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RESEARCH ARTICLE

A comparative study on *in-vitro* antioxidant, anticancer and antimicrobial activity of the methanol extracts of the roots of four species of *Aristolochia* L. from southern Western Ghats of India

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Abstract

'Green medicine', the drugs derived from plants attained a wide spread interest, as believed to be safe and dependable, compared with costly synthetic drugs that have adverse effects. The present study is an attempt to evaluate the antioxidant, anticancer (cytotoxic) and antimicrobial effects of the methanol extract of the roots of four ethnomedicinally important *Aristolochia* species from southern Western Ghats. Antioxidant activity was evaluated by three separate methods: DPPH free radical scavenging, reducing power assay, NBT superoxide radical scavenging assay. Out of four *Aristolochia* species tested, *A. bracteolata*, *A. tagala* and *A. krisagathra* recorded significant antioxidant activity. Anticancer activity of the extracts was tested against Breast cancer cell lines (MDAM B-231), Cervical cancer cell lines (HeLa), Lung cancer cell lines (A 549) and Colon cancer cell lines (HTL 116) using MTT method. Methanol extract of *A. krisagathra* recorded significant activity and best activity was recorded against HeLa and MDAM B-231 with IC_{50} value of 25 µg/ml. Antimicrobial activities was recorded by minimum inhibitory concentration and agar disc diffusion method. The maximum antimicrobial activity was found in the methanol extract of *A. indica*, which inhibited all the ten tested bacteria in relatively low concentration below 125µg/ml, except *Salmonella typhi* and *Staphylococcus simulans*. The extract of the *A. tagala* also recorded relatively good antibacterial activity which inhibited all of the tested bacteria. To the contrary, the extracts of the candidate species showed poor antifungal activity. The results of the present study showed that *Aristolochia* species demonstrated a strong antioxidant, anticancer and antibacterial activity and deserve detailed pharmacological studies.

Introduction

Plants are considered as one of the main sources of biologically active compounds. In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs against diseases and disorders, the potential of bioactive plants or their extracts to provide new and novel compounds for disease treatment and prevention is still enormous [1].

Reactive oxygen species (ROS) are continuously formed as a by-product of metabolisms in aerobic organisms and are also produced on exposure to tobacco smoke, ozone, radiations, organic solvents and other environmental pollutants [2]. ROS play an important role in various physiological processes in humans, including;

energy production, phagocytosis, cellular signal transduction, cell proliferation, differentiation and apoptosis. On the other hand, overproduction of ROS can induce oxidative damage to all biomolecules (lipids, carbohydrates, proteins, enzymes, DNA and RNA) and acts as a mediator of numerous diseases and disorders, e.g. inflammation, arthritis, diabetes, arteriosclerosis, cancer, genotoxicity, and neurological disorders such as Alzheimer's disease [3]. Antioxidants are very essential for preventing degenerative reactions produced by free radicals and reactive oxygen species, which have been concerned with many diseases and in food deterioration and spoilage [4]. However, the safety of some of the synthetic antioxidants used in the food industry has been questioned, because recent studies recognized they might be carcinogenic [5]. Hence, there is an emerging interest in natural antioxidants, which might help to prevent oxidative damage [6].

In this background, several studies have been carried out on natural sources to unravel the components which possess antioxidant properties and with low cytotoxicities. Natural antioxidants are generally more desirable for consumption than the synthetic one such as butylated hydroxyanisole (BHA) which was reported to be carcinogenic to humans. Recently, many studies have been carried out on the antioxidant properties of phenolic compounds which have aroused increasing interest in the isolation of such compounds present in the plants [7].

Infectious diseases are still one of the major threats to public health, despite the tremendous progress made in human medicine. Their impact is particularly large in under developing countries due to the relative unavailability of medicines, uncontrolled use and the emergence of widespread multi drug resistance [8]. Plant-based antimicrobials represent a vast untapped source for drugs and hence have enormous therapeutic potential. Therefore, interest in higher plants those possess antimicrobial activity has increased in recent years [8].

Aristolochia L. of family Aristolochiaceae is a genus of herbaceous perennials, evergreen, vines that consists of 120 species distributed in the tropical and warm regions of old world [9]. They possess pungently aromatic tuberous roots. In traditional Chinese medicine *Aristolochia* species are used for certain forms of acute arthritis and edema. In southern India, several tribes used the root of *A. bracteolata*, *A. indica* and *A. tagala* against poisonous bites, skin diseases etc. They were not so far subjected for a comprehensive antioxidant, anticancer and antimicrobial studies and compared.

