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In Vitro evaluation of antioxidant and cytotoxic activities of the leaf extracts of *Aristolochia indica*

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Abstract

The present study was carried out to analyse the physical to chemical constituents and to evaluate the antioxidant and cytotoxic activity of the leaf extract of *Aristolochia indica in vitro.* Preliminary phytochemical analysis revealed the presence of flavonoids, tannins, alkaloids, quinines, cardiac glycosides, terpenoid, triterpenoids, phenol, steroids and phytosterols. Total phenolic content is higher in methanol extract when compared to other extracts (9700 mg GAE/L). *In vitro* antioxidant potential of five different concentrations of (50, 100, 150, 200 and 250 µg/ml) methanolic leaf extract of *Aristolochia indica* was studied. DPPH radical scavenging activity, reducing power assay, total antioxidant activity, hydrogen peroxide scavenging activity and nitric oxide radical scavenging activity were evaluated. Dose dependant antioxidant activities of plant extracts were comparable to that of standard ascorbic acid were observed, *In vitro* cytotoxicity study against MCF -7 cell line by MTT assay using Doxorubicin as standard showed that the plant extract remarkably inhibited the growth of cancer cell in a dose dependant manner. It is concluded that the methanolic extract of *A. indica* leaves has significant antioxidant and cytotoxicity activities which might be due to the presence of phytochemicals such as phenolic compounds.

Keywords: Antioxidant, *Aristolochia indica*, Cytotoxicity, Phenolic compound, Phytochemical.

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INTRODUCTION

Free radicals can be defined as reactive chemical species having a single unpaired electron in an outer orbital (Riley, 1994). This unstable configuration creates energy which is released through reactions with adjacent molecules, such as proteins, lipids, carbohydrates, and nucleic acids. The majority of free radicals that damage biological systems are oxygen-free radicals, and these are more generally known as "Reactive Oxygen Species" (ROS). These are the main byproducts formed in the cells of aerobic organisms, and can initiate autocatalytic reactions so that molecules to which they react are themselves converted into free radicals to propagate the chain of damage. ROS can be (i) generated during UV light irradiation and by X-rays and gamma rays, (ii) produced during metal catalyzed reactions, (iii) are present in the atmosphere as pollutants, (iv) are produced by neutrophils and macrophages during inflammation, and (iv) are by-products of mitochondrial catalyzed electron transport reactions, and various other mechanisms (Cadenas, 1989).

"Oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents (Lushchak, 2014). Human Body is constantly bombarded by exogenous factors such as ultraviolet rays and tobacco smoke that cause oxidative stress. Such stress can also arise from drugs (including anticancer drugs) that are used in medical practice. In addition to those exogenous sources, endogenous sources of oxidative stress include those derived from activities of mitochondria or microsomes and peroxisomes in the electron transfer system and those from the enzyme NADPH present in macrophages and neutrophils as a mechanism of protection against infection.

The term "antioxidant" refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved highly complex antioxidant systems (enzymic and nonenzymic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. An ideal antioxidant should be readily absorbed, and quench free radicals, and chelate redox metals at physiologically relevant levels. It should also work in both aqueous and/or membrane domains and affect gene expression in a positive way.

Cancer is the second leading cause of death in the world after cardiovascular diseases. Half of men and one third of women in the United States develop cancer

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during their lifetime. Today, millions of cancer people extend their life due to early identification and treatment. Cancer is not a new disease and has afflicted people throughout the world. Breast cancer is a malignant tumor (a collection of cancer cells) arising from the cells of the breast. Although breast cancer predominantly occurs in women, it can also affect men. The most frequent cancer type in females in the Western world is breast cancer, with a lifetime risk of the order of 1/10. Breast cancer does not always produce symptoms; women may have cancers that are so small they do not produce masses that can be felt or other recognizable changes in the breast. When symptoms do occur, a lump or mass in the breast is the most common symptom. Cancer treatment produces lot of side effects to the patients and further damages their health. For this reason, in the recent years there has been a gradual revival of interest in the use of medicinal plants for cancer treatment.

