PRELIMINARY PHYTOCHEMICAL AND *INVITRO* CYTOTOXIC ACTIVITY OF THE LEAVES OF *Asparagus racemosus* Willd., (Liliaceae)

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Abstract

Asparagus racemosus Willd.(Shatavari) is a well known plant in Ayurvedic systems of medicine. 'Shatavari' is a reputed classical drug and said to possess therapeutic properties as Rasayana drugs of Ayurveda. The present study is aimed at the development of phytochemical parameters and *invitro* cytotoxic activity of various extracts of the leaves of *Asparagus racemosus* plant. The plant material was successively extracted with solvents of increasing polarity namely n-Hexane, chloroform, ethyl acetate and ethanol in a soxhlet extractor. Preliminary phytochemical tests, fluorescence analysis, thin layer chromatography and high performance thin layer chromatographic studies were carried out for all the extracts. Chloroform, ethyl acetate and ethanol extracts were screened for *invitro* cytotoxic activity by MTT assay using EAC (Ehrlich's Ascites Carcinoma) cell line. The preliminary phytochemical analysis of various extracts of the leaves showed the presence of carbohydrates, tannins, phenolic compounds, sterols and flavonoids. The chloroform and ethyl acetate extracts showed significant cytotoxic activity when compared to ethanol extract against the above mentioned cancer cell line.

Key words: *Asparagus racemosus*, phytochemistry, *invitro*, MTT assay.

INTRODUCTION

Medicinal plants are the nature's gift to human being to make disease free healthy life. *Asparagus racemosus* Willd., (Liliaceae) is one such plant is selected for the present work. *Asparagus* is a Greek word for "stalk" or "shoot" [(1)]. In Ayurveda, this amazing herb is known as the "Queen of herbs", because it promotes love and devotion [(2)]. It is an important monocot medicinal plant which is distributed in tropical and subtropical forest and in central parts of India, *Asparagus racemosus* Willd., is a perennial shrub, with a tuberous root-stock, stems covered with recurved spines, linear leaves arranged in a tuft, white flowers which is sweet-scented appears in October [(3)]. It contains adventitious root system with tuberous roots. These tuberous roots after proper processing and drying are used as ayurveda medicine, with the name of Shatavari. Its leaves are reduced to form cladodes. Branches contain spines on them [(4)].

Asparagus racemosus is used to treat various diseases such as ulcer, dyspepsia and debility. In Indian medicine it is well known as an antispasmodic, aphrodisiac, demulcent, diuretic, galactogogue, nervine tonic and refrigerant. It is also used in the treatment of diarrhoea, rheumatism, diabetes, brain complaints, jaundice, urinary disorders, blood diseases, cough and bronchitis [(5-7)]. The aerial parts are used as spasmolytic, anticancer, antiarrhythmic, antibacterial and anti-fungal [(8)].

CHEMICAL CONSTITUENTS

A lot of chemical analysis has been carried out on the roots of *Asparagu racemosus*. The major reported constituents includes steroidal saponins, shatavarin I, shatavarin II, shatavarin IV alkaloids, proteins, starch and tannins. Isoflavones including 8-methoxy-5,6,4'-trihydroxyisoflavone 7-O- beta-D-glucopyranoside and Asparagamine, a polycyclic alkaloid were also isolated [(9)]. The leaves contains steroid-diosgenin along with rutin; a flavanoid- glucoside-quercetin-2-glucuronide, ferulic, caffeic and chlorogenic acides. The flowers contains quercetin, hyperoside and rutin; fruit contanins glycosides of quercetin, rutin, hyperoside, fully ripe fruit contanins cyanidine-3-galactoside and cyanidine-3-glucorhanoside. The shoots of the plant contains a bitter principle, 22-spirostan-3β-ol and 22-*iso*-spirostan-3β-ol, sarsapogenin, rhamnose, xylose and glucose [(10)].

MATERIALS AND METHODS

Collection and authentication of the plant

The fresh and healthy leaves of *Asparagus racemosus* Willd., were collected from Ariyaperumbakkam Village, Kanchipuram Dist., Tamil Nadu, India during the month of August 2014. The plant was identified and authenticated by Dr. Jayaraman, Director, Plant Anatomy and Research Centre, Thambaram (PARC/2014/2316). A voucher specimen has been reserved in the Department of Pharmacognosy, COP, MMC, Chennai.

