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Phyto-chemical analyses and antimicrobial activity in partially purified protein derived from roots of *Aristolochia indica* L. : An Indian Birthwort being practiced in the Traditional Medicine System

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Abstract- *Aristolochia indica*, L. an Indian Birthwort is a twisting perennial plant with an ancient history of ethno-medicinal use. The focal objective of the study intended to make accessible the contemporary information on the traditional uses of root part (core focus), its chemical constituents, pharmacological importance, antimicrobial efficacy and DPPH radical scavenging activity in the root extract of *A. indica.* In addition, the rationale was to accentuate the prospective uses of this plant root as a further scope of research in terms of newer phyto-medicine or as a lead factor with a configure of functional drug. Phyto-chemical analyses were done in the root extract of *A. indica*, then, the buffer extraction from roots of *Aristolochia indica,* L. was obtained and the protein was concentrated by adding known amount of ammonium sulphate to the extract. Then, the extract was subjected for fractionation through sephadex G-75 Column. As per the accomplished research literature, the diverse chemical compounds including aristolochic acids, aristolactam, phenanthrenes, alkaloids, lignans, steroids and terpenes are revealed in the different parts of the plant. In the present study, the generated data on phytochemical constituents were logically analyzed. Subsequently, in the protein extraction processes, three different peaks of protein, P1, P2 & P3 were obtained and the Peak - 3 samples was subjected for refractionation through the same column. As a result, P4, P5 & P6 were obtained and the protein of highest peak (P5) was directed to assess for different bio-molecules like, total protein, poly phenol and total sugar. Further, the antimicrobial activity of P5 sample which was obtained from the refractionation of *Aristolochia* root extract was tested against *Escherichia coli* strain using disc diffusion method. It showed significant inhibitory effect as compared to the standard antibiotic Gentamycin. The preliminary data have confirmed that the most significant activity that supports the traditional use of plant-root as a key component in their drug formula of therapeutic system. The inclusive data pertaining to the dose of crude root extracts and its protein component needs to be purified for ascertaining the drug functionality and its mechanism.

Key words: *Aristolochia indica*, (Eshwari balli), Phyto-chemical analyses, Protein purification, Antimicrobial activity, DPPH activity

INTRODUCTION

Ethno-medicinal plants are the very basis for the accomplishment of several number of extremely efficient drugs. These traditional medicinal plants are functional for healing as well as for therapeutic role of many serious human diseases because of the presence of phyto-chemical constituents.[1-5] Phyto-chemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various ailments and

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disorders. These active Phyto-chemical constituents are the primary and secondary compounds and play a vital role in preventing various diseases such as, the antidiuretic, anti-inflammatory, anti-analgesic, anticancer, antiviral, antimalarial, antibacterial and antifungal activities of the medicinal plants are due to the presence of the above-mentioned secondary metabolites.[6-9] However, ethno-medicinal plants are used for discovering and screening of the phyto-chemical constituents which are very much helpful for the manufacturing of new drugs and they are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs.[10-17] Plants are naturally constructed with various other phyto-constituents and active molecules such as vitamins, terpenoids, phenolic acids, lignin, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, and other metabolites, which are rich in antioxidant activity.

The *Aristolochia indica* L. – an Indian Birthwort belongs to the family Aristolochiaceae. This medicinal plant is bitter and poisonous and generally contains alkaloids; a few are medicinal and are formerly reputed to be useful in the treatment of snake bites.[18,19]

Dried stem and root are used as traditional drug. It should be used in minimal doses; the drug promotes digestion and controls menstruation. In higher doses, it may prove lethal. It is used as a stimulant, tonic and emmenagogue in fever and some gynaecological problems. In moderate doses, it is used as a gastric stimulant and in minor ailments of children such as flatulence and dyspepsia. Roots of *Aristolochia* are used for the treatment of snake bites. The Powdered root of *Aristolochia indica,* L. with honey is given for Leucoderma. The juice of the leaves is regarded as a specific for cobra poisoning and it is also used for cough. It is a purifier of blood and hence useful in skin diseases.

