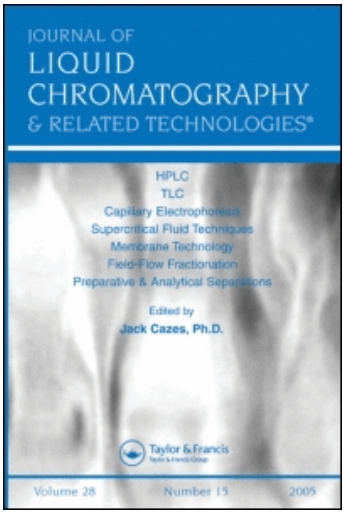

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Evaluation of *Asparagus racemosus* on the Basis of Immunomodulating Sarsasapogenin Glycosides by HPTLC*

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Abstract: The manuscript describes a HPTLC procedure for resolution of constituents, identification and quantification of selected sarsasapogenin glycosides in *Asparagus racemosus* plant extracts. Shatavarin-IV and immunoside, new sarsasapogenin glycosides were employed as external standards. The analytical procedure will prove handy for identification and quality control of the Rasayana drug on the basis of immunomodulating sarsasapogenin glycoside content.

Keywords: Sarsasapogenin glycosides, *Asparagus racemosus*, Immunomodulation, HPTLC

INTRODUCTION

The commercial Ayurvedic crude drug, Shatavari consists of dried decorticated roots of *Asparagus racemosus* Willd (liliaceae). Several therapeutic attributes have been mentioned in the classical ayurvedic literature for this drug. It is useful in cases of threatened abortion and as a galactogogue, it is also considered a powerful Rasayana drug capable of improving

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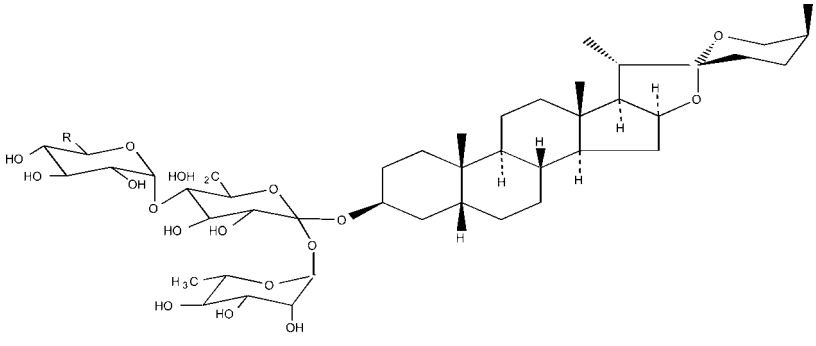
memory, intelligence, physical strength, and maintaining youthfulness and as a reputed drug for diseases caused by the morbidity of *Vata, Pita*, and *Kapha*,[1] to mention a few. The volatiles of *Asparagus racemosus* showed excellent inhibition of spore germination in some of the fungi.[2] Chemical constituents reported from the plant material include steroidal glycosides, a novel polycyclic cage type pyrrolizidine alkaloid, asparaginine,[3] and a 9,10-dihydrophenanthrene derivative.[4] The immuno-pharmacological evaluations of the aqueous extract of the plant showed optimum stimulatory response at (100 mg/kg p.o. × 5 days) in immunocompromised animals (Cyclophosphamide). The two sarsasapogenin glycosides viz., Shatavarin-IV and a new isolate, immunoside (Fig. 1) showed significant immunomodulation activity against specific T-dependent antigen in immunocompromised animals, in doses corresponding to their concentration in the aqueous extract, i.e., 0.15 mg/kg p.o. for Shatavarin-IV and 0.006 mg/kg p.o. for immunoside.

Non-standardized herbal extracts have not found acceptance in the global market and, therefore, there is a need for chemically standardized herbal extracts on the basis of isolated constituents, preferably bioactive ones. In view of the importance of the plant in traditional Indian medicine and its apparent potential in the global market as a positive health promoter, it was decided to carry out chemical profiling on the basis of bioactive glycosides. In the present communication, we report herein on immunomodulatory activity of the aqueous extract, as well as the pure constituents isolated from it, followed by the development of a sensitive selective HPTLC method for the quantization of Shatavarin-IV and immunoside in the extract.



Immunoside (R= -CH_3) **1**

Shatavarin IV (R= -CH_2OH) **2**

Figure 1. Molecular structures of immunoside and Shatavarin-IV.

EXPERIMENTAL

Plant Material

Asparagus racemosus Willd (Liliaceae) was supplied by Zandu Pharmaceutical Ltd. Bombay. A voucher specimen (RJM/0001) is deposited in the herbarium of Regional Research Laboratory, Jammu.

