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Original Article

IN VITRO FREE RADICAL SCAVENGING AND ANTICANCER POTENTIAL OF *ARISTOLOCHIA INDICA* L. AGAINST MCF-7 CELL LINE

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ABSTRACT

Objective: To investigate the *in vitro* free radical scavenging and anticancer potential of *Aristolochia indica* L. leaves and stem against MCF-7 cell line.

Methods: Phytochemicals were analysed in chloroform (leaves) and aqueous extracts (stem) of the *Aristolochia indica* by using standard methods. *In vitro* antioxidant studies were carried out for the chloroform and aqueous extracts of the *Aristolochia indica* using various free radical models such a DPPH, Reducing power assay, hydrogen peroxide (H_2O_2) scavenging,. *In vitro* cytotoxic assay such as MTT assays were carried out against MCF-7 cell line.

Results: Preliminary phytochemical screening revealed the presence of flavonoids, tannins, glycosides, phenol, saponins, in chloroform extract of leaves and aqueous extract of stems. The results revealed that the chloroform extract has significant antioxidant potential than aqueous extract. The result revealed that the chloroform extracts of *Aristolochia indica* L showed pronounced anticancer activity against Ehrlich Ascites Carcinoma (MCF-7) cell line than ethanol extract.

Conclusion: The result of the present study concluded that the chloroform extract of *Aristolochia indica* L has significant antioxidant and anticancer activity then the aqueous extract. The potential antioxidant and anticancer activity of *Aristolochia indica* L might be due to the presence of phytochemicals.

Keywords: *Aristolochia indica* L, Ehrlich Ascites Carcinoma, Free radicals, Antioxidant.

INTRODOUCTION

Cancer is the abnormal growth of cells in our bodies that can lead to death. Cancer cells usually invade and destroy normal cells. These cells are born due to imbalance in the body and by correcting this imbalance, the cancer may be treated. Cancer is a leading cause of mortality, and it strikes more than one–third of the world's population and it's the cause of more than 20% of all deaths [1]. Breast cancer is one of the most leading cancers among women globally and the leading cause of cancer deaths in women. Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [2]. Breast cancer occurs in humans and other mammals. While the overwhelming majority of human cases are in women, breast cancer can also occur in men. Some women have HER2-positive breast cancer. HER2 refers to a gene that helps cells grow, divide, and repair themselves. In those with mutations in the breast cancer susceptibility genes *BRCA1* or *BRCA2*, or who have a family history of breast cancer, use of modern oral contraceptives does not appear to affect the risk of breast cancer [3].

MCF-7–Michigan Cancer Fountation-7. It is the breast cancer cell line isolated in 1970 from 69 years old Caucasian woman. It is a malignant tumor that starts in the cells of the breast. It is found mostly in women. MCF7 cells have been extensively used as the model for breast cancer and breast cancer therapy. However, different sources of MCF7 show difference in response to 17beta-estradiol, resulting from activation or inhibition of insulin-like growth factor I (IGF-1). Therefore, the different responses of MCF7 should be realized due to the expression of IGF-1. Hence it is delineated that, MCF7 is suitable candidate as reference materials in quality control for HER2 testing. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. [4]. A considerable part of the current knowledge on breast carcinomas is based on *in vivo* and *in vitro* studies performed with *cell lines* derived from breast cancers. These provide an unlimited source of homogenous self-replicating material, free of contaminating stromal cells, and often easily cultured in simple standard media. The first breast cancer cell line described, BT-20, was established in 1958. The Michigan Cancer Foundation is now known as the Barbara Ann Karmanos Cancer Institute [5].