In India, it is declared that traditional healers use 2500 plant species, and 100 species of plants serve as natural principles of medicine**.** With a view to increasing the wide range of medicinal usages, the present day entails new drugs with more potent and desired activity with less or no side effects against particular disease.[20-25] *Aristolochia* is an important genus in the family of Aristolochiaceae. The genus *Aristolochia* consists of about 400 species of herbaceous perennials, under shrubs or shrubs bearing essential oils and is widespread across tropical Asia, Africa, and South America. Apart from these *Aristolochia* species has been used extensively in the traditional Chinese medicine. Its diverse biological functions include hypertension relief, leukocyte enhancement, rheumatism relief, edema therapy, as well as analgesic and diuretic effects.[13] Various *Aristolochia* species have been used in herbal medicines since antiquity in obstetrics and in the treatment of snakebite, festering wounds, microbial infections and tumors and they remain in use particularly in Chinese herbal medicine.[26-29]

However, the ongoing growing recognition of medicinal plants is not only due to the above reasons, but also including an escalating faith in herbal medicine. Besides, allopathic medicines may cure a wide range of diseases; however, its high prices and side-effects are causing many people to return to herbal medicines which have fewer side effects.[5] So, the instant rising demand of plant-based drugs is unfortunately creating heavy pressure on some selected high-value medicinal plant populations in the nature due to over-harvesting.

Hence, keeping the above fact in consideration, *Aristolochia indica,* L. – a very important ethno-medicinal plant with a core focus on root part; was selected for phyto-chemical analyses, and for antimicrobial activity against *Escherichia coli* respectively.

MATERIALS & METHODS

The ethno-botanical survey was made at B.R Hills- a religious hillock where the plant was collected with the help of traditional healers and subjected for taxonomical classification with the distinguished expert; Dr. Prasajit Mukherjee, Plant Taxonomist, Ranchi University, Ranchi, Jharkhand, India.

The taxonomical details[24] of *Aristolochia* are as follows.

Scientific classification

Kingdom	:	Plantae
Order	:	Piperales
Family	:	Aristolochiaceae
Subfamily	:	Aristolochioideae
Genus	:	*Aristolochia*
Species	:	*A. indica*

Further, the study was carried out at Department of Biotechnology, PES University (RR Campus), Bengaluru, Karnataka, India. Some specific analysis was done at Department of Studies in Molecular Biology, University of Mysore, Karnataka, India.

MATERIALS

The collected root of *Aristolochia indica* and its ethno-pharmacological practices were validated with the Government Ayurvedic Medical College, Mysore, Karnataka. The dried root of *Aristolochia indica* Lin is about 10-20 cm long and 1.5–5 cm in diameter, cylindrical, tapers. It externally appears to be grayish brown, rough and longitudinally wrinkled and can be easily broken.[12,30] Inside is starchy, exhibiting alternately greyish brown and whitish radial lines. It is extremely bitter in taste (Plate-1a-e). The Pure culture of *Escherichia coli* was obtained from the authorized hospital, Mysore (Fig.2a & b).

PLATE-1

Fig. 1a. *Aristolochia indica*, L.- A habit

Fig. 1b. Showing Leaf & flower of *A. indica*, L.

Fig. 1c. Duck Flower of *A. indica*

Fig. 1d. Dry pods and seeds of *A. indica*

Fig. 1e- Showing root samples and powdered root of *Aristolochia indica*, L.