Materials and methods

Plant Material

The roots of the four candidate species of *Aristolochia* such as *A. bracteolata, A. indica, A. krisagathra* and *A. tagala* were collected from natural habitats in southern part of Western Ghats. The voucher specimen of each species (Anilkumar 40635, Pazhavoor, Tamil Nadu, India; Anilkumar 40634, Kavanadu, Kollam, Kerala, India; Anilkumar 40630, Ponmudi, Thiruvananthapuram, Kerala, India and Anilkumar 40631, Ponmudi, Thiruvananthapuram, Kerala, India respectively) were deposited in the Herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala, India (TBGT).

Preparation of the extracts

The methanolic extracts of the powdered dried root of the four *Aristolochia* species were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -20°C for further studies. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments.

Antioxidant activity

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of crude extracts was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH). The method used was similar to that previously used by Ibanez et al. [11] and Dorman et al. [12] with suitable modifications. The method involves the reaction of methanol extract with the stable DPPH in 0.1 mM methanol solution. The reaction mixture contained 300 µl of test compound of varying concentrations (10–100 µg/ml) and 2 ml of DPPH solution. After 10 minutes, the change in absorbance was recorded at 517 nm in a spectrophotometer (Systronic, India) against a blank, which did not contain the test compound. Then the absorbance was measured at 517 nm in spectrophotometer. Butylated hydroxyanisole (BHA) was used as positive control. The DPPH radical concentration was calculated using the following equation:

DPPH scavenging effect (%) = [(A0−A1)/A0] ×100

Where A0 is the absorbance of the negative control and A1 is the absorbance of reaction mixture or standards.

Reducing Power Assay

The reducing power assay of crude extracts was determined according to the method of Oyaizu [12]. Initially 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) were added to different

concentrations of methanol extract (1 ml). The reaction mixture was allowed to incubate at 50°C for 20 minutes. Then 2.5 ml of trichloroacetic acid (10%) was added to the reaction mixture, which was then centrifuged at 9500 rpm for 10 minutes. The upper layer of solution (2.5 ml) was recovered and mixed with 2.5 ml distilled water and 2.5 ml $FeCl_3$ (0.1%). The absorbance of the solution was measured at 700 nm in spectrophotometer (Systronic, India). Higher absorbance of the reaction mixture indicated that the reducing power is increased. Butylated hydroxyanisole (BHA) was used as positive control.

NBT Superoxide Radical Scavenging Assay

The scavenging activity of the methanol extract towards superoxide anion radicals was measured by following the method of Liu et al. [13]. The superoxide anion was generated in 3 ml of Tris-HCL buffer (100 mM, pH 7.4) containing 750 µl of NADH (936 µm) solution and 300 µl of different concentrations (10–100 µg/ml) of extract. BHA was used as positive control. The reaction was initiated by adding 750 µl of PMS (120 µm) to the mixture. After 5 minutes of incubation at the room temperature, the absorbance was measured at 560 nm. The percent NBT decolourization of the sample was calculated by the equation:

Percent inhibition = (A0 − A1/A0) × 100,

Where A0 is the absorbance of the negative control and A1 is the absorbance of the reaction mixture. The IC_{50} values (the amount required to inhibit superoxide radical formation by 50%) of extracts was calculated.

Anticancer (Cytotoxic) activity

Cell lines

The following cell lines were used in the study: 1. Breast cancer cell lines (MDAM B-231), 2. Cervical cancer cell lines (HeLa), 3. Lung cancer cell lines (A 549) and 4. Colon cancer cell lines (HTL 116) purchased from National Centre for Cell Science, Pune, India and maintained in DMEM supplemented with 10% FBS with antibiotics and antimycotics at 37ºC in a CO_2 incubator. Both cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) fetal bovine serum, l-glutamine (3 mM), streptomycin (100 mg/ml), penicillin (100 IU/ml), and 25 mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Treatment of cell lines

Stock solutions (100 mg/ml) of extracts, made in dimethyl sulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. Cancer cells (5000 cells per well) were seeded into 96-well microtiter plates, and 24 hours later, after the cell adherence, five different, double diluted, concentrations of investigated compounds were added to the wells. Final concentrations applied to target cells were 200, 100, 50, 25 and 12.5 µg/ml, except to the control wells, where only nutrient medium was added to the cells. Nutrient medium was RPMI 1640 medium, supplemented with l-glutamine (3mM), streptomycin (100 µg/ml) and penicillin (100 IU/ ml), 10% heat inactivated (56°C) fetal bovine serum (FBS) and 25 mM HEPES, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 hours.