Extraction and Isolation of Markers

Air-dried plant material (4000 g) was ground to a coarse powder and extracted with deionised water at 98°C for 2 hrs. The extraction process was repeated thrice using total water (28 + 16 + 16 L, three extractions) in 1 : 15 ratio w/v with respect to the plant material. The pooled aqueous extract was centrifuged, clear supernatant was evaporated to dryness on a wiped film evaporator at 50 ± 5°C, residue (1.48 kg) was dissolved in de-ionised water (8.0 L), and the resulting solution was extracted with $CHCI_3$, EtOAc, and n-BuOH (6 × 2 L each), successively. $CHCl_3$ and EtOAc extracts were 0.8 and 1.2 gm, respectively, whereas n-BuOH extract residue (160 gm) was rich in quantity and chemical composition. n-BuOH extract (150 gm) was subjected to adsorption chromatography. It was dissolved in a minimum quantity of MeOH and adsorbed on SiO_2 gel, 100–200 mesh (200 g). The solvent was completely removed to get free flowing material. A glass column of 1.5″ dia. was packed with 600 gm SiO_2 gel, (100–200 mesh) in $CHC1_3$. The adsorbed extract was charged in the column. The column was eluted successively with $CHCI_3$ (8.0 L), 3% MeOH in $CHC1_3$ (8.0 L), 5% MeOH in $CHC1_3$ (8.0 L), 10% MeOH in $CHC1_3$ (8 L), 15% MeOH in $CHCl_3$ (8 L) and then with MeOH. The pooled 5 and 10% MeOH in $CHC1_3$ fractions (32.0 gm) was rechromatographed using a 100–200 mesh SiO_2 gel column (1 : 20 ratio) and eluted with $CHC1_3$: MeOH mixtures of increasing polarity. In all, 240 fractions of 200 mL each were collected, fractions 32–54 eluted with 5% MeOH in $CHC1_3$ were pooled on the basis of TLC pattern using EtOAc : MeOH : H_2O (75 : 13.5 : 10) as developing solvent, and the spots were visualized by spraying the plate with 1% cerricammonium sulfate followed by heating in an oven at 110° for 5 min. Immunoside (104 mg) was crystallized from MeOH as amorphous powder, Rf, 0.44; m.p. 275°C (MeOH); $[\alpha]_D^{21}$: −90.2° (c 0.50, Py); $C_{45}H_{74}0_{16}$. FAB-MS: $m/z = 893$ $[M + Na]^+$, ^1H and ^{13}C-NMR.[5] Similarly, from the fractions that were eluted in 15% MeOH in chloroform, another sarsasapogenin glycoside was isolated and identified as Shatavarin-IV on the bases of reported physical constants and spectral data.

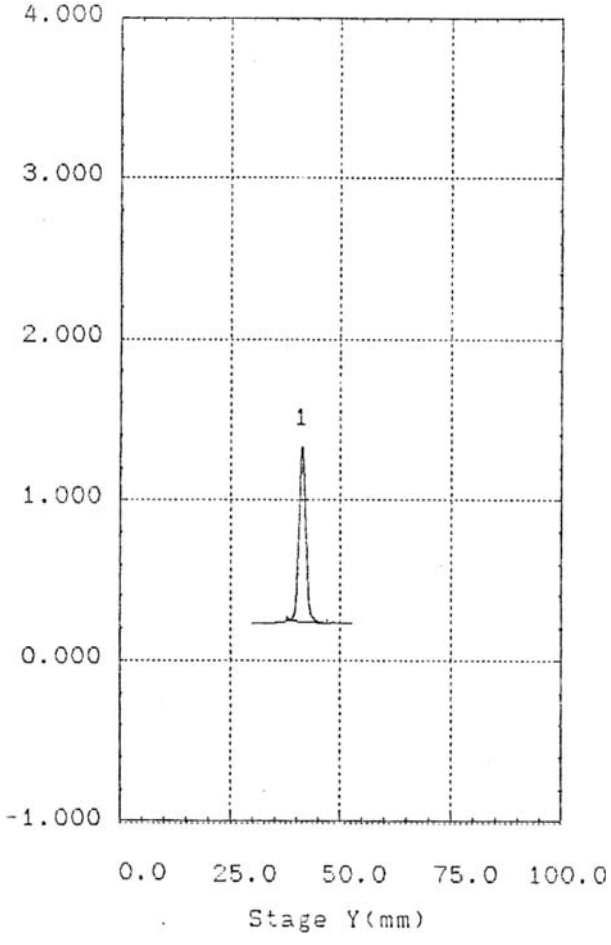


Figure 2. HPTLC profile of Shatavarin-IV.