The management of breast cancer depends on various factors, including the cancer. Breast cancer is usually treated with surgery which may be followed by chemotherapy or radiation therapy, or both [6] but severe side effects associated with it. Plant use in treating diseases is as old civilization [7] and traditional medicines are still a major part of habitual treatments of different maladies [8]. In recent times and due to historical, cultural, and other reasons, folk medicine has taken an important place especially in developing countries where limited health services are available. Plants are considered as one of the main sources of biologically active materials. Recent records reported that medicinal herbs are used by 80% of the people living in rural areas as primary healthcare system [9]. It has been estimated that about 50% of the prescription products in Europe and USA are originating from natural products including plants or their derivatives [10]. Keeping in mind, the aim of the present study to investigate the *in vitro* free radical scavenging and Anti cancer potential of *Aristolochia indica* L. stem.

The *Aristolochia indica* L. is used to treat cholera, fever, bowel, troubles, ulcers, leprosy, poisonous bites [11], and also used as emmenagogue, abortificient, antineoplastic, antiseptic, anti-inflammatory, antibacterial, antioxidant and phospholipase A_2 inhibitor [12, 13]. The plant has been used in skin diseases. The fresh juices of the leaves are popular antidote to snake poison [13]. The leaves and bark are used in intermittent fever. It is used ethanomedically as an antitumor, anti-inflammatory, antibacterial and antioxidant and antimicrobial [13]. The extracts are found have various medicinal activities like, immunomodulatory activity and antitumor activity [14].

MATERIALS AND METHODS

Collection of plant material

One kilogram of fresh leaves and stems of *Aristolochia indica* were collected from Thanjavur district. The collected plant materials were

authenticated by the Department of Botany, Government Arts College, for Men Kumbakonam, Thanjavur district, Tamil Nadu.

Preparation of plant extracts

Chloroform extraction

The leaves of *Aristolochia indica* were shade dried and powdered as coarse particles using blender. 50 gms of coarse powder was extracted with chloroform (260 ml) in soxhlet extractor for 16 to 18 hours. Then the extract was filtered and concentrated using rotary flash evaporator.

Aqueous extraction

10g of MCF-7h leaves and stems powders of *A. indica* was extracted with 180 ml of distilled water by cold maceration method. They were kept in a sterile condition for 72 hours. The sample is filtered and evaporated using boiling water bath for 30 minutes.

Phytochemical screening of various extracts

Preliminary phytochemical screening of drug powder and various extracts were carried out as per the standard textual procedure [15].

***In vitro* antioxidant activity**

Antioxidant activity measured by using DPPH radical scavenging assay method Reducing Power assay[16], Hydrogen peroxide radical scavenging activity [17].

***In vitro* cytotoxity assay [18]**

Methods

The human breast cancer cell line (MCF 7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 °C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples.

They were initially dispersed in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48h of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to MCF-7h well and incubated at 37 °C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl 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.

Non-linear regression graph was plotted between % Cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software.

RESULTS AND DISCUSSION

Plant have provided a sources of inspiration for novel drug compounds as plant derived medicines have made significant contribution towards human health, phytomedicines can be used for the treatment of disease as done in case of Unani and Ayurvedic system of medicines or it can be the base for the development of a medicine, a natural blue print for the development of a drug [19].

For over thousands of years, natural plants have been used as a valuable source of medicinal agents with proven potential of treating infectious disease and with lesser side effects compared to synthetic drug agents [20].

Table 1 represents the qualitative analysis of chloroform extract of leaves and aqueous extract of stems of *Aristolochia indica.* Preliminary phytochemical screening revealed the presence of flavonoids, tannins, glycosides, phenol, saponins, in chloroform extract of leaves and aqueous extract of leaves, and stems. Alkaloids have been the source of much human interest and fascination both in terms of scientific research and cultural usage. Often highly rMCF-7tive in small amount, their effect on the human physiology is most notable on the nervous system [21].

Phytochemicals are referred to as phytonutrients. These are compounds present in plant derived-foods that induce biological activities in the body. They have various saluvrious functions in the body. For example, thus phytonutrients promote the function of the immune system, act directely against bacteria and viruses, reduces inflammation and are also associated with the treatment and prevention of cancer, cardiovascular disease and many other maladies affecting the health or well being of an individual [22].

