International Journal of Research and Development in Pharmacy and Life Sciences
Available online at http//www.ijrdpl.com
June - July, 2016, Vol. 5, No.4, pp 2255-2263
ISSN (P): 2393-932X, ISSN (E): 2278-0238

Research Article

ISOLATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS OF *ASPARAGUS RACEMOSUS* AS MARKERS

Sajjad Pasha[1], Salma Khanam[2], Zeeshan Afsar[3]*

1. Department of Pharmacognosy, Farooqia College of Pharmacy, Mysore.
2. Department of Pharmacognosy, Al-Ameen College of Pharmacy, Bangalore.
3. Department of Pharmacognosy, Farooqia College of Pharmacy, Mysore.

***Corresponding author's Email:** zeeshanafsar@gmail.com

(Received: April 24, 2015; **Accepted:** May 25, 2016**)**

ABSTRACT

In the present study an attempt was made to isolate and characterize the phytoconstituents from *Asparagus racemosus* and utilize them as marker compounds. Powdered root of the drug was extracted and the butanolic extract was used for the isolation process. Isolation of the phytoconstituents was done by Column chromatography using gradient elution with different stationary phases and mobile phases of varying polarity. Two compounds were isolated and characterized by NMR spectroscopy. A HPLC method was developed to estimate the isolated two compounds. The simplicity of isolation and HPLC analysis of the compounds may be utilized as markers in standardization of preparations containing *Asparagus racemosus*.
Keywords: *Asparagus racemosus*, Marker Compounds, Column Chromatography, TLC, Shatavarins, MPLC, HPLC, ^{1}H NMR, ^{13}C NMR.

INTRODUCTION

India has a rich medicinal plant flora of some 2500 species of which at least 150 species are used commercially on a fairly large scale. Asparagus racemosus is also one of the commonly used medicinally important herbs. Asparagus racemosus (Liliaceae) commonly called as Shatavari is an herb growing widely throughout India. Asparagus racemosus is a perenial climber with fasciled finger-like clustered tuberous roots producing cupious amount of small spinescent pine-like leaves. It bears tiny white flower in small spike forming sub globose berries containing black seeds during autumn season[1].

Shatavari is used traditionally for treatment of many diseases. Tubers, leaves and fruits of Shatavari are used in gonorrhea, piles, diabetes, rheumatism, cough, diarrhoea, dysentry, gastric trouble and headache, and also for increasing lactation. In ayurveda and siddha the plant is used in formulations as nutritive tonic, anabolic & galactogogue. The demand for roots of this plant has considerably increased in the herbal industries. Many Ayurvedic formulations like 'Phalagrita', 'Narayana Taila' and 'Root powder' etc are known to contain Shatavari extract as one of the ingredients[2].

Shatavari and its extract have been scientifically investigated for different properties like anti-oxidant[3], anti-diarrrhoeal[4], anti-oxitocic[5], anti-tumor[6], immuno-modulation[7], anti-inflammatory[8], anti-tussive[9], galactogouge[10]. It also has effect on reproductive[11], cardiovascular[12], and respiratory systems[13].

A number of phytoconstituents belonging to different classes have been isolated and characterized from Asparagus racemosus using aqueous & hydro-alcoholic solvents. The roots contain four steroidal saponins like Shatavarin I - IV, isoflavonoids, asparagamine A (a polycyclic alkaloid), racemofuran, 9, 10-dihydrophenanthrene derivative racemosol. Polysaccharide and mucilage. Diosgenin and quercitin –3-glucuronide are present in the leaves. Sitosterol, stigmasterol, sarasapogenin, sitosterol beta-D-glucoside, two spirostanolic and furostanolic saponins are present in the fruits. Shatavarin I and IV are the major steroidal saponin glycosides[14-17].

Markers constitute chemically defined constituents, which can serve as a powerful tool for standardization of finished form of herbal preparations. HPTLC analysis of selected sapogenins in Asparagus racemosus has been reported[18]. However no other compounds of Shatavari have been reported as markers. Hence there is a need to isolate, purify and quantify the phytoconstituents as marker compounds from Asparagus racemosus in order to standardize its extracts and formulations.

In the present study an attempt was made to isolate, characterize and quantify the chemical constituents from the extracts of roots of Asparagus racemosus using simple chromatographic techniques, HPLC profiling was also done in order to characterize them as markers.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The dried plant roots of Asparagus racemosus were obtained from Natural Remedies PVT LTD, Bangalore and authenticated by NISCAIR, Delhi. (Voucher no. NISCAIR/RHM/2000/F-3/237); the roots were then sun dried, powdered and stored in airtight containers for further use.

2.2 Extraction of Plant material

The roots of Asparagus racemosus were subjected for extraction using static extractor. The powdered root was refluxed with fresh methanol (1:3 drug to solvent ratio) for one and half hour at temperature not exceeding 650 C and the extract was strained through muslin cloth to obtain the 1st extract. The marc was again subjected for second time extraction and the extract obtained was strained through muslin cloth to yield 2nd extract. The same procedure was followed for third wash to obtain the 3rd extract. The 1st, 2nd, 3rd extracts were combined together and concentrated to obtain a thick paste.