Aristolochia indica is belonged to *Aristolochiacae* family, distributed throughout the low hills and plains of India from Nepal to the south of Konkan. It is common in dry and deciduous forests. The leaves are simple, alternate, short petiole, the blade ovate or somewhat wedge shaped. It has flowers which are greenish white in colour, while fruits are round or oblong and hexagonal, 2.5-4.0 cm long and six chambered which contain numerous winged compressed seeds (Das *et al*., 2010). The root is pungent, bitter, alexiteric, emmenagogue, useful in "triodes", pain in the joints, bowel troubles of children. The plant is used to treat cholera, fever, bowel troubles, ulcers, leprosy and poisonous bites (Krishnaraju *et al*., 2005; Kanjilal *et al*., 2009). *indica* has been reported to have antimicrobial (Kumar *et al*., 2006), antidiabetic (Sanjay Kumar *et al*., 2012) and anti inflammatory (Das *et al*., 2010) activity.

MATERALS AND METHODS

Collection of plant material

Leaves of *A. indica* were collected from in and around Madurai, Tamil Nadu, India. The collected samples were carefully kept in polythene bags. These plant samples were authenticated by Dr. S.John Britto, Director, RAPINAT Herbarium, St. Joseph's College, Tiruchirappalli and a voucher specimen was deposited in the Department of Biochemistry, S.T.E.T Women's College, Mannargudi (Voucher No: 001). Collected plant materials were cleaned, and they were shade dried. Then dried plant materials were coarsely powdered using a mechanical grinder. The powder was stored in air tight and light resistant container for further analysis.

Preparation of extraction

10gm of the powdered sample was extracted with 85% methanol using soxhlet extraction method. The filtrate was condensed using rotary vaccum evaporator at 45° C. The dried extract was dissolved in dimethyl sulfoxide solution (DMSO) (Sigma) at concentration of 100 mg/ml and kept as a stock sample. The obtained paste form of the extract was used for further analysis.

Preliminary phytochemical screening

Phytochemical analysis of the powder of *A. indica* leaves was conducted as per standard procedures (Harborne, 1998).

Total Phenol Content (TPC)

Different extracts like hexane, chloroform, ethyl acetate, methanol and water were subjected to determine the concentration of phenolics content using spectrophotometric method (Singleton and Rossi, 1965).

Antioxidant Assay

Antioxidant properties of the leaf extract of *indica* were determined by various *in vitro* assays such as DPPH radical scavenging assay (Blois, 1958), Reducing power assay (Oyaizu, 1986), Total antioxidant activity (Mitsuda *et al*., 1966), Hydrogen peroxide scavenging activity (Ruch *et al*., 1989) and Nitric oxide radical scavenging activity (Nathan and Xie, 1991).

Cytotoxicity Assay

Cytotoxic activity of *A. indica* was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against breast cancer cell line MCF-7 (Mosmann, 1983).

RESULTS

Preliminary phytochemical analysis

Phytochemicals analysis of the leaf extract of *A. indica* was performed and the results revealed the presence of tannins, saponins, flavonoids, alkaloids, quinines, cardiac glycoside, terpenoid, triterpenoids, steroids, phytosteroids and absence of carbohydrate, glycoside, coumarins, phlobatannins and anthraquinones (Table 1).

Determination of Total Phenolic Content (TPC)

The total phenolic content of the different extracts of *A. indica* was measured using the Folin-Ciacalteu (FC reagent) method and the results are shown in Table 2. The total phenolic content of the extracts ranged from 1000 to 9700 mg GAE/L. Methanol extract of *t*he leaves of *A. indica* showed the highest phenolic content (9700 mg GAE/L), followed by water (5800 mg GAE/L), acetate (1900 mg GAE/L), hexane (1500 mg GAE/L) and chloroform (1000 mg GAE/L) extracts. Based on the high phenolic