HPTLC Analysis

In order to enrich the aqueous extract with the sarsasapogenin glycosides, i.e., Shatavarin-IV and immunoside, three batches (2 g each) of the aqueous extract were dissolved in 20 mL of dematerialized water and the resulting solutions were extracted with n-BuOH (3×10 mL). The n-BuOH extracts (0.219, 0.195, and 0.220 g) were dissolved in methanol (5 mg/mL, HPLC grade) in the case of Shatavarin-IV, and 15 mg/2 mL in the case of immunoside. A solution of each extract (10 μL) was applied on a precoated SiO_2 gel plate (Merck) of 0.25 mm thickness, with the help of CAMAG Linomat IV along with the standards, which were also dissolved in MeOH (HPLC grade,

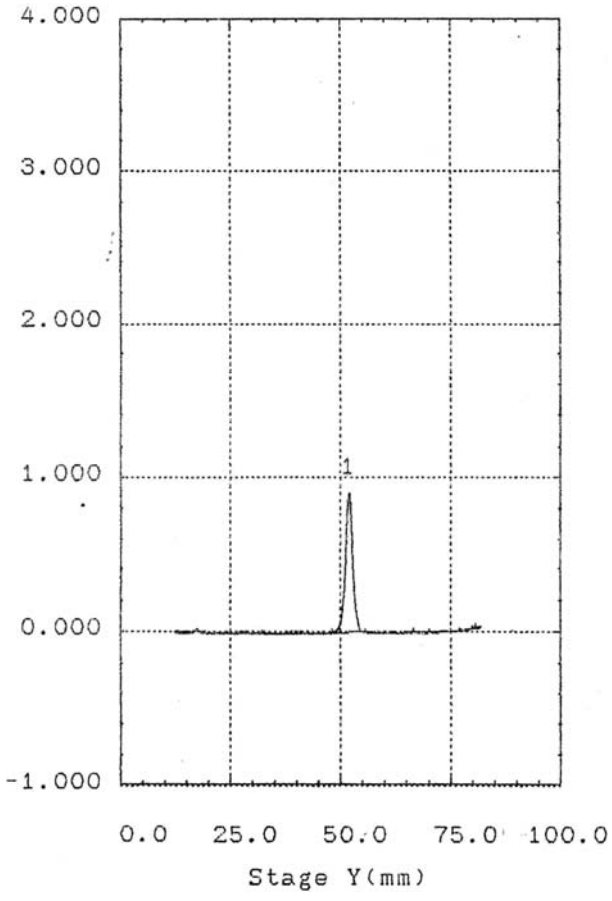


Figure 3. HPTLC profile of Immunoside.

1 mg/2 mL). The chromatoplates were developed with the mobile phase consisting of EtOAc : MeOH : H_2O :: 75 : 13.5 : 10. The spots were visualized by spraying the plates with ceric ammonium sulphate followed by heating at 10°C for 5′. The plates were scanned by a flying spot scanning densitometer (CS-9301PC, Shimadzu) at 450 nm by linear (scan) reflection (photo) mode. The concentration of the marker compounds, i.e., Shatavarin-IV (Fig. 2) and immunoside (Fig. 3) in the extract (Fig. 4) was determined by using the standard curves generated for the compounds (Table 1).

Bioevaluations

Strong immunorestorative activity of the aqueous extract has been accounted for by pure chemical entities, i.e., Shatavarin-IV and immunoside. The

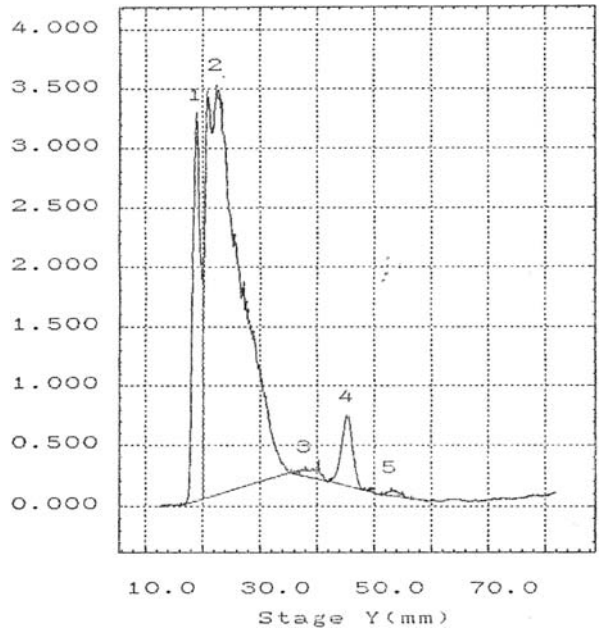


Figure 4. HPTLC profile of n-BuOH extract of *Asparagus racemosus*.

aqueous extract displayed maximum activity (humoral as well as CMI) at an optimum dose of 100 mg/kg body weight in mice by an oral route, while a similar degree of activity has been observed for Shatavarin-IV and immunoside at doses representing their concentrations in the extract (Table 2).

