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Hytochemical analysis and *in vitro* cytotoxic activity of various extracts of *Indigofera astragalina*

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ABSTRACT

In the present study, cytotoxic activities of various extracts of Indigofera astragalina were evaluated by short term cytotoxic assay and antiproliferative assay models. The extracts produce potent cytotoxic effect against Human cancer cell lines viz. Human cervical cancer (HeLa), lung cancer (A549), breast cancer (MCF7) and liver cancer (HepG2) cell lines. Hexane and water extracts showed moderate activity whereas ethanol, chloroform and ethyl acetate extracts showed potent activity against the tested cell lines. At the same time all the tested extracts showed 3-4 fold high IC50 values for the normal human dermal fibroblast cell line. This indicates the selective cytotoxicity towards the cancer cells. Preliminary phytochemical analysis of these extracts revealed the presence of alkaloids, flavonoids, glycosides, polyphenolics and saponins. The anticancer activity of the extracts may be due to the rich amount of these phytochemicals. Therefore, the plant could be considered as a very good anticancer source with renowned therapeutic potential.

Key words: *Indigofera astragalina*, cytotoxicity, Trypan blue dye exclusion assay, MTT assay, phytochemicals.

INTRODUCTION

Plants, vegetables and herbs used as food and in the folk and traditional medicine have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development (Graham et al., 2000; Liu, 2004). Despite a number of plant extracts used against diseases in traditional medicine, only a few of them have been scientifically explored. Scientific studies on plants used in ethnomedicine led to the discovery of many valuable drugs. Some examples of plant derived drugs are taxol, camptothecin, vincristine and vinblastine (Suffiness and Douros, 1982).

Indigofera astragalina (Leguminosae), is a small trailing herb distributed throughout India. Various parts of this medicinal plant were used in Indian System of Medicine to treat various illnesses such as rheumatism, arthritis, inflammation, tumor and liver diseases. Proximate and mineral composition of the leaves of *Indigofera astragalina* was determined and the results revealed that the plant contains essential nutrients like vitamin C and iron. The plant was also have high calorific value and considered as safe for consumption (Gafar and Itodo, 2011). *Indigofera astragalina* leaves are used to treat diarrhoea and the results highlighted the traditional uses of the plant

(Achuthanand Shukla et al., 2013). Several members of the species of *Indigofera* like *Indigofera trita, Indigofera aspalathoides* etc. are used traditionally for a wide variety of ethnomedical properties such as antitumor, hepatoprotective, antioxidant, anti-inflammatory and analgesic (Kumar et al., 2007; Kumar et al., 2008; Senthil Kumar et al., 2009; Rajkapoor et al., 2004). Based on the literature review and ethnopharmacological details, the plant *Indigofera astragalina* was selected to screen its various extracts for the cytotoxic activities using *in vitro* systems.

MATERIALS AND METHODS

Plant material and Extraction

Entire plants of *I. astragalina* were collected from Tiruchengode, Tamilnadu. The plant was authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen is preserved in our laboratory for future reference (Voucher No.: P. Chem. IA 003). The plant material was shade dried, pulverized and extracted (500 g) with the solvents of increasing order of polarity viz. hexane, chloroform, ethyl acetate and ethanol using soxhlet extractor for 72 h. The residue obtained after extraction with ethanol was extracted with water by cold maceration process for 72 h. The prepared extracts were filtered and concentrated to dryness under reduced pressure and controlled temperature in a rotary evaporator and yield was calculated. The extracts were stored in a refrigerator until further use.

Preliminary phytochemical screening

Prepared plant extracts of *I. astragalina* were analyzed for the presence of various phytochemical constituents employing standard procedures (Wagner et al., 1984). Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

In vitro anticancer activity

Tumor cells and inoculation

Normal Human Dermal Fibroblast cells (NHDF), Human Cervical Cancer cells (HeLa), Human Liver Cancer cells (HepG2), Human Lung cancer cells (A549) and Human Breast Cancer cell lines (MCF7) were obtained from National Centre of Cell Sciences (Pune, India). The cultures were maintained in Dulbecco's modified eagles medium (DMEM) containing 10 % inactivated calf serum and were grown in 25cm^2 tissue culture flasks (Tarson Products Ltd, Kolkatta, India) until confluent and used for cytotoxic assays. EAC cells were obtained from Amala Cancer Research Centre (Trissur, Kerala, India). The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. Tumor cells aspirated from the peritoneal cavity of mice were washed with normal saline and were used for further studies.