2.3 Fractionation of the extract

The methanolic extract was fractionated with butanol and water to get saponin rich fraction, further the butanolic extract was used for isolation by column chromatography using gradient elution method.

2.4 Optimization of TLC method for identification of the constituents

Different solvents combinations were tried for developing a TLC system in order to identify the constituents in the extract and those showing maximum separation were selected as the mobile phase. The following solvents exhibited maximum separation, hence were used for the development of the TLC system.

1. Chloroform : methanol : water, (64 : 50 : 5)
2. Chloroform : GL. acetic acid : methanol : water, (64 : 32 : 12 : 4)
3. Chloroform : methanol : water, (7 : 2.6 : 0.4)
4. Chloroform : methanol : water, (7 : 3 : 0.5)
5. Ethyl acetate: methanol: water, (75: 15: 10).

2.5 Isolation of phytoconstituents by column chromatography

The butanolic extract was used for isolation using column chromatography by gradient elution using solvents of increasing polarity. A total of eighty seven fractions were collected from twelve different columns. The solubility profile and melting point of the isolated compounds were determined.

2.6 Determination of melting point and solubility profiles and of the isolated compounds

The isolated compounds were analyzed for their melting point using capillary tube and their solubilitiy in solvents such as methanol, ethanol, demineralized water, dimethyl sulfoxide and pyridine in the concentration of 2mg/ml was determined in order to facilitate their further analysis by HPLC and characterization.

2.7 HPLC analysis of the isolated compounds

The isolated compounds were then subjected for the HPLC analysis for identification and purity assessment. A HPLC

method was developed and standardized with the following parameters.

Column	: Chromosil column
Mobile Phase	: 50% Acetonitrile in HPLC water.
Elution	: Gradient
Flow rate	: 1 ml/ min & 1.5ml/min
Detector	: ELSD
Injection volume	: 100µl
Instrument	: Shimadzu

2.8 Characterization of the isolated compounds:

The isolated compounds were characterized using ^{1}H NMR and ^{13}C NMR at the Indian Institute of Science (IISC), Bangalore.

2.9 Quantification of isolated compounds in the different parts of the same plant

The isolated compounds were used as markers for the estimation of extracts prepared from different parts of Shatavari by HPLC method.

3. RESULTS

3.1 Isolation of compounds using column chromatography

The butanolic extract was used for isolation using column chromatography by gradient elution using solvents of increasing polarity. Out of eighty seven fractions which were collected from twelve different columns, two pure compounds namely AR-SH-01(Compound-1) and AR-SH-04 (Compound-2) were isolated which were having percentage purity of 100% and 94.52% respectively. The TLC of these two compounds is shown in figures 1 and 2 respectively.

3.2 Melting point and Solubility profiles of the isolated compounds

The melting point of isolated compound-1(AR-SH-01) was found to be 274 - 278^{0}C while melting point of compound-2 (AR-SH-04) was found to be sharp at 180^{0}C. The solubility profiling revealed that these compounds were soluble in Methanol, DMSO, Ethanol and Pyridine but sparingly soluble in demineralized water. The physical characters showed that compound-1 was white colored fine powder and compound-2 was white colored crystalline powder. Both were having a pungent odor.

3.3 HPLC analysis of the isolated compounds

HPLC method for analyzing the isolated compounds was developed and standardized. Compound 1 and compound 2 were subjected to HPLC analysis for assessing their purity. The HPLC Chromatogram of the compound 1 showed single distinct peak with 100% purity. The retention time of this peak was 31.539 min. The HPLC chromatogram of compound 2 showed the presence of a distinct peak with 94.5286% purity along with two minor peaks having 1.4355% and 4.0359% purity respectively. The retention times of two minor peaks were 1.421 and 18.967 min. whereas the major peak showed retention time of 20.7 min. The chromatograms of these two compounds are shown in figures 3 and 4 respectively.

3.4 Characterization of Isolated compounds

The two isolated compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and are shown in Fig. 5-6 and Fig. 7-8 respectively.

3.5 Interpretation of compound-1:

From the ^{1}H and ^{13}C NMR spectra's, structure of compound-1 was interpreted and it was proposed to have a chemical name3-O-{[β-D-glucopyranosyl(1→2)][α-Lrhamnopyranosyl-(1→4)] -β-D-glucopyranosyl} - (25S) – 5 β - spirostan-3β-ol 4. The 1H and 13C NMR spectrum in DMSO-d6 revealed the presence of two quaternary methyl groups (δ 0.85 and 0.97 ppm) corresponding to the angular methyl group of a steroidal sapogenin as well as two tertiary methyl groups [δ 1.10 (J 7.1 Hz) and δ 1.19 (J 6.9 Hz)]. The presence of another tertiary methyl group at 1.67 ppm (d- J 6.2 Hz) and three anomeric protons signals at δ 4.87 (d, J 7.6 Hz), δ 5.48 (d, J 7.8), δ 5.78 (d, J 1.4 Hz) suggested the presence of three monosaccharides including one deoxyhexose. Based on this the structure was found to have molecular formula $C_{45}H_{74}O_{17}$.