Figure- 1

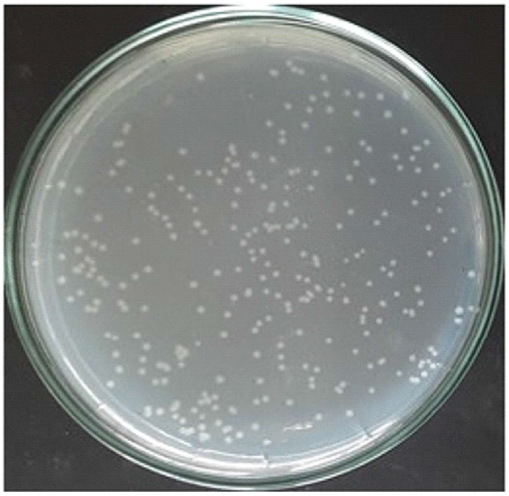


Fig.2a. Growth of *E. coli* on Nutrient Agar Medium

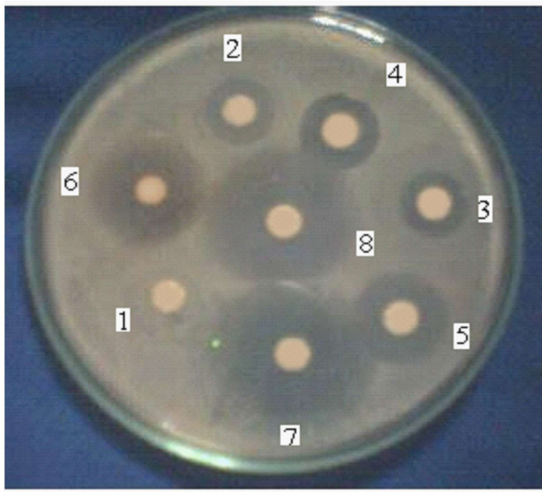


Fig.2b. Inhibitory effect of root extract of *A. indica* sample against *E.coli*

Bovine Serum Albumin (BSA), sodium carbonate, copper sulphate, sodium potassium tartarate**,** Folin ciocalteau reagent, phenol, sulphuric acid, gallic acid, beef extract, yeast extract, peptone, NaCl and Agar.

Other requirements: Sephadex G-75 column from commercial sources of reputed brand and Glass wares, plastic wares etc. All the Chemicals used in this experiment were of analytical grade obtained either from Merch, Sigma, SRL, Ranbaxy, Ranchem, Glaxo, Himedia etc.

METHODOLOGY

I. Extraction from roots of *Aristolochia indica*, L.

Aristolochia indica, L**.** roots were washed, shade dried and powdered. 50gms of powdered root was mixed well with 200ml of 0.1M citrate phosphate buffer of pH 6.8 and left overnight at 4°C. The supernatant was decanted. The residue was squeezed using a muslin cloth. The liquid obtained was mixed with supernatant. Then it was centrifuged at 5000 rpm for 1hour at 4°C. The optical density was measured at 280nm and the absorbance spectrum was taken for the clear extract between the range 200 to 400 nm.[31]

Note: The optical density of the extract was adjusted to approximately 1 by diluting the extract sample using distilled water.

II. Protein extraction and purification process

Salting out of Protein

To the 100ml supernatant thus obtained, 76g of ammonium sulphate was added to precipitate the protein and left overnight at 4°C. This solution was then centrifuged at 5000rpm for 1hr at 4°C. The supernatant was discarded and the pellet was dissolved using 50ml of distilled water.

Resolution of *Aristolochia indica*, L. root extract by Gel filtration on Sephadex G-75 column

The 2ml of the concentrated protein solution obtained after ammonium sulphate precipitation was loaded onto a Sephadex G-75 column (1.5cm x 55cm) which was pre equilibrated with 0.1M NaCl and the flow rate was adjusted to 20ml/hr and 2ml fractions were collected in each tube. The protein elution was monitored at 280nm using a spectrophotometer.

Re-chromatography on Sephadex G-75 Column

The pooled fraction of peak III from Sephadex G-75 column was applied onto Sephadex G-75 column pre-equilibrated with 0.1M NaCl. Elution was carried out in the same solution. The rechromatography was carried out at room temperature. The flow rate was adjusted to 20ml/hr and 2ml fractions were collected in each tube.