Table 1: Phyto-constituents of *Aristolochia indica* leaves and stems extract

S. No.	Phyto-constituents	Chloroform extract (leaves)	Aqueous extract (leaves)	Aqueous extract (stems)
1.	Alkaloids	-	-	-
2.	Flavanoids	+	+	+
3.	Tannins	+	+	+
4.	Glycosides	+	_	+
5.	Phenols	+	_	+
6.	Saponins	+	+	+
7.	Terpenoids	+	_	+
8.	Carbohydrate	_	+	+
9.	Protein	_	+	+
10.	Phlobatannins	_	+	_
11.	Anthroquinone	_	_	_
12.	Cardiac glycoside	_	+	_
13.	Aminoacid	+	+	+

(+) Present, (-) Absent

To determine the efficacy of natural antioxidants either as pure compounds or as plant extract, a great number of *invitro* methods have been developed in which antioxidant compounds act by several mechanisms.

Table 2 represents the free radical scavenging activity of leaves and stems were found to be increased with increasing concentration of plant extract. The results showed that the aqueous extract of stems of *A. indica* shows the increased scavenging activity when compare to chloroform extract of leaves.

Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep and spicy foods as well as physical stress, cause depletion of immune system oxidants, change in gene expression and include abnormal proteins, Oxidation process is one of the most important route for producing free radicals in food, drugs, and even living systems. Catalase and hydroperoxidase enzymes convert hydrogen peroxide and hydroperoxides to non-radical forms and functions as natural antioxidants in human. Due to depletion of immune system natural antioxidants in different Maladies, consuming antioxidants as free radical scavengers may be necessary [23].

Table 2: DPPH radical scavenging activity of chloroform extract of leaves and aqueous extract of stems of *A. indica*

S. No.	Concentration (µg/ml)	DPPH radical scavenging activity %		
		Standard	Leaves	Stem
1	200	50±3.50	10±0.7	33.33±2.33
2	400	58±4.06	16.66±1.17	43.33±3.03
3	600	66±4.62	23.33±1.63	55±3.85
4	800	70±4.90	43.33±3.03	65±4.55
5	1000	75±5.25	48.33±3.38	66.66±4.67

Values were expresses as mean±SD for triplicates

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain rMCF-7tions. The potential of the antioxidant constituents of plant materials for the maintenance of health and protectionfrom coronary heart disease and cancer is also raising interest among scientists and food manufacturers as consumers move toward functional foods with specific health effects [24]. The antioxidative effect is mainly due to phenolic components, such as flavonoids.

Antioxidant potential of various extracts *Vitex negundo* Linn at different concentration. The result shows the maximum DPPH scavenging activity of ethyl acetate extract of *Vitex negundo* Linn appeared to be as standard vitamin c with a maximum inhibition of 98.96% at 250µg/ml which is comparable to 77.92% for vitamin c at the same concentration. The antioxidant properties of plants have been linked to their therapeutic and protective effects in many diseases such as parkinson's disease, cancer, cardiovascular disorders, bacterial and viral infections and inflammation [25].

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [26] and is usually used as a substrate to evaluate the antioxidant activity of a compound [27]. Based on the data obtained from this study, DPPH radical scavenging activity of AS extract showed IC_{50} value 182.31±0.31 µg/ml. while ascorbic acid showed the value of 30.12±0.11µg/ml. it was revealed that AS extract did show the proton donating ability and could serve as free radical inhibitor or scavenger. In fact, the radical scavenging capability of phenolic compounds are due to their hydrogen donating ability/number of hydroxyl group present, which in turn is closely related both to the chemical structure and spatial conformation, that can modify the rMCF-7tivity of the molecules [28]. In the present study this possibility is supported by the estimation of total phenol [29]. Which was found to be present in the *Aristolochia indica* species extracts.