Preparation of test samples

For cytotoxicity assays, various extracts of *I. astragalina* were dissolved in dimethyl sulfoxide (DMSO) and the volume made up to 10 ml to obtain a 1000 µg/ml stock solution. Serial two-fold dilutions were made using DMSO to get lower concentration.

Short term cytotoxic activity

Short term cytotoxicity study of various extract of *I. astragalina* was determined by using trypan blue dye exclusion method (Sunila and Kuttan, 2004). EAC cells were cultured in peritoneal cavity of healthy albino mice by injecting a suspension of tumor cells (1 x 10^6 cells/ml) intraperitoneally. The cells were aspirated aseptically from the peritoneal cavity of the mice on day 15 and washed with normal saline and centrifuged for 15 min at 1500 rpm in a cooling centrifuge. The pellet was resuspended with normal saline and the process was repeated until to get a clear supernatant. Finally the cells were suspended in a known quantity of normal saline and the cell count was adjusted to 1 x 10^6 cells/ml. Then, 0.1 ml of this cell suspension was distributed in to Eppendrof tubes and exposed to 0.1 ml of various concentrations of plant extracts (500 – 31.25 µg/ml) and incubated at 37 ºC for 3 h. After 3 h, the trypan blue dye exclusion test was performed to determine the percentage cytotoxicity and the IC_{50} value was calculated.

Antiproliferative studies on human cancer cell lines

Stock cells of normal and human cancer cells were cultured in RPMI-1640 and DMEM supplemented with 10 % calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5 % CO_2 at 37 ºC until confluent. The cells were dissociated with 0.2 % trypsin and 0.02% EDTA in PBS. The cytotoxic assay was carried out by adding 0.1 ml of cell suspension containing 10,000 cells to each well of a 96-well microtitre plate

(Tarson, Kolkatta, India) and fresh medium containing various concentrations of extracts were added at 24 h after seeding. Control cells were incubated without the extracts and with DMSO. The microtitre plates were incubated at 37 ºC in a humidified atmosphere with 5 % CO_2 for a period of 72 h. The percentage cytotoxicity was determined by the standard MTT assay method and IC_{50} value was calculated (Vijayan et al., 2003).

RESULTS AND DISCUSSION

Various solvent extracts of *I. astragalina* was prepared and the percentage yield was calculated and the results are presented in Table 1. Among the extracts, maximum yield was obtained by using ethanol and water. Prepared extracts were analyzed for the presence of various phytochemical constituents and the results were presented in table 2.

Table 1. Extractive Yields of various Extracts of *Indigofera astragalina*

Name of the extract	*Indigofera astragalina*		
	Colour	Nature	Yield (%)
Hexane	Dark green	Solid mass	2.3
Chloroform	Greenish yellow	Solid mass	1.8
Ethyl acetate	Yellowish green	Solid mass	6.7
Ethanol	Brownish yellow	Semi solid mass	10.3
Water	Brown	Dry powder	8.6

Preliminary phytochemical screening of the plant extracts showed the presence of various phytochemical constituents. Terpenoids, phytosterols, alkaloids were present in hexane and chloroform extracts. Ethyl acetate extract contains terpenoids, amino acids, flavonoids and saponins whereas phytosterols, carbohydrates, glycosides, alkaloids, terpenoids, tannins, saponins, proteins and amino acids were present in ethanol extract. Aqueous extract consist of many polar constituents like carbohydrates, glycosides, tannins, flavonoids and saponins. Gums and mucilage were absent in all the prepared extracts. The results clearly showed that the presence of each phytoconstituents depends upon the solubility of phytochemical constituents in the particular solvents. Many of the constituents were extracted by ethanol.