III. Estimation of bioactive constituents in *Aristolochia indica*, L.

The root extract of *Aristolochia indica*, L. was obtained using 0.1M citrate phosphate buffer of pH 6.8. Protein was concentrated by adding known amount of ammonium sulphate to the extract. Ammonium sulphate precipitated *A.indica*, L. root extract and refractionated sample were used for estimation of total protein[32], total sugar[33] and total poly-phenol[34] respectively.

IV. Determination of Antimicrobial activity

The peak III fraction of crude sample was further re-chromatographed on Sephadex G-75 column. The fraction obtained was tested for antimicrobial activity against E-coli by Disc diffusion method. The concentration of the extract used 5µg to 50µg. Gentamycin was used as standard antibiotic in the centre of the plate (10µg).

V. DPPH free radicals Scavenging Activity

The free radicals scavenging activity of Root extract of *Aristolochia indica*, L. was measured as per the standard procedure by decreased the absorbance of test solution of DPPH (2,2-Diphenyl-1- picryl hydrazyl). The absorbance was determined at 517 nm and from these values corresponding percentage of inhibitions were calculated. Then a % inhibition was analyzed against concentration and IC50 was calculated using the formula. The experiment was performed in triplicate and average absorption was noted for each concentration. The positive control, ascorbic acid was maintained.[35]

VI. Statistical analysis

The data for phyto-chemical and antimicrobial parameters were analyzed and expressed as mean ± standard deviation. The IC50 values were calculated from linear regression analysis. Results were processed by a computer program, Microsoft Excel (2007).

RESULTS

In the present investigation, all the obtained data are represented in the form of plates, tables and graphs respectively.

Plant material

The details on medicinal plant species *Aristolochia indica* is explored compressively in the data base. The plant is expansively distributed in Nepal, India, Sri Lanka and Bangladesh respectively. The plant herb is found all over the plains and low hilly part of India especially in tropical and subtropical regions like BR Hills, Chamaraja Nagara district, Karnataka and also in the low country part from west Bihar, Orrisa, Puri, Bengal, Konkan and popular of districts of southern India. It is reported to be endangered species in northern part of India including Gujarat and Rajasthan.

Phyto-chemical study

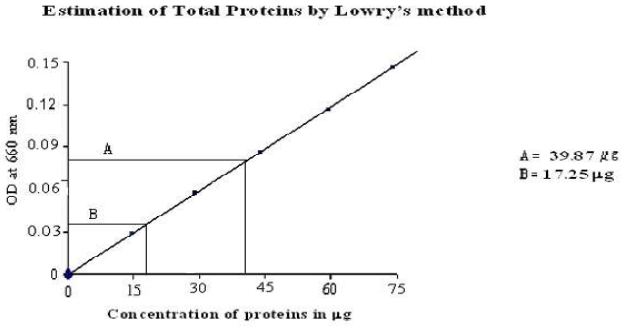
Phyto-chemicals are biologically active compounds presents in plants used for food and medicine. Phyto-chemical characteristics of the root extract of *A. indica* investigated are summarized in Table 1. Preliminary phyto-chemical study reveals the presence of glycosides, carbohydrate in all the three hexane, ethyl acetate and methanol extracts, presence of steroids in hexane ethyl acetate and methanol extract, presence of flavonoids and terpenoids in hexane and methanol extract, presence of tannins and phenol in methanol extract, presence of coumarins in hexane extract followed by absence of quinones, cardiac glycosides, anthraquinones, alkaloids, saponins, and protein respectively. All the extracts were subjected to further analytical tests for the quantification of phyto-chemical constituents.[9,13]

Table 1. Phyto-chemical analysis in root extract of *Aristolochia indica* Linn.