Table 3 represent the antioxidant property of *Aristolochia indica* extract was determined through FRAP using ascorbic acid as standard. The results showed that the chloroform extract of leaves of *A. indica* shows the increased scavenging activity when compare to aqueous extract of stems.

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [30]. Moreover, it has been reported that the phenol and polyphenolic compound (flavonoids) constituents of the plant possess antioxidant properties mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential [31]. Our results suggest that the antioxidant activity of AS extract might be attributed to the phenolic and flavonoids, which detected by phytochemical analysis in our study.

Table 4 represent the antioxidant property of *Aristolochia indica* extract was determined through H_2O_2 using ascorbic acid as standard. The results showed that the chloroform extract of leaves of *A. indica* shows increased scavenging activity when compare to aqueous extract of stems.

The scavenging effect of sugiol against hydroxyl radicals was investigated by Vivek *et al.* using the Fenton rMCF-7tion. The percentage inhibition of sugiol, ascorbic acid and BHA on hydroxyl radical scavenging was found to be 85.04%, 73.79%, 70.02%, respectively. The results showed significant antioxidant activity in a concentration-dependent manner ($P<0.05$). The ability of the sugiol to quench hydroxyl radicals seems to be directly related to the prevention of propagation of lipid peroxidation; because sugiol seems to be a good scavenger of active oxygen species, it will thus reduce the rate of the chain rMCF-7tion.

It is recognized that antioxidant (mainly polyphenolic) compounds from plant extracts can act by either free radical Scavenging singlet oxygen quenching chelating of transitional metal such as iron [32], as well as a reducing agents and activator of antioxidative defense enzyme systems to suppress radical damage in biological system.

Table 3: Reducing power of chloroform extract of leaves and aqueous extract of stems of *A. indica*

S. No.	Concentration (µg/ml)	Reducing power activity %		
		Standard	Leaves	Stems
1	200	50±3.5	66.66±4.67	33.33±2.33
2	400	66±4.62	75±5.25	60±4.20
3	600	75±5.25	80±5.60	71.42±5.00
4	800	80±5.60	85.71±6.00	80±5.60
5	1000	83±5.81	88.88±6.22	83.3±5.83

Values were expresses as mean±SD for triplicates

Table 4: Hydrogen Peroxide Scavenging activity of Chloroform and Aqueous extract of *Aristolochia indica* Leaves and stems

S. No.	Concentration (µg/ml)	% of Hydrogen peroxide		
		Standard	Leaves	Stems
1	200	0.12±0.008	0.13±0.009	0.14±0.010
2	400	0.08±0.006	0.11±0.008	0.11±0.008
3	600	0.06±0.004	0.08±0.006	0.09±0.006
4	800	0.05±0.004	0.06±0.004	0.05±0.004
5	1000	0.04±0.003	0.03±0.002	0.03±0.0002

Values were expresses as mean±SD for triplicates

Flavonoids are among the best candidates for mediating the protective effect of diets which are found in fruits and vegetables with respect to colorectal cancer. Study shows relative activity being as quercetin>apigenin>fisetin>kaempferol. Quercetin belongs to the flavonoids group due to its powerful antioxidant activity. Previous studies showed that quercetin may help to prevent cancer, especially prostate cancer[33]. Scambia *et al.* reported quercetin inhibited human breast cancer cells (MCF-7 and MDA-MB231) significantly [34].

Du *et al.* explained mechanism of breast cancer inhibition by quercetin. In ginger quercetin is abundant flavonoid compound. Antioxidant activity of quercetin was believed to have cytoprotective role against oxidative stress. It seemed that quercetin not only protects cells from free radical damage through antioxidant effect but also motivates apoptotic cell death via pro oxidant activity and inhibits tumurigenesis. Hence, anticancer power may be related to quercetin content in those varieties. In addition, flavonoid compounds could probably be responsible for the anticancer activity of *A. indica.* Further research is required to untangle the specific bioactive compounds responsible for the anticancer properties of the extracts of *A. indica.*

These results are in accordance with [35] who investigated the cytotoxicity effects of water extracts from leaves and branches of Philadelphus coronaries(HydrangMCF-7eae), against A431 cells (human skin carcinoma cell line) and the human breast cancer adenocarcino effects were observed against MCF-7 cell line. A431 were sensitive but their sensitivity was less in comparison with MCF-7 cell line.