3.6 Interpretation of compound-2:

From the ^{1}H and ^{13}C NMR spectra's, structure of compound-2 was interpreted and it was proposed to have a chemical name 3-O-{[β-D-glucopyranosyl(1→2)][α-Lrhamnopyranosyl - (1→4)] - β-D-glucopyranosyl}-26-O-(β-d-glucopyranosyl) (25S) - 5 β -furostan-3 β, 22α, 26triol 3. The ^{1}H and ^{13}C NMR spectrum in DMSO-d6 revealed the presence of two quaternary methyl groups (δ 0.86 and 0.94 ppm) corresponding to the angular methyl group of a steroidal

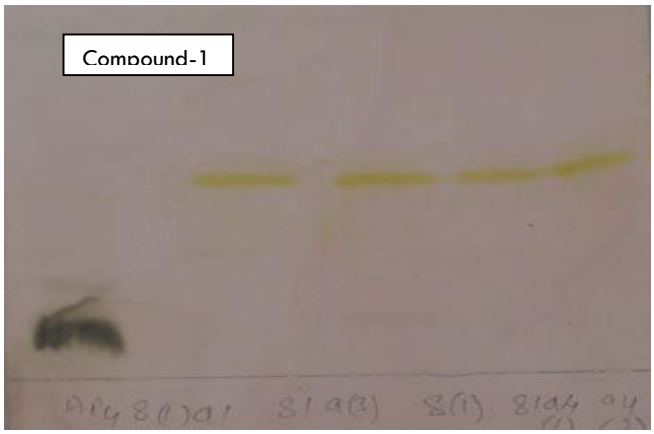


Figure 1: TLC chromatogram of fractions collected from column II showing distinct spots of compound-1

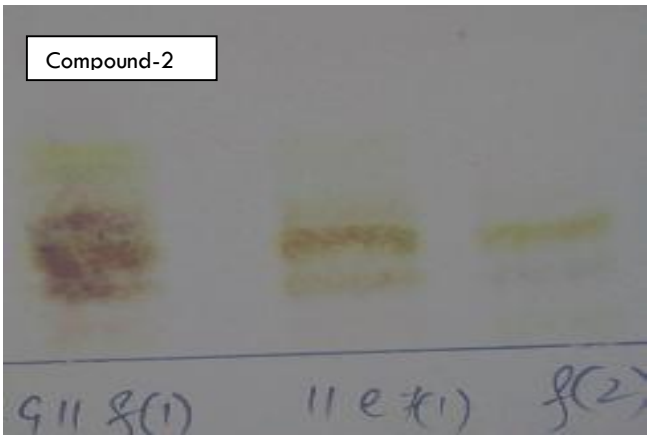


Figure 2: TLC chromatogram of fractions collected from column VII showing distinct spot of compound-2 (f2)

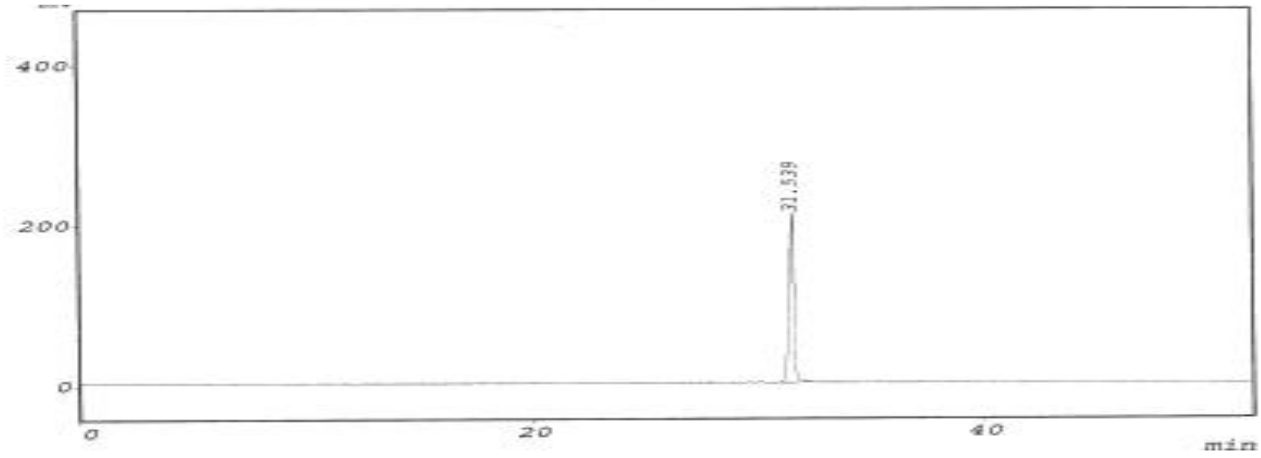
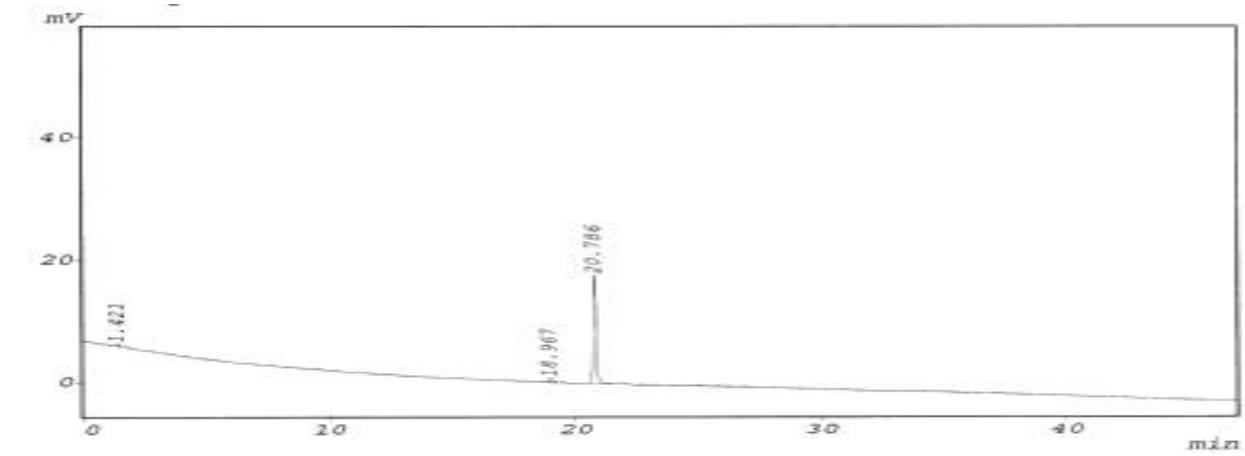