Preparation of extracts [11]

Asparags racemosus leaves were properly washed with tap water and then rinsed with distilled water. The rinsed leaves were shade dried and powdered. The powdered material was subjected to solvent extraction with n-Hexane, chloroform, ethyl acetate and ethanol in Soxhlet apparatus at room temperature. Each time before extracting with next solvent, the powdered material was dried. The filtrate was concentrated by using rotary vacuum evaporator. The extract obtained with each solvent was weighed and the percentage was calculated in terms of dried weight of the plant material. The colour and consistency of the extracts were also noted. These extracts were used for phytochemical analysis.

Preliminary phytochemical screening [12-14]

A systematic and complete study of crude drugs includes a complete investigation of both primary and secondary metabolites derived from plant metabolism. Different qualitative tests were performed for establishing profiles of various extracts for their nature of chemical composition. The extracts obtained were subjected to chemical tests for identification of various phytoconstituents as per the methods given by Harborne.

Quantitative estimation [15-17]

Quantitative estimation of phytoconstituents like tannins, flavanoids and phenolic compounds were carried out for all the extracts.

Fluorescence [18-19]

Fluorescence characteristics of the powdered leaf and extracts were observed in daylight and UV light (254nm and 365nm).

Thin layer chromatography [20-22]

All the extracts were subjected to thin layer chromatographic studies using silica gel G as stationary phase and as Ethyl acetate: Chloroform: Ethanol (5:3:2) and Ethyl acetate: chloroform(6:4) as mobile phase. The extracts were spotted on the stationary phase using capillary tube. The sample spots were dried in the air and plates were developed and viewed under UV light at 254 and 365nm.

HPTLC [23, 24]

The HPTLC studies were carried out for ethyl acetate extract. It was performed on a 10 × 10cm pre-activated HPTLC silicagel 60 F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with nitrogen flow (CAMAG, Switzerland), 8mm from the bottom. Densitometric scanning was performed on CAMAG scanner II. The plates were developed using toluene:ethylacetate:methanol:formicacid (6:3:1:0.2). Chromatographic finger print was developed for detection of phytoconstituents present in the ethyl acetate extract and R_f values were tabulated.

INVITRO STUDIES [25-27]

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The cells (10000cell/well) were plated in 96 well flat bottom titre plates. After 24hrs of incubation at 37^0C in 5% CO_2 atmosphere, different concentration of chloroform, ethyl acetate and ethanol extracts (18.75, 37.50, 75, 150 & 300µl/ml) were added and incubated. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using GraphPad Prism software.

RESULTS AND DISCUSSION

Table 1. Percentage yield of successive extracts of leaves of *Asparagus racemosus* Willd.,

S.No	Extract	Method of Extraction	Physical nature	Colour	Percentage yield (%W/W)
1	n-Hexane	Continuous extraction using Soxhlet apparatus	Semi solid	Yellowish brown	3.5%
2	Chloroform		Semi solid	Green	4.89%
3	Ethyl acetate		Semi solid	Yellowish brown	3.9%
4	Ethanol		Semi solid	Brown	4.3%

QUALITATIVE PHYTOCHEMICAL ANALYSIS

Table .2. Preliminary phytochemical screening of powdered leaves and extracts

S.NO	Test	Powdered leaf	n-Hexane	Chloroform	Ethyl acetate	Ethanol
1	**Alkaloids**	-	-	-	-	-
2	**Carbohydrates**	+	-	-	-	+
3	**Flavanoids**	+	+	+	+	+
4	**Glycosides**	-	-	-	-	-
5	**Phenolic compounds**	+	-	+	+	+
6	**Proteins**	+	-	-	+	+
7	**Quinones**	-	-	-	-	-
8	**Saponins**	+	-	+	+	+
9	**Steroids**	+	-	+	-	+
10	**Tannins**	+	-	-	+	+
11	**Terpenoids**	+	-	-	+	-
12	**Volatile oils**	-	-	-	-	-

Note: + ve indicates positive result, whereas – ve indicates negative result

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

The phytoconstituents like tannins, flavanoids and phenolic compounds were estimated quantitatively and tabulated in Table 3

