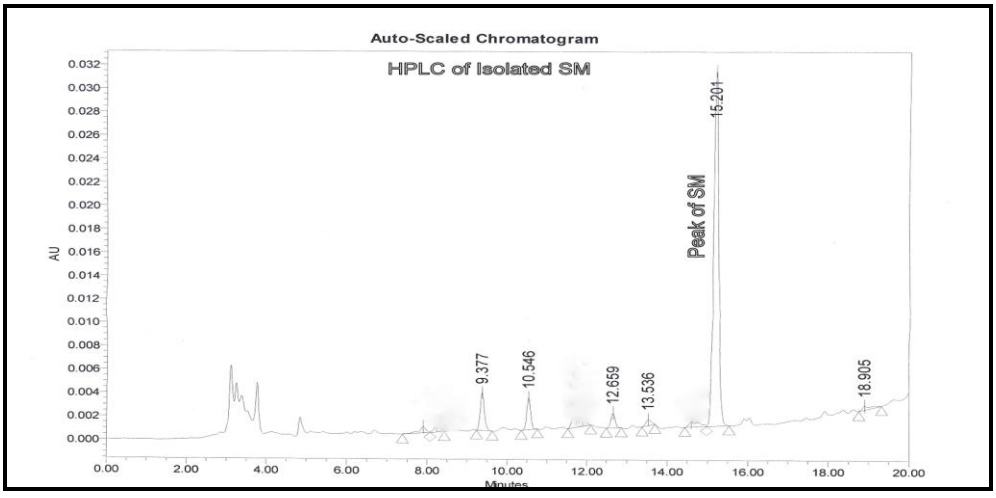

Fig. 2: HPLC chromatogram of isolated saponin (SM) of *M. oleifera* pods

Characterization of compound IS_1 (SM) by spectral studies

Infra red Spectophotometry (IR)

Data of IR spectrum (KBr, cm^{-1}) exhibited absorption in the range from 3722.3 cm^{-1} to 770.0 cm^{-1}(fig 3). IR spectrum exhibited a long and sharp peak in the range of 3722-3598.9 cm^{-1} for hydroxyl group (-OH) without involvement in hydrogen bonding. The IR spectrum exhibited a broad peak in the range of 3400 cm^{-1} -2400 cm^{-1} for acidic group (3233.2 cm^{-1} and 2363.4 cm^{-1} for carboxylic acids) and 3140.2 cm^{-1} which clearly verifies the presence of alkynes.

A sharp peak at 1645.8 cm^{-1} indicated the presence of (C=C) group in the extracted compound. Involvement of this group in this compound is geometrically cis, which is inferred by one very sharp peak at 770.0 cm^{-1}. The sharp peaks in the range of 1300 cm^{-1} -1000 cm^{-1} at 1220.2 cm^{-1} and 1112.0 cm^{-1} indicated the presence of –C=O (esters) and >C-O (ethers) groups in the compound. Further the presence of a peak at 1017.4 cm^{-1} is clear evidence for the presence of another ester group (C=O-CH_3) in the isolated compound. In IR spectrum, aliphatic C-H stretching was observed at 2963 cm^{-1}. Thus, IR spectrum showing peaks around 1220-1017 cm^{-1} are due to presence of O-CH_3 group.

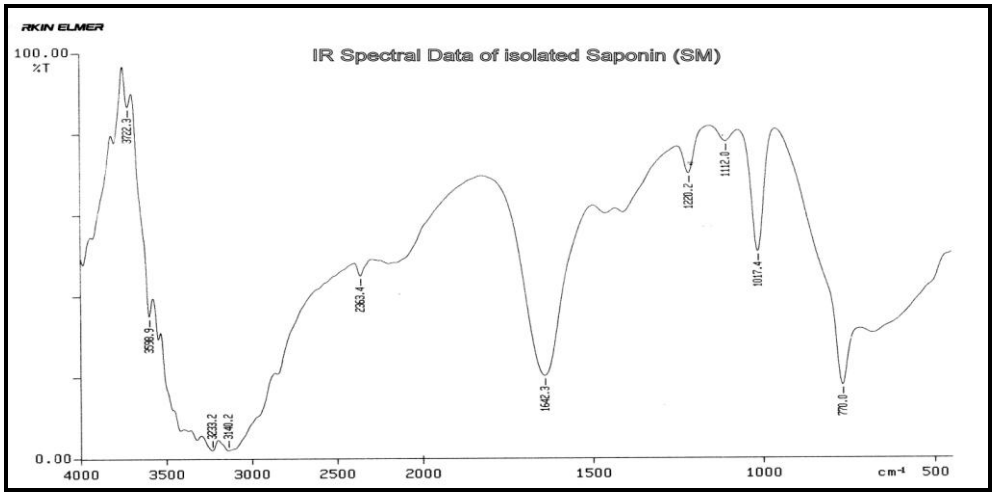

Fig. 3: IR spectral data of isolated saponin (SM)