Figure 3: HPLC chromatogram of compound-1



*** Peak Report ***

NO	TIME	AREA	CONC
1	1.421	2223	1.4355
2	18.967	6249	4.0359
3	20.786	146367	94.5286
		154839	100.0000

Figure 4: HPLC chromatogram of compound-2

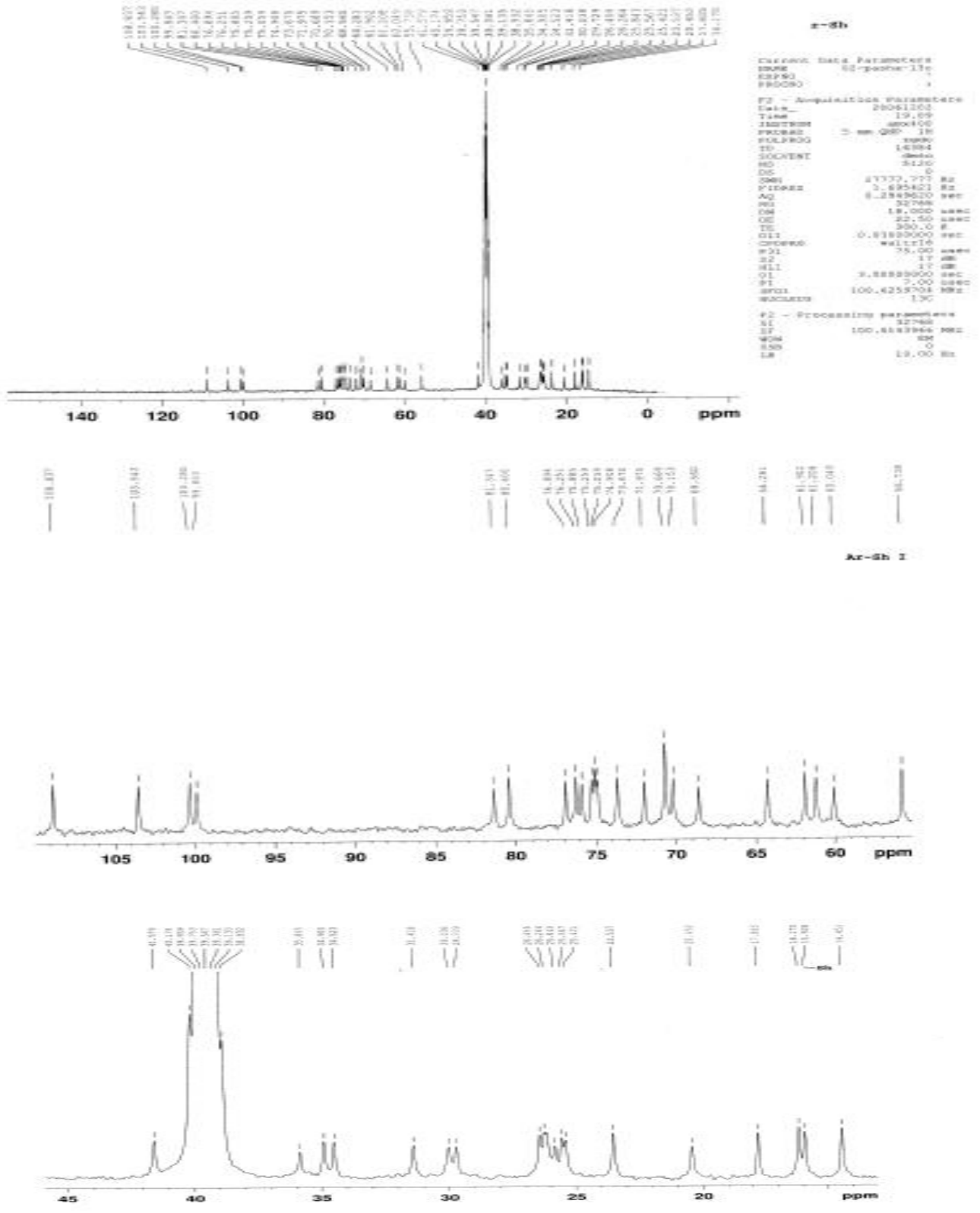


Figure: 5 ^{13}C NMR of compound-1

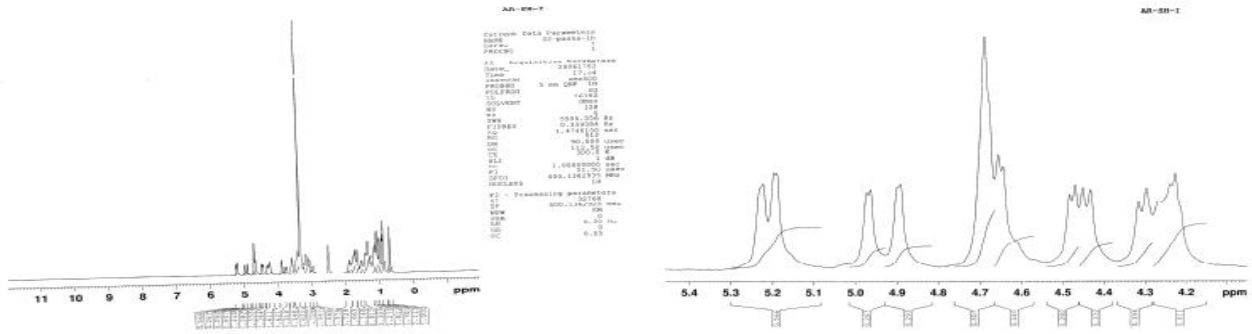


Figure: 6 ^{1}H NMR of Compound-1

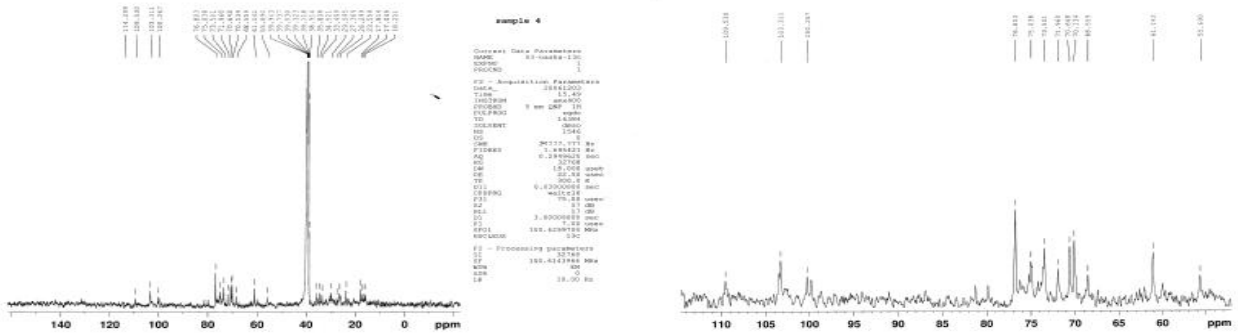