Phyto-chemicals	Aqueous	Ethyl acetate	Hexane	Methanol
Protein	+	-	-	-
Carbohydrates	-	+	+	+
Coumarins	+	-	+	-
Carotenoid	-	+	+	+
Steroids	+	+	+	-
Quinones	+	-	-	-
Phenols	+	-	-	+
Terpenoids	-	-	+	+
Tannins	+	-	-	+
Anthraquinone	+	-	-	-
Flavonoids	+	-	+	+
Alkaloids	+	-	-	-
Glycosides	+	+	+	+
+: Present, -: Absent				

Estimation of Protein

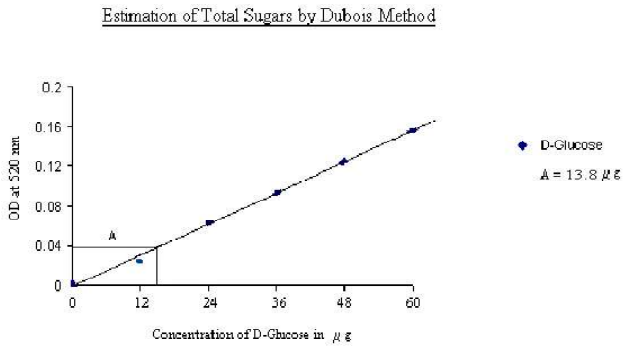
The total protein content of the sample was estimated by method of Lowry *et al.*, (1951)[32] using Bovine Serum Albumin (BSA) as standard reference. 20µl of the ammonium sulphate precipitated sample yielded 39.87µg of protein. 1 ml of sample contains 1993.5 µg or 1.993mg of protein. 2ml of the sample with protein concentration of 3.986mg was loaded on to Sephadex G-75 column. About 200l of P5 sample was estimated for total protein which yielded 17.25µg of protein (Graph 1).



Graph - 1 Showing total content of Protein in root extract of *Aristolochia indica*, L.

Estimation of total sugar

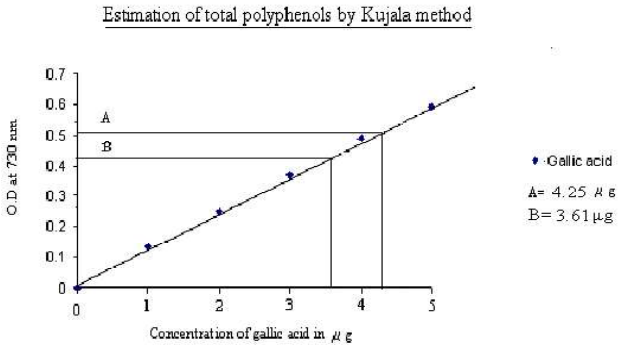
The total sugar concentration was estimated by Dubois method[33] using glucose as standard reference. The total sugar content of the ammonium sulphate precipitated protein extract was found to be 690µg/ml. (Graph - 2).



Graph - 2 Showing total content of Sugars in root extract of *Aristolochia indica*, L.

Estimation of total polyphenol

Total content of phenolics was determined according to the method of Folin ciocalteau reaction[34] with minor modifications, using gallic acid as standard. The polyphenol content of the ammonium sulphate precipitated protein extract was found to be 212.5µg/ml. The protein content of the P3 sample resulted in net yield of 180.5µg/ml (Graph -3).

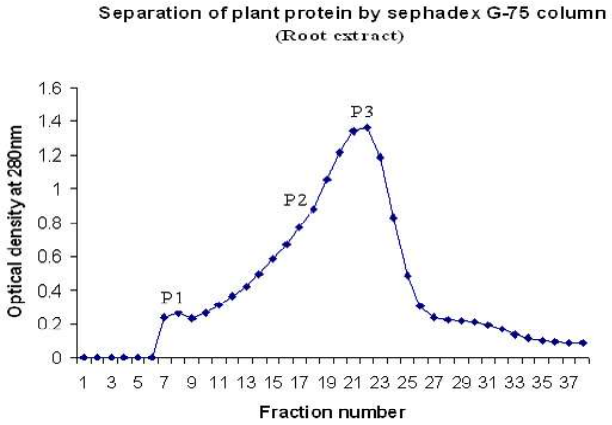


Graph -3 Showing total content of Polyphenols in root extract of *Aristolochia indica*, L.