Tabel 3. Amount of Tannins, Flavanoid and Phenolic compounds present in extracts

S.No	EXTRACTS	TOTAL TANNIN CONTENT	TOTAL FLAVANOID CONTENT	TOTAL PHENOLIC CONTENT
1.	Chloroform	14.73±0.37µg/mg	4.67±0.56 µg/mg	40.31±0.49 µg/mg
2.	Ethyl acetate	16.58±0.45µg/mg	7.98 ±0.48µg/mg	60.85±0.29 µg/mg
3.	Ethanol	19.34±0.33µg/mg	34.44 ±0.62µg/mg	73.78±0.53 µg/mg

FLUORESCENCE ANALYSIS

The Fluorescence analysis of leaf powder and various extracts were carried out and given in Table 4

Table 4. Fluorescence characteristic of powdered leaves

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Light green	Green	Dark green
2.	Powder + water	Yellowish green	Yellowish brown	Green
3.	Powder + NaoH	Yellowish brown	Dark brown	Dark green
4.	Powder + HCl	Yellowish brown	Dark brown	Dark green
5.	Powder + Acetic acid	Dark brown	Brown	Yellowish green
6.	Powder + Alc.KOH	Brown	Brownish green	Green
7.	Powder + KOH	Light green	Brown	Dark green
8.	Powder + Sulphuric acid	Dark brown	Dark black	Dark green
9.	Powder + Nitric acid	Yellowish green	Light brown	Yellowish green
10.	Powder + Iodine	Yellowish green	Brown	Green
11.	Powder + $Fecl_3$	Brown	Dark brown	Green
12.	Powder +Ammonia	Light green	Greenish black	Dark green
13.	Ethanol	Greenish black	Brown	Green

Table 5 . Fluorescence analysis of various extracts

S.NO	Extracts	Day light	Short UV	Long UV
1	n-Hexane	Yellowish brown	Brown	Dark brown
2	Chloroform	Green	Yellowish brown	Dark green
3	Ethyl acetate	Yellowish brown	Brown	Dark black
4	Ethanol	Brown	Green	Dark green

THIN LAYER CHROMATOGRAPHY OF EXTRACTS

Table 6. Thin layer chromatography various extracts

S.No	Extracts	Solvent system	No. Of Spots	R_f value
1.	n-Hexane	Ethyl acetate: Chloroform:Ethanol (5:3:2)	3	0.48 0.51 0.53
2.	Chloroform	Ethyl acetate: Chloroform:Ethanol (5:3:2)	4	0.36 0.39 0.46 0.49
3.	Ethyl acetate	Ethyl acetate: chloroform(6:4)	4	0.45 0.50 0.52 0.53
4.	Ethanol	Ethyl acetate: chloroform(6:4)	3	0.29 0.31 0.32

HPTLC

High performance thin layer chromatography (HPTLC) finger printing was performed with the ethyl acetate extract of *Asparagus racemosus* Willd., The chromatographic conditions were carried as detailed in

material and method of this study. There were 9 peaks observed with different R_f values and different heights. Percentages of areas were also obtained from the chromatogram.

Table 7 HPTLC profile of ethyl acetate extracts

S.NO	Extract	Solvent system	Number of spots	R_f values
		Toluene:Ethylacetate:Methanol:Formicacid (6:3:1:0.2)	9	10, 22, 27, 32, 33, 38, 63, 70, 73

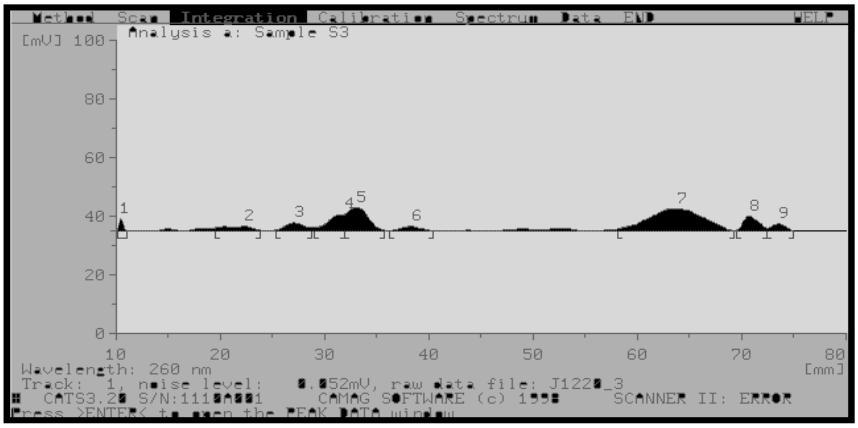

Fig HPTLC finger print for ethyl acetate extract of *Asparagus racemosus* Willd.,

DISCUSSION

In India most of the traditional knowledge on medical plants is in the oral form carried over generations to generations without any standard inventory. Necessary steps are needed for proper documentation, systematic regulation and widespread application. Since herbal medicines are prepared from materials of plant origin, they are prone to contamination, deterioration and variation in composition. Hence, before proceeding to clinical studies, scientists need to authenticate plants and also to detect their potency. A lot of analytical techniques have been developed for quality control of drug from plant origin.