Figure: 7 ^{13}C NMR of compound-2

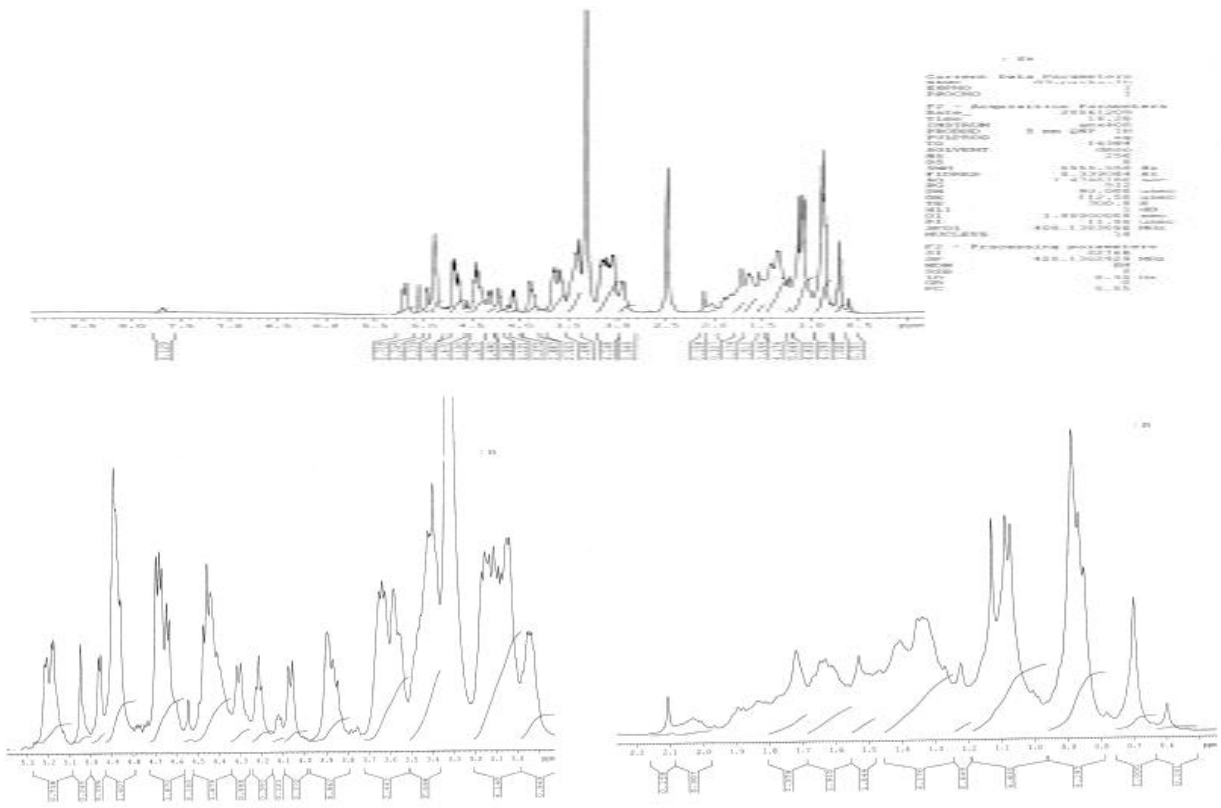


Figure: 8 ^{1}H NMR of compound-2

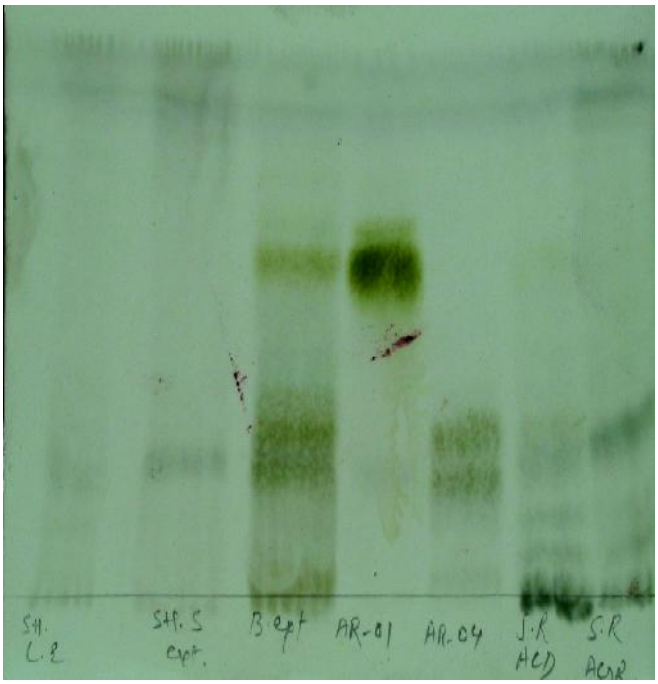
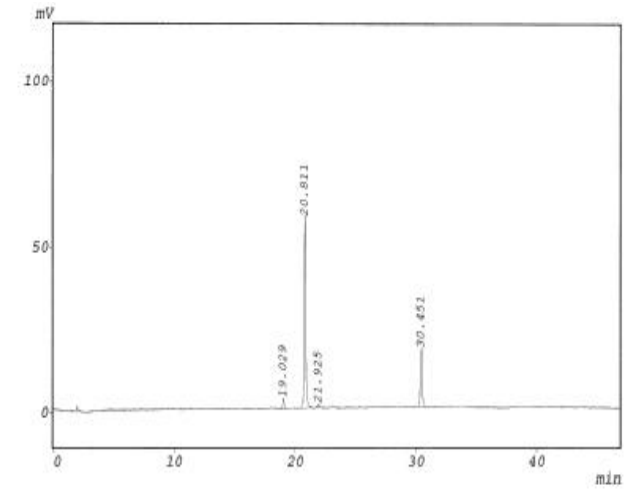