Table.1. Phytochemical screening of leaf extracts of *Aristolochia indica*

S.No.	Phytochemical	Results
1	Carbohydrate	-
2	Tannins	+
3	Saponins	+
4	Flavonoid	+
5	Alkaloid	+
6	Quinines	+
7	Glycoside	-
8	Cardiac glycosides	+
9	Terpenoid	+
10	Triterpenoids	+
11	Phenol	+
12	Coumarins	-
13	Steroids and phytosteroids	+
14	Phlobatannins	-
15	Anthraquinones	-

Table.2. Total Phenolic Content in leaf extracts of *A. indica* in different solvents

S.No.	Extracts	TPC (mg GAE/L)
1	Hexane	1500
2	Chloroform	1000
3	Ethyl acetate	1900
4	Methanol	9700
5	Water	5800

Table.3. DPPH scavenging assay of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	DPPH scavenging	
		Plant extract	Ascorbic acid
1	50	16.2± 1.1	18.0 ± 1.90
2	100	21.0± 0.9	14.7 ± 1.00
3	150	24.2± 0.8	28.7 ± 2.01
4	200	28.5 ±0.6	30.0 ± 0.40
5	250	33.5 ±0.8	35.3 ± 0.70

concentration, methanolic extract was selected for antioxidant and cytotoxicity study.

Antioxidant Assay

DPPH free radical scavenging activity

It was observed that methanolic extract of *A.indica* at the concentration of 50, 100, 150, 200 and 250 µg/ml significantly scavenged DPPH free radical to an extent of 16.2, 21, 24.2, 28.5 and 33.5% respectively, in a concentration dependent manner. The DPPH radical scavenging reaction in ascorbic acid ranged from 18.0 to 35.3% (Table 3).

Reducing power assay

Reducing power of methanol extract of *A.indica* leaves is represented in Table 4. Reducing power of the extract displayed an increase with increasing concentrations, as indicated by the increase in the absorbance of the reaction mixture. At 50,100,150, 200 and 250µg/ml of plant extract with the reducing power around 7.55, 12.8, 17.5, 21.8 and 25.2%, respectively. Reducing power of standard was 39.7% at 250µg/ml which was higher than that of plant extract at the same concentration.

Table. 4. Reducing power assay of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	Reducing power in	
		Plant extract	Ascorbic acid
1	50	7.55± 0.6	19.0 ± 0.9
2	100	12.8± 0.4	24.7 ± 1.25
3	150	17.5± 0.7	30.7 ± 0.64
4	200	21.8 ±0.5	34.25 ± 0.8
5	250	25.2 ±1.3	39.7 ± 0.85

Total antioxidant activity

A. indica extract has remarkable total antioxidant activity and the results of antioxidant capacity of the extract were represented in Table 5. An increasing trend in the antioxidant activity was observed with the increasing concentration of plant extract (50, 100, 150, 200 and 250µg/ml) and the values were 19.8, 23.5, 25.3, 31, and 34.3%, respectively. Antioxidant activity of standard was found to be 20.7 to 37.6% at different concentrations.

Hydrogen peroxide scavenging activity

Scavenging capacity on hydrogen peroxide by methanolic extract of *A. indica* is presented in the Table 6. The extract showed remarkable scavenging activity which was found to be 17.2, 22.7, 26.5, 32.4 and 37 % at 50, 100, 150, 200, and 250µg/ml, respectively. Highest value of 37% was at 250µg/ml. The extract showed a hydrogen peroxide scavenging activity in a concentration dependent manner.