The methanolic fraction of *Ononis hirta*(aerial parts)and *Inula viscose* (flowers) which exhibited the highest anti proliferative potential among the five active plants with IC50 values of 27.96 and 15.78µg/ml respectively, previous studies reported the antiproliferative activity of *Inula graveolens*, *Inula helenium* and *Inula cappa* [36].

Table 5 represent the result of cytotoxicity evaluation of chloroform extract of *A. indica* was examined by MTT assay using breast cancer cell line (MCF-7). The extend of cytotoxicity can be measured by MTT dye reduction assay. The percentage viability was analyzed by MTT assay after treatment of chloroform extract of *A. indica* at 18.75 µg/ml, 37.5µg/ml,75µg/ml,150µg/ml and 300µg/ml concentration. The plant extract showed inhibitory effect at (IC_{50}347 µg/ml).

A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [37]. The anticancer activities of chloroform extract of *A. salvifolium* are probably due to the presence of alkaloid, phenolic compounds, flavonoids as well as terpenoids.

Table 5: Anticancer activity of chloroform extract of *Aristolochia indica* leaves against MCF-7 cell lines by MTT assay

S. No.	Concentration of test (µg/ml)	% of cell inhibition	Concentration of standard (µg/ml)	% of cell inhibition	Control
1.	18.75	0.09±0.006	0.001	14.02±0.98	0.36±0.02
2.	37.5	4.46±0.312	0.01	35.24±2.46	0.379±0.02
3.	75	13.75±0.963	0.1	51.27±3.58	0.35±0.02
4.	150	29.69±2.07	1	75.59±5.29	0.36±0.02
5.	300	43.80±3.06	10	92.53±6.47	0.36±0.02

Values were expresses as mean±SD for triplicates

Hamelers *et al.*, [38] represent MCF-7 is one of the commonly used breast carcinoma cell line. It is relatively resistant to cisplatin tratment. Morphology of this cell line exhibits epithelial like cell including the ability to process estradiol via cytoplasmic estrogen receptors and domes formation. This cell line has oncogene, and induce tumor in nude mice. MCF-7 cell line expresses both estrogen and progesterone receptors whereas the expression of Her2/neu is absent. The cells can be suppressed by catechinhydrate, product from plant sources such as green tea, through TP53/caspase mediated apoptosis. Some vasoactive peptides such as endothelin 1 is found at low level in MCF7 while in SKBR3 it is expressed at the higher level. This expression might corrrlate with high invasiveness phenotype in breast cancer [39].

The results of the present study established that the phytochemical analysis revealed that the presence of tannin, flavonoids, Terpenoids, alkaloids, carbohydrate, protein and glycosides in chloroform and aqueous extracts. The antioxidant activity of *Aristolochia indica* L. was evaluated using DPPH (2,2Diphenyl picryl hydrazyl), FRAP (ferric reducing antioxidant power), and H_2O_2 radical scavenging activity. The chloroform extract of the plant showed promising antioxidant activity. The cytotoxicity of plant sample was evaluated in human breast cancer cell line (MCF-7 Michigan Cancer Foundation-7) by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)assay using taxol as standard. The plant extract showed inhibitory effect at (IC_{50}347 µg/ml). From the above results, it is concluded that the chloroform extract of *Aristolochia indica* L has significant antioxidant and anticancer activity then the aqueous extract.

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CONFLICT OF INTERESTS

Declared None

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