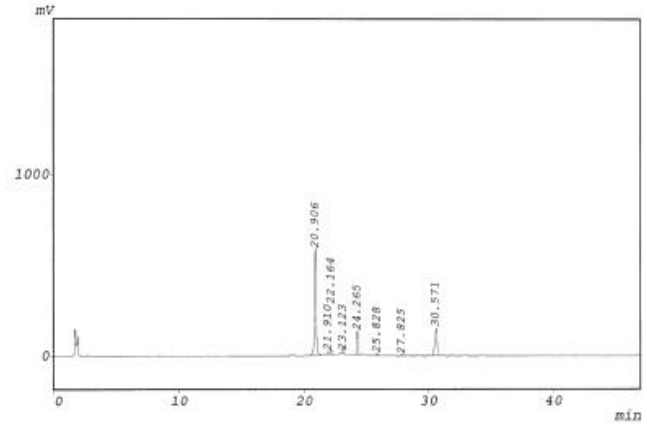
Figure no-9: TLC chromatogram of different extracts along with isolated compounds.
Solvent system (chloroform: methanol: water - 7:3:0.5), detection with Anisaldehyde reagent



** Peak Report ***

NO	TIME	AREA	CONC
1	19.029	21445	2.9959
2	20.811	507459	70.8928
3	21.925	7739	1.0811
4	30.451	179169	25.0302
		715812	100.0000

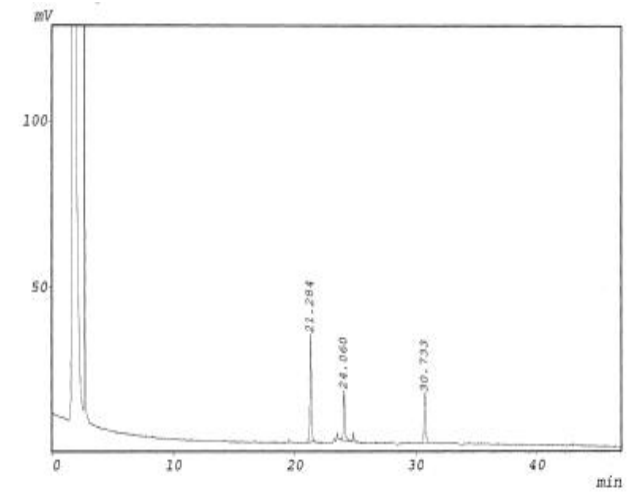
Figure no-10: HPLC chromatogram of the mixture of compound 1 and compound 2



** Peak Report ***

NO	TIME	AREA	CONC
1	20.906	5320008	60.7371
2	21.910	119191	1.3608
3	22.164	196299	2.2411
4	23.123	289326	3.3032
5	24.265	798629	9.1177
6	25.828	81390	0.9292
7	27.825	32497	0.3710
8	30.571	1921742	21.9400
		8759081	100.0000

Figure no-11: HPLC chromatogram of the butanolic extract showing the presence of markers compounds 1&2



** Peak Report ***

NO	TIME	AREA	CONC
1	21.284	260924	49.7605
2	24.060	116665	22.2492
3	30.733	146770	27.9903
		524359	100.0000

Figure no-12: HPLC chromatogram commercial root extract showing the presence of markers compounds 1&2

sapogenin as well as two tertiary methyl groups [δ 1.01 (J 6.8 Hz) and δ 1.131 (J 6.8 Hz)]. The presence of another tertiary methyl group at 1.67 ppm (d- J 6.3 Hz) and four anomeric protons signals at δ 4.85 (d, J 7.8 Hz), δ 5.46 (d, J 7.8), δ 5.92 (br s) and δ 4.81 (d, J 7.6 Hz) suggested the presence of four monosaccharides including one deoxyhexose. Based on this the structure was found to have molecular formula $C_{51}H_{86}O_{23}$.

3.7 Quantification of isolated compounds in the different parts of the same plant

Authenticated dried roots, stem and leaves of Asparagus racemosus were subjected to extraction and the extracts were subjected to TLC. The root extract and butanolic extract were found to contain compound 1 and compound 2 as shown in figure no-9. The HPLC of the mixture of compound 1 and compound 2 was carried out and the chromatogram is shown in Figure no-10. All the extracts were subjected to HPLC. The chromatograms are shown in Figure 11-12. Hence results suggested that the HPLC analysis of the extracts also indicated the presence of these two marker compounds.

4. DISCUSSION AND CONCLUSION

Shatavari is used in traditional system of medicine for treatment of many diseases. A number of studies on isolation of chemical constituents[22-25] and pharmacological activities[35-46] of Shatavari and its extracts have been reported. Shatavarins have been isolated as biologically active compounds. But there are scanty reports on estimation using HPLC[18]. Many industries are using this drug for preparing different formulations. Hence an attempt was made to isolate, purify and characterize the compounds which can be used as markers and can serve as a powerful tool for standardization of its extracts and formulations.