Nitric oxide scavenging activity

Methanol extract of *A.indica* showed remarkable dose dependent inhibitory effect on nitric oxide radical scavenging activity (Table 7) and the values were 19.8, 23.5, 25.3, 31.02 and 36.99% at 50, 100, 150, 200

Table.5. Total antioxidant activity of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	Total antioxidant activity in %	
		Plant extract	Ascorbic acid
1	50	19.8 ± 0.47	20 .7 ± 1.02
2	100	23.5 ± 0.31	24.91 ± 0.25
3	150	25.3 ± 0.60	26.7 ± 0.64
4	200	31.0 ± 0.42	33. 50 ± 1.20
5	250	34.3 ± 1.03	37.60 ± 1.20

Table.6. Hydrogen peroxide scavenging activity of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	Hydrogen peroxide scavenging activity in %	
		Plant extract	Ascorbic acid
1	50	17.2 ± 1.7	18.30 ± 2.9
2	100	22.7 ± 1.2	22.90 ± 1.70
3	150	26.5 ± 0.8	29.4 ± 0.84
4	200	32.4 ± 0.5	34.00 ± 1.80
5	250	37.0 ± 0.4	39.9 ± 0.45

Table.7. Nitric oxide radical scavenging activity of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	Nitric oxide scavenging activity in %	
		Plant extract	Ascorbic acid
1	50	19.80 ± 0.4	22.72 ± 1.9
2	100	23.50 ± 0.3	25.7 ± 1.25
3	150	25.30 ± 0.6	28.7 ± 0.64
4	200	31.02 ± 0.4	34.25 ± 0.8
5	250	36.99 ± 1.03	37.7 ± 0.12

Table.8. MTT assay of methanolic leaf extract of *A.indica*

S. No.	Concentration (µg/ml)	Cytotoxicity in %	
		Plant extract	Standard Doxorubicin
1	MCF-7 untreated	-	-
2	5	15 ± 0.01	61 ± 0.13
3	10	24 ± 0.18	70 ± 0.21
4	15	30 ± 0.19	77 ± 0.25
5	20	38 ± 0.23	80 ± 0.33
6	25	43 ± 0.28	85 ± 0.36
7	30	50 ± 0.33	90 ± 0.41

and 250µg/ml, respectively. The reduction value of 37.3% was recorded at the highest concentration of 250µg/ml. Scavenging activity of plant extract towards nitric oxide was almost similar to the standard ascorbic acid at similar concentration (37.7%).

Cytotoxicity assay

In the present study, different concentrations of 5, 10, 15, 20 and 25 µg/ml of methnolic extract of *A. indica* was examined for its cytotoxicity effect by MTT assay against breast cancer cell line (MCF-7). Standard Doxorubicin was also evaluated in order to compare the efficacy of anticancer activity of selected plant extract. Percentage of cytotoxicity of plant extract was found to be 15, 24, 30, 38, 43 and 50% (Table 8) at 5, 10, 15, 20, 25 and 30 µg/ml of plant extracts, respectively. The percentage of cytotoxicity of standard was 61, 70, 77, 80, 85 and 90, respectively, at similar concentrations.

DISCUSSION

Herbal drugs referred as plant materials or herbals, involve the use of whole plants or parts of plants, to treat injuries or illnesses. Herbal drugs are use of therapeutic herbs to prevent and treat diseases and ailments or to support health and healing. These are drugs or preparations made from a plant or plants and used for any of such purposes. There are many herbal products offered to treat the symptoms of a broad range of problems, from depression to cold and flu. World Health Organization (WHO) has distinct herbal drugs as complete, labeled medicinal products that have vigorous ingredients, aerial or secretive parts of the plant or other plant material or combinations. In India, Ayurvedic medicine has used many herbs such as turmeric possibly as early as 1900 BC. Earliest Sanskrit writings such as the Rig Veda and Atharva Veda are some of the earliest available documents detailing the medical knowledge that formed the basis of the Ayurveda system. In the present study, menthanol extract of *A. indica* leaves was prepared which was subjected to phytochemical screening and in *vitro* antioxidant and cytotoxicity activity were evaluated.

Phytochemical analysis is of paramount importance in identifying new source of therapeutically and industrially valuable compounds (Ambasta *et al*., 1986). In the present study, qualitative phytochemical screening revealed the presence of flavonoids, tannins, phenols and alkaloids which are very important plant constituents because of their free radical scavenging ability (Kumaran and Karunakaran, 2001). Plants produce a large number of secondary metabolites as bioactive compounds such as alkaloids, tannins, flavonoids, sterols and

terpenes, etc., that have a major role in nutrition, physiology and control of diseases.