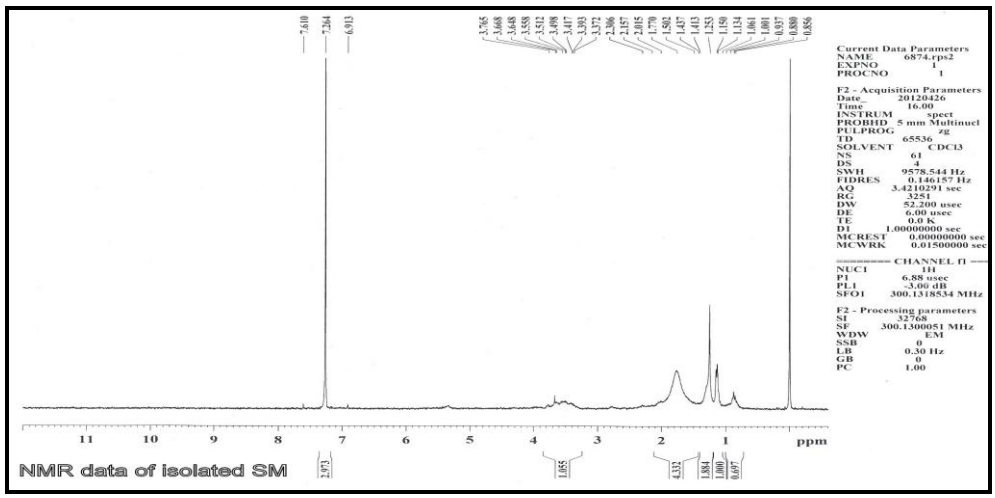

Fig. 4: ^{1}H NMR spectral data of isolated saponin (SM)

Nuclear Magnetic Resonance Spectophotometry (NMR)

^{1}H NMR spectrum of isolated compound revealed a one strong solvent ($CDCl_3$) peak at 7.264 ppm . PMR spectrum of this compound gives a peak around δ 3.65 ppm due to the presence of –OH group and methoxy group. In ^{1}H NMR spectrum a less intensive peak appears at 5.3 ppm which is due to (C=C) group and this result agree with IR spectral data of this isolated compound (fig 4).

DISCUSSION

In recent years, although technology and medicine have developed extensively due to decrease in natural richness and other drawbacks, some countries have made it obligatory to use natural products for many goals [24], India is one amongst them. For this reason we have chosen an important medicinal plant *Moringa oleifera*, which is a table food with incredible medicinal properties. In the above studies, the saponins were extracted from *Moringa oleifera* pods by thin layer chromatography and HPLC. FTIR and NMR were carried out to investigate unknown saponin present in plant extract.

A variety of techniques can be used to determine and estimate the presence of such phytochemical compounds, including saponins. Chromatography techniques are the most useful and popular tools used for this purpose. Chromatography is an analytical technique dealing with the separation of closely related compounds from a mixture [20]. Various chromatography methods like Thin layer chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) are commonly used.

Thin layer Chromatography in one and two-dimensional (1/2D) modes is a powerful technique, which has been used successfully in the separation, and determination of a large number of saponins in plant extracts [25]. Most frequently, silica gel plates are used and developing system consist of chloroform-methanol-water or chloroform-glacial acetic acid-methanol-water mixture for saponins [25]. This method enabled determination of large number of samples and did not require any tedious clean-up steps prior to analysis and is highly recommended in pharmaceutical quality control practice.

High-performance liquid chromatography is the most powerful and the most frequently used technique for saponin determination due to the fact that it can deal effectively with non-volatile, highly polar compounds. It has been used extensively for determination of both aglycones and intact saponins. The separations are performed usually on normal (silica gel) and reversed-phase (C_8, C_{18}) columns, of which C_{18} has been definitely preferred. The detection at lower wavelengths, however, limits the selection of solvents and the gradients that can be used. Since acetonitrile gives much lower absorption at lower wavelengths than methanol, the selection of acetonitrile–water gradients is the mode of choice.

In the above studies, saponin was extracted from *Moringa oleifera* dried pods and then separated and characterized by thin layer chromatography, HPLC, FTIR and 1H NMR. Elango and Jadhav [26]performed analysis on *Moringa oleifera* saponins and reported the presence of 13 saponin components at 254nm with R_f values in the range of 0.01-0.87. Our results are in accordance with these

results. Jung *et al* [27]had performed isolation of saponins from *Pleurospermum kamtschaticum* and showed their inhibitory effect on nitric oxide, prostaglandin E2 and tumor necrosis factor in TLC mobile phase of chloroform-methanol-water (7:3:1, lower phase). The same solvent system is used in the present study to isolate saponin from *Moringa oleifera* pods.

In conclusion, we can state that the present study revealed the presence of saponins in *Moringa oleifera* dried pods which were confirmed by various characterization studies. Since, saponins contains a wide range of medicine and pharmacological properties, they can be exploited more in future for further studies.

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