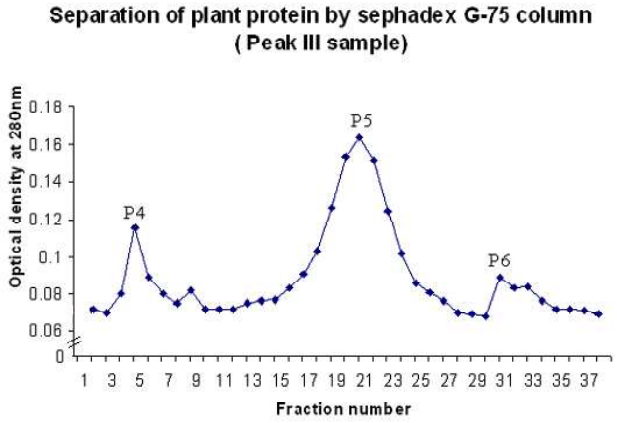
Extraction of protein

Root extract of *Aristolochia indica*. L was obtained using 0.1M citrate phosphate buffer of pH 6.8 (Plate-1e). The protein was concentrated by adding known amount of ammonium sulphate to the extract. Ammonium sulphate precipitated A. indica root extract was fractionated through Sephadex G-75 column.

Fractionation of the ammonium sulphate precipitated *A. indica* root extract through Sephadex G-75 column (Graph - 1) resolved into three peaks namely; P1, P2 and P3. The peak obtained could probably indicate mixture of proteins or proteins with many subunits. Fractions number 23 and 24 were pooled separately and re-fractionated through Sephadex G-75 (Graph 4). Re-fractionation of fraction no. 23 and 24 yielded 3 major peaks labeled Peak 4 (P4) Peak 5 (P5) Peak 6 (P6) (Graph -5).



Graph - 4 Sephadex G 75 column chromatography of ammonium sulphate precipitated` *A. indica* root extract



Graph - 5 Re-chromatography of P3 sample on Sephadex G -75 Column

Antimicrobial activity

Anti-microbial activity of P5 sample which was obtained from the refractionation of *Aristolochia indica,* L. root extract was tested against *E.coli* strain by Disc diffusion method.

2, 4, 6, 8, 10.12,14 µl of samples showed 0, 12, 13, 14, 15, 16, 29mm diameter of zone of inhibition respectively (Table 2). Antibiotic Gentamycin showed 26mm diameter of zone of inhibition. Thus, it was seen that Root extract of *Aristolochia indica* showed significant inhibitory effect

compared to antibiotic Gentamycin *i.e*., 29mm in sample-7 compared to sample-8 (gentamycin) The IC50 value was found to be most significant (Fig- 2b and Table-2).

Table 2. Antimicrobial activity of root extract of *Aristolochia indica*, L. against *E.coli*

Sl. No.	Amount of sample added	Zone of inhibition (Diameter in mm)
1	2 µl	0.0
2	4 µl	12.0
3	6 µl	13.0
4	8 µl	14.0
5	10 µl	15.0
6	12 µl	16.0
7	14 µl	29.0
8	Gentamycin	26.0

DPPH free radical scavenging activity:

Aqueous root extract of the *Aristolochia indica* (L) displayed significant antioxidant activity where the IC50 was 26.14 mg/mL, against DPPH free radical was recorded through standard calibrations (Table 3).