REFERENCES

1. Phytochemical investigation of selected medicinal plants of India. 1992. Cen Cou Res in Ayur and Sid. New Delh: pp. 43-46.
2. Tiwari L. 2005; New Findings Vindicate Efficacy of Shatavari. Traditional Medicine: pp.1-5.
3. Jayashree PK, Krutin KB, Thomas PA, Venkatachalan SR. 2000; Antioxidant properties of A. racemosus against damage induced by gamma-radiation in the rat liver mitochondria. J Ethnopharmacol. 71: pp.425-435.
4. Venkatesh N, Thyagraj V, Narayan S, Arul A. 2005; Antidiarrhoeal potential of A.racemosus wild root in laboratory animals. J Pharm Pharmaceu Sci.8(1): pp.39-45.
5. Michael T. 2002; Antioxitocic action of saponin isolated from A. racemosus on uterine muscle. www.phytomedicine.com
6. Yo Shao, Chee KC, Chi TH, Stephen AG, and Mou TH. 1996; Anti-tumor activity of the crude saponin obtained from A.racemosus. Can Lett. 104(1): pp.31-36.
7. Manish G, Sham Diwanay, Sunil G, Yojana S, Patwardhan B, and Patki P. 2004; Immunoadjuvant potential of A. racemosus aqueous extract in experimental system. J Ethnopharmacol. 9: pp.251-255.
8. Mandal SC, Maiti TK, Pal M, Saha BP. 1998; Evaluation of Anti-inflammatory activity of A. racemosus wild root extracts. Nat Prod Sci. 4: pp.230-233.
9. Mandal SC, Ashok Kumar CK, Mohan LS, Saha BP, Murugeshan S, Sinha S, and Lakshmi SM. 2000; Anti-tussive effect of A. racemosus root against sulfur dioxide induced cough in mice. Fitoterap. 71(6): pp.686-689.
10. Patel AB, and Kanikkar UK. 1969; A. racemosus wild-form Bordi, as a galactogogue in buffaloes. Ind Vet J. 48(9): pp.718-721.
11. Pandey SK, and Sahay A. 2001; Effect of A.racemosus on the liver of pregnant and non-pregnant rats. Ind Drugs. 38(3): pp.132-136.
12. Roy RN, Bhagwager S, Chavan SR, Dutta NK. 1971; Preliminary pharmacological studies on extracts of root of Asparagus racemosus wild. J Res Ind Med. 6: pp.132-138.
13. Bhagwager S, Dutta NK, Chavan SR, Roy RN. 1971; Effect on respiratory system with alcoholic extract of the roots of Asparagus racemosus wild. J Res Ind Med. 6: pp.132-138.
14. Khan AGR, and Subramanian SS. 1968; Isolation of phytosterol, hyperoside from the flowers. From the fruits and roots of Asparagus racemosus they isolated rutin and steroidal sapogenin. Cur Sci. No.10: pp.287-289.
15. Langde AB, Bose JL. 1970; Structure of Disaccharide from Asparagus racemosus (Shatamuli). Ind J Chem. 8: pp.588-589.
16. Ravikumar PR, Soman R, Chetty GI, and Sukh Dev. 1987; Chemistry of Ayurvedic crude drug: part VI-Shatavari-I: Structure of Shatavarin-IV. Ind J Chem. 26B: pp.1012-1017.
17. Jayashree J, Joshi J, and Sukh Dev. 1988; Chemistry of Ayurvedic crude drug: part VIII: Shatavari-2: Structure elucidation of bioactive Shatavarin-I and other glycosides. Ind J Chem. 27B: pp.12-16.
18. Satti N, Suri K, Amina, Rauf A, Prabhu, Dutt, Suri O, Musarat, Qazie. 2006; Evaluation of Asparagus racemosus on the basis of immunomodulating

sarasapogenin glycosides by HPTLC. J Liq Chromatog. 29(9): pp.219-227.
19. Kokate CK, Purohit AP. Pharmacognosy. Nirali Prakashan, Pune. 1996; 23rd edition: pp.1-5.
20. Hostettmann K, Marston A, Wolfender JL. Phytochemistry of plants used in traditional medicine. 1st edition: pp.254-260.
21. Kamboj VP. 2000; Herbal medicine. Cur Sci. 78: no.1.
22. Colegate SM, Molynevx RJ. 2000; Bioactive natural products (detection, isolation and structural elucidation). CRC press: pp.351-353.
23. Mukherjee PK. 2002; Quality control of Herbal drugs. Bus Hor. New Delhi. 1st edition: pp.19-102.
24. www.planetherbs.com/article/standardization%20edited%203.html
25. www.satveda.com/shatavari
26. Phytochemical investigation of certain medicinal plants used in Ayurveda. 1990. Cen Cou Res in Ayur and Sid. New Delhi: pp.257-258.
27. Subramanian SS. 1968; Chemical components of Asparagus racemosus. Cur Sci. 10: pp.287-288.
28. Kanitkar UK, Dange PS, Pendse GS. 1969; Pharmacognostic studies on Hithreto Unrecorded form of Asparagus racemosus. J Res Ind Med. 3: 2. pp.123-135.
29. Krishnamurthy R, Chandorkar MS, Kalzunkar BG, Pathak JM and Rajendra Gupta. 2004; Agrpnomic manipulations for improving the productivity of Shatavari. J Med and Arom pla. Sci. 26: pp.740-745.
30. Chhetri RB. 2006; Trends in ethnodomestication of some wild plants in Meghalaya, Northeast India. Ind J Trad Know. 5(3): pp.342-347.