Therefore it is very important to undertake phytochemical investigation along with biological screening to understand therapeutic dynamics of medicinal plant and also to develop quality parameter.

In this analysis, different polarity of phytoconstituents were sorted out from the coarsely powdered leaves of *Asparagus racemosus* Willd., by using solvents of increasing polarity like n-Hexane, chloroform, ethyl acetate and ethanol in soxhlet apparatus. Successive extractive values revealed the solubility and polarity particulars of the metabolites in the powder. Percentage yield of various extracts were as follows, n-hexane (3.5%w/w), chloroform (4.89w/w), ethyl acetate (3.9w/w) and ethanol (4.35%w/w).

Qualitative preliminary phytochemical analysis were performed with different chemical reagents to detect the phytoconstituent's nature and their presence in each extract and powder. The n-hexane extract showed the presence flavanoids. The chloroform extract showed the presence of flavanoids, phenolic compound, saponins and steroids. Ethyl acetate extract showed the presence of flavanoids, phenolic compounds, proteins, tannins and terpenoids. The ethanolic extract showed the presence of carbohydrates, steroids, flavanoids, phenols, proteins, saponnis and tannins.

The quantitative phytochemical analysis for chloroform, ethyl acetate and ethanol extracts were performed. The total phenolic content was found to be 40.31±0.49, 60.85±0.29 &73.78±0.53µg/ml%w/w. The total tannin content was found to be 14.73±0.37, 16.58±0.45&19.34±0.33µg/ml%w/w. The total flavanoid content was found to be 4.67±0.56, 7.98±0.48&36.44±0.62µg/ml%w/w.

Fluorescence analysis for leaf powder and extracts were carried which is an important qualitative diagnostic tool to detected the presence of chromophore in the drug under UV and day light. There was no characteristic florescence observed with either in the powder or in the extracts.

Thin layer chromatographic analysis of all the extracts were carried out using different solvent systems. The hexane extract showed 3 spots, where as 4 spots were found in chloroform extract. Ethyl acetate extract showed 4 spots and ethanol extract showed 3 spots. TLC was performed for the identification of different components in the extracts qualitatively. HPTLC was done for ethyl acetate extract and the finger print of the extract showed 9 spots with the R_f value of 0.10, 0.22, 0.27, 0.32, 0.33, 0.38, 0.63, 0.70 and 0.73. Since

secondary metabolites are responsible for the therapeutic activity, this study will be helpful in the selection of extract for pharmacological activity.

The *invitro* anticancer study for chloroform, ethyl acetate and ethanol extracts were carried out by MTT assay. The extracts were screened for its cytotoxicity against HeLa cell line at different concentration to determine the IC_{50} value. The percentage growth inhibition was found to be increased with the increasing concentration of test compound. The IC_{50} value of Chloroform, Ethyl acetate and Ethanol extracts on the HeLa cell line were found to be 62.587, 87.837, 297.9 µg/ml and R^2 values were 0.9993, 0.9999, 0.9953 respectively. The chloroform and ethyl acetate extract showed significant *invitro* anticancer activity against HeLa cell line were taken for further *invivo* anticancer studies.

CONCLUSION

The preliminary phytochemical analysis and *invitro* cytotoxic activity were studied in the present investigation. The leaf powder was extracted with different solvents and the extracts were phytochemically analysed. All the extracts were screened for *invitro* cytotoxic activity, chloroform and ethyl acetate extracts showed significant cytotoxic activity which may be due to the presence of one or more phytoconstituents present in the extracts which might be responsible for the anticancer activity. From the present study it can be concluded that chloroform and ethyl acetate extracts of leaves of *Asparagus racemosus* showed significant *invitro* anticancer activity which can be effectively used for the treatment of cancer.

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