Determination of total phenolic content (TPC)

Different parts of the plant have different phytochemical compound, which could contribute to different pharmacological effect of each part. Among the different phytochemicals, phenolic compounds are ubiquitous secondary metabolites in plants. They are known to have antioxidant activity and it is likely that the activity of these extracts is due to these compounds. The antioxidant activity of the phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Osawa, 1994). It is also suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ~1.0 g was daily ingested from a diet rich in vegetables and fruits. Epidemiological studies confirmed that phenolics, namely flavonoids, appear to be beneficial compounds in various stages of carcinogenesis (Tanaka *et al*., 1998; Krishnaraju *et al*., 2005).

They have a complementary and overlapping mode of action, including antioxidant activity, scavenging free radicals and modulation of carcinogen metabolism that alter important cellular and molecular mechanisms related to carcinogenesis, a multistep process involving the transformation, survival, proliferation, invasion, angiogenesis, and metastasis of the tumor cells. In this study, total phenolic content of different extracts of *A.indica* was determined. Concentrations of phenolic content were expressed in terms of gallic acid equivalent (GAE). Among the extracts, methanol extract exhibited higher range of TPC (9700mg GAE/L) than other extracts.

Antioxidant assay

Ultraviolet light, ionizing radiation, chemical reactions and metabolic processes can induce the production of reactive oxygen species in the cells. These reactive oxygen species can cause lipid peroxidation, DNA damage, protein peroxidation and cellular degeneration in the cells and can induce numerous diseases. Certain phytoconstituents are commonly found in both edible and non edible plants, and have multiple biological effects, including antioxidant activity (Beyer, 1994).

Antioxidants play a central role in defending free radical species which are formed from various biochemical reactions in normal system. High amounts of free radical molecules cause oxidative stress in cell which results in destructing important macromolecules like DNA, lipids and proteins. The mutilation of macromolecules leads to inflammation and several deteriorating disorder such as Parkinson's disease, aging, diabetes, atherosclerosis, ischemic heart disease, immunosuppression, and reduced membrane fluidity (Hoffmann, 2004).These free radicals are the main culprits in lipid per oxidation.

In various diseases, there is extreme production of O^{2-}, OH radicals as well as non – free radical's species (H_2O_2) and activation of phagocytes, which can harm severely tissues. Peroxide and –OH radical formed from lipid peroxidation, result side effects in the body such as heart attacks and stroke. Phytodrug having antioxidant property is nontoxic or has less side effects than synthetic compounds. In this concern, our attempt is to search out an herbal drug to substitute synthetic ones. In the present study, different concentrations of plant extract were subjected to analyses *in vitro* antioxidant activity using various models such as DPPH free radical scavenging assay, reducing power assay, total antioxidant activity, hydrogen peroxide scavenging activity and nitric oxide scavenging activity**.** Antioxidant assay was also performed for standard ascorbic acid to compare the efficacy of the selected plant..

DPPH radical scavenging activity

The DPPH free radical is a stable free radical, which has been widely used for estimating the free radical-scavenging activities of plant extract. In free radical scavenging activity, DPPH accepts an electron or hydrogen radical to become stable diamagnetic molecule. Phyto constituents of the extract are possibly involved in their free radical reactions by reducing the stable DPPH radical to yellowish coloured diphenylpicryl hydrazine derivative (Mosmann, 1983).

In the present study, dose dependent DPPH radical scavenging activity was observed in plant extract. Highest scavenging activity was at 250 µg/ml in the value of 33.5%. There was moderate activity towards DPPH scavenging when compared to ascorbic acid. Ascorbic acid is acting as a chain breaking antioxidant activity impairs with the formation of free radical in the process of formation of intracellular substance throughout the body, including collagen, bone matrix and tooth dentine (Beyer, 1994).