Table 2. Preliminary phytochemical analysis of various extracts of *I. astragalina*

Name of the Phyto-chemical	Name of the extract				
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
Carbohydrates	-	-	-	+	+
Glycosides	-	-	+	+	+
Alkaloids	-	+	+	+	-
Terpenoids	+	+	-	+	-
Phytosterols	+	+	-	+	-
Flavonoids	-	-	+	+	+
Phenolics	-	-	+	+	+
Tannins	-	-	-	+	+
Saponins	-	-	-	+	+
Proteins & amino acids	-	-	-	+	+
Fixed oils & fats	+	+	-	-	-
Gums & mucilages	-	-	-	-	-

(+) Present (-) Absent

Table 3: Short Term Cytotoxicity Studies of Various Extracts of *Indigofera astragalina* against EAC Cell Line by Trypan Blue Dye Exclusion Method

Name of the Extract	IC_{50} (µg/ml)*
Hexane	282.37
Chloroform	194.41
Ethyl acetate	162.94
Ethanol	110.68
Water	240.66

**Average of three determinations, three replicates*

All the prepared plant extracts were screened for their cytotoxic property against animal and human cancer cell lines. Hexane and aqueous extracts show moderate activity against EAC cell lines in trypan blue dye method. Ethanol, ethyl acetate and chloroform extracts exhibit good activity and the results are displayed in table 3.

In MTT assay, the percentage cytotoxicity progressively increased in a concentration dependent manner. The IC_{50} of various extracts of *I. astragalina* showed good activity against all the human cancer cell lines used. However the IC_{50} values against the normal human dermal fibroblast cells (NHDF) were found to be very high when compared to that of cancer cell lines. This indicates that the extracts possess selective cytotoxicity against the cancerous cell lines, but is safer towards the normal cells (Table 4).

Table 4. *In Vitro* Cytotoxic Activity of Various Extracts of *I. astragalina*

Name of the Extract	IC_{50} Value (µg/ml)*				
	A549	HeLa	MCF7	HepG2	NHDF
Hexane	270.3 ± 14.4	272 ± 15.1	230 ± 13.1	222 ± 10.8	473.6 ± 19.6
Chloroform	174 ± 9.2	192 ± 8.19	171.3 ± 6.03	183.7 ± 6.7	423.7 ± 11.2
Ethyl acetate	140.3 ± 16.56	159 ± 7.1	143 ± 12.53	126.7 ± 6.1	430 ± 9.17
Ethanol	127 ± 14.53	125.7 ± 12.7	131.7 ± 5.5	108 ± 9.17	451 ± 11.2
Water	184.7 ± 8.6	179.3 ± 15.5	195.3 ± 10.1	177 ± 34.3	526.7 ± 34.3

**Average of three determinations, three replicates; IC_{50}, Drug concentration that produce 50% cell death following 72 h of drug exposure.*

The systemic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging, antiangiogenic and antimutagenic (Weber et al., 1996; Fotsis et al., 1997).

Alkaloids, terpenoids and phytosterols like compounds are commonly found in both edible and medicinal plants and they have been reported to have various biological effects including cytotoxic and anticancer activity. The cytotoxic activities of these compounds are mainly due to their interaction with DNA, microtubules and cell cycle. Various extracts of *Indigofera astragalina* showed moderate to strong cytotoxic activities in various *in vitro* systems tested. Moreover the plant extracts also contain terpenoids, alkaloids, glycosides, saponins and tannins. The cytotoxic effect of various extracts of *I. astragalina* is may be due to the presence of various phytochemical compounds present.

CONCLUSION

In conclusion, the results obtained from short term cytotoxicity study and antiproliferative activity reveals that various extracts of *Indigofera astragalina* have significant cytotoxic activity against all the animal and human cancer cell lines studied. Further studies in our laboratory are in progress for the isolation and identification of phytochemical compounds and to ensure the medicinal properties of the plant *in vivo* correlate with its anticancer activity.

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