RESULTS AND DISCUSSION

Two standards, i.e., Shatavarin-IV and immunoside (1 mg/2 mL), from 1 to 5 ug were spotted on the precoated silica gel plates having 0.25 mm thickness along with the n-BuOH extracts solution; 5 mg/mL in Shatavarin-IV and 15 mg/mL in the immunoside in triplicate. The method described utilizes precoated silica gel plates as stationary phase and EtOAc : MeOH : H_20 :: 75 : 13.5 : 10 as mobile phase, which gives good separation for both the standards in the extracts. The method was found to be accurate, precise, and sensitive, and suitable for application to the routine quality control analysis and quantitative determination of Shatavarin-IV and immunoside in the n-BuOH, as well as aqueous extract of the plant and its pharmaceutical preparations.

The immunopharmacological evaluations of the aqueous extract of the plant showed optimum stimulatory response at (100 mg/kg p.o. × 5

Table 1. Quantitative analysis of Shatavarin-IV and immunoside by HPTLC

	Shatavarin-IV (%)			Immunoside (%)				
S.No.	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	X %	SD
n-BuOH extract	1.506	1.380	1.360	—	—	—	1.41	0.0079
Aqueous extract	0.165	0.135	0.150	—	—	—	0.15	0.015
n-BuOH extract	—	—	—	0.048	0.062	0.052	0.054	0.0072
Aq. extract	—	—	—	0.005	0.006	0.005	0.0053	0.00057

Table 2. Effect of Aq. extract of *A. racemosus*, immunoside & Shatavarin-IV on Humoral & Cell mediated immune response in immunecompromised mice

Treatment	Dose mg/kg p.o.	Antibody titre mean ± S.E.	Change compared to control (%)	DTH mean ± S.E.	Change compared to control (%)
Normal control	—	6.50 ± 0.50	—	0.78 ± 0.04	—
Cyclophosphamide CY	250	3.60 ± 0.21	45* ↓	0.68 ± 0.22	27* ↓
LEV	2.5	7.01 ± 0.22	89** ↑	1.80 ± 0.24	130** ↑
Aq. extract + CY	50	5.5 ± 0.26	48** ↑	1.6 ± 0.15	100** ↑
Aq. extract + CY	100	7.5 ± 0.26	102*** ↑	1.8 ± 0.37	125*** ↑
Aq. extract + CY	200	8.0 ± 0.26	116*** ↑	1.7 ± 0.33	112*** ↑
Immunoside + CY	0.0006	5.60 ± 0.33	56*** ↑	0.80 ± 0.14	48** ↑
	0.0125	6.41 ± 0.16	78*** ↑	0.91 ± 0.42	76** ↑
Shatavarin-IV + CY	0.0375	8.50 ± 0.34	131*** ↑	1.16 ± 0.17	48** ↑
	0.075	8.60 ± 0.21	134*** ↑	1.24 ± 0.17	76** ↑
	0.15	6.80 ± 0.16	85** ↑	1.26 ± 0.04	85** ↑
	0.30	5.70 ± 0.28	55** ↑	0.93 ± 0.05	36** ↑

Lev: Levamisole; DTH: Delayed type hypersensitivity.
Number of observations = 12.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
Treatment schedule.
0 day = Sensitization with 0.2 mL of 5×10^9 SRBC/mL i.p.
0–4 day = Drug treatment.
4 day = Challenge with 20 μL of 5×10^9 SRBC/mL into right hind foot pad for DTH reaction only.
5 day = Measurement of foot thickness/Haemagglutination antibody titre.

days) in immunocompromised animals (Cyclophosphamide). The two sarsasapogenin glycosides, viz., Shatavarin-IV and a new isolate, immunoside showed significant immunomodulation activity against a specific T-dependent antigen in immunocompromised animals, in doses corresponding to their concentration in the aqueous extract, i.e., 0.15 mg/kg p.o. for Shatavarin-IV and 0.006 mg/kg p.o. for immunoside.

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