Reducing power assay

Reducing power is an indicative of reducing agent having the availabilities of atoms which can donate electron and react with free radicals and then convert them into more stable metabolites and terminate the radical chain reaction. The reducing ability of a compound generally depends on the presence of reductants, which have been exhibited anti oxidative potential by breaking the free radical chain and

donating a hydrogen atom. The reducing power of plant extract was analyzed by electron donation ability (Blazovics *et al*., 2003). In the present study, dose dependent reducing power was observed in plant extract which was less than that of standard. Highest reducing power was observed (25.2%) at 250µg/ml. Antioxidant activity may be probably due to phenolic compounds present in the extract.

Total antioxidant capacity

Antioxidant activity is tremendously important potentiallity which possesses the ability to protect the body from damage caused by free radical induced oxidative stress. Antioxidant activity of plant extract is the most efficient way of combating tissue injuries, undesired transformations and preventing health risks (Sindhu *et al*., 2010). In the present study, dose dependent total antioxidant activity was observed in the plant extract and the values were range of from 19.8 to 34% at 50-250µg/ml. Total antioxidant capacity of plant extract was almost similar to that of standard.

Hydrogen peroxide scavenging activity

Hydrogen peroxide, although not a radical speies, plays a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally occurring iron complexes inside the cell believed to react with H_2O_2 *in vivo* to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects (Miller *et al*., 2000). Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with $Fe^{2+,}$ and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Hydrogen peroxide scavenging activity of *Aristolochia indica* was evaluated and the results revealed that remarkable scavenging activity of plant extract towards H_2O_2 radical in a dose dependent manner. Highest value 37% was obtained at highest concentration of 250µg/ml.

Nitric oxide scavenging activity

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. NO is generated in biological tissues by specific nitric oxide synthesis (NOSs), which metabolizes arginine to citrulline with the formation of NO via a five electron oxidative reaction (Ross, 1993). These compounds are responsible for altering the structural and functional behavior of many cellular components. In the present study, highest nitric oxide scavenging activity was observed at 250µg/ml and percentage of reduction value was 37.3%. Incubation of solutions of sodium nitroprusside in PBS at 25Úc for 2 h resulted in linear time dependent nitrite production, which is reduced by the tested plant extract.

Cytotoxicity assay

Cancer is one of the diseases that occurs in both developed and developing countries and is the leading cause of death. An extensively used treatment for cancer is chemotherapy and one of the major drawbacks is the toxicity that is caused to the normal cells due to the inability of the chemical drugs to differentiate between normal and cancerous cells (Balamurugan *et al*., 2014). Globally, more than 20% enhancement in breast cancer since 2008 with a newly diagnosed cases of 1.7 million in 2012; and there are 6.3 million women alive with breast cancer with 14% increase in mortality rate in preceding 5 years.

Ongoing research is being done throughout the world to seek out effective treatments for cancer, including the use of plants to relieve and treat cancer patients. This treatment makes use of the compounds naturally present in plants that are known to inhibit or kill carcinogenic cells. Hence, the present study has been designed to evaluate *in vitro* cytotoxicity activity of medicinal plant *Aristolochia indica* by MTT assay. Five different concentration (5, 10, 15, 20 and 25µg/ml) of the leaves extract of *Aristolochia indica* were subjected to evaluate *in vitro* anticancer activity against MCF-7 cell lines by MTT assay. Standard Doxorubicin was also evaluated to compare the efficiency of anticancer activity of plant extract. The results showed plant extract decreased cell viability and inhibit cancer cell growth in a dose dependent manner. Maximum percentage of cytotoxicity was found to be at 30µg/ ml percentage cytotoxicity of plant extract which was less than that of standard.

CONCLUSION

In conclusion, the methanol extract of *Aristolochia indica* leaves exhibits good antioxidant and cytotoxicity activities which might be due to the presence of phenolic compounds. Further studies are needed to examine underlying mechanisms of antioxidant and cytotoxic effects of *Aristolochia indica*.

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