Table 3. Analysis of Antioxidant activity of *Aristolochia indica,* L

Extract Sample	Concentration (µg/mL)	Inhibition (%)	IC50 (µg/mL)
Root aqueous extract of *Aristolochia indica*, L	5	2±0.016	186.46
	10	1±0.014	
	25	14±0.035	
	50	32±0.031	
	75	66±0.038	
	100	85±0.044	
Standard Ascorbic acid	5	18±0.015	8.2
	10	41±0.020	
	25	62±0.024	
	50	96±0.048	
	75	97±0.042	
	100	96±0.056	

DISCUSSION

Even though, the pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has been increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents.[18,28,36] The use of plant extracts with known antimicrobial properties can be of great significance in therapeutic treatments. Thus, according to the present investigation, *Aristolochia indica*, L. plant root can be used as an antimicrobial agent against *E.coli*. This has been supported by Shafi *et al*., (2002)[26]; Konning *et al*., (2004)[11]; Kumar *et al*., (2011)[19].

The roots of *Aristolochia indica*, L. have been found to contain - an alkaloid, aristolochine, $C_{17}H_{11}O_3N$, a yellow bitter principle isoaristolochic acid $C_{17}H_{11}O_7N$ and allantoin and thus, the presence of above constituent might have influenced the antimicrobial activity and also as a therapeutic agent.[7]

In the present investigation, the samples were obtained from the re-fractionation of root extract through Sephadex G- 75 column. The sample contained 86.25µg /ml of protein, 690.0 µg /ml of total sugar and 180.5µg/ml of poly phenols. The root extract of *Aristolochia indica*, L. showed significant inhibitory effect compared to antibiotic Gentamycin. It also showed different inhibitory effect at different concentrations. This is in accordance with the earlier reports of Almagboul *et al.*, (1985)[1]; Arora and Bharadwaj (1987)[2]; Mau *et al*., (2001)[20]; Prashanth Kumar *et al.*, (2006)[16] and Panduranga Murthy *et al.*, (2011)[37].

Phyto-chemical screening shows that flavonoids content is significantly superior in root of *Aristolochia indica*. Polyphenolic compounds, like flavonoids, tannins and phenolic acids have the antioxidant activity. Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer.[23] An antioxidant is any substance that, when present at low concentrations significantly prevents oxidation of cell content like protein, lipid, carbohydrates and DNA. The presence of bio-molecules like protein, sugar and phenols in the root extract of *Aristolochia indica*, L. indicates the efficacy of bioactive constituents and thus inhibits the growth of *E.coli*.

SUMMARY & CONCLUSION

Plants and their compounds with anti-inflammatory and anti-hepatotoxic properties are known to neutralize microbes. *Aristolochia indica* possesses both these properties and hence was studied for antimicrobial property against *E.coli*, followed by DPPH radical scavenging activity.

Plant root extract was obtained from 0.1M citrate phosphate buffer of pH 6.8. To the root extract obtained, known amount of ammonium sulphate was added in order to salt out the protein. The concentrated protein solution obtained after ammonium sulphate precipitation was passed through Sephadex G-75 to separate the molecules based on their molecular mass. It resolved into three peaks namely

P1, P2 & P3. The peak-3 sample was again subjected for re-fractionation through the same Sephadex G-75. It resolved into three peaks. Third peak (P5) sample which was found to contain homologous protein was tested for antimicrobial activity against *E.coli* by disc diffusion method. It reveals that, when compared to the standard antibiotic, Gentamycin, the inhibitory effect of root extract of *Aristolochia indica,* L. has been observed and was significantly superior over antibiotic treatment. Besides, other biomolecules like, total Protein, total sugar and Polyphenol content was estimated by Lowry, Dubois and Kujala method respectively.

It can be concluded that, the extract of root protein of *Aristolochia indica*, L. showed better Antimicrobial activity against *E.coli* and exhibited significant antioxidant activity. The presence of bioactive molecules in the plant root validate that it may be a good source of cancer chemotherapeutic agent. Hence, further research is also requisite at isolation of lead constituents and detection in line with molecular level to confirm all the parameters studied.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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