Determination of Cell Survival (MTT Test)

The effect of extracts on cancer cell survival was determined by MTT test (microculture tetrazolium test), according to Ohno & Abe [14], 72 hours upon addition of the compounds, as described earlier. Briefly, 20 µl of MTT solution (5 mg/mL PBS) were added to each well. Samples were incubated for further 4 hours at 37˚C in 5% CO_2 and humidified air atmosphere. Then, 100 µl of 10% SDS were added to extract the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 hour later. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided with control optical density (the A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. As a positive control cis-diamminedichloroplatinum (Cis-DDP) was used. All experiments were done in triplicate.

Antimicrobial Activity

Microorganisms and Media

The following bacteria were used as test organisms in this study: Gram positive bacteria: *Bacillus subtilis* MTCC 2756, *Staphylococcus aureus* MTCC 902, *S. epidermis* MTCC 435 and *S. simulans* MTCC 3610; Gram

negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642, and *Salmonella typhi* MTCC 3216. Bacterial cultures were maintained on Müller Hinton agar substrates (Hi-media, Mumbai, India). The following fungi were selected for testing the antifungal activity: *Aspergillus flavus* MTCC 183, *Candida tropicalis* MTCC 184, *C. albicans* MTCC 277, *Trichophyton rubrum* MTCC 296 and *Fusarium oxysporum* MTCC 284. All cultures were stored at 4°C and subcultured every 15 days.

The sensitivity of microorganisms to methanol extracts of the investigated species was tested by determining the Minimal Inhibitory Concentration (MIC). Bacterial inoculi were obtained from bacterial cultures incubated for 24 hours at 37°C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standards to approximately 10^8 CFU/ml and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by CLSI.

Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) was determined by the broth microdilution method using 96-well micro-titer plates. A series of dilutions with concentrations ranging from 1 to 2000 µg/ml for extracts was used in the experiment against every microorganism tested. The starting solutions of extracts were obtained by measuring of a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts were prepared in Müller-Hinton broth for bacterial cultures and PD broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin was defined as the MIC for the tested microorganism at the given concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria. A DMSO solution was used as a negative control for the influence of the solvents. All experiments were performed in triplicate.

Antibacterial Assay by Agar Diffusion

Agar disc diffusion technique was used to determine the antibacterial activity of extracts using Mueller Hinton agar medium [15]. The test cultures maintained in nutrient agar slant at 4°C were sub-cultured in nutrient broth to obtain the working cultures approximately containing 1×10^6 CFU/ml. The methanol extract (50 µg/ml) were incorporated in a 6 mm sterile disc. Mueller Hinton agar plates were swabbed with each bacterial strain and the test discs were placed. Ciprofloxacin discs (5 µg/disc) were used as positive control. Plates were incubated overnight at 37°C. Clear, distinct zone of inhibition was visualized surrounding the discs. The determinations were done in duplicates. After 24 hours of incubation, the plates were examined if there is any inhibition zone. The diameters of the inhibition zones produced by each of the concentrations of the solutions were measured in millimeters and interpreted using the CLSI 2006 [16], zone diameter interpretative standards.

Antifungal Activity

MIC of the extract was determined using potato dextrose agar media against the standard fungicide bavistin by the poisoned food technique [17], against *A. flavus*, *F. oxysporum*, and *T. rubrum*. A stock solution of 2000 µg/ml of the test extracts was prepared, which was further diluted with methanol to give the required concentrations ranged from 1000 to 1 µg/ml. One tube was used as solvent control. For *C. albicans*, the broth dilution method [18], was adopted using potato dextrose broth against the standard fungicide amphotericin B. All experiments were done in triplicate for each treatment against each fungus.

Agar Disc Diffusion Method

In vitro antifungal activity of the extracts was measured using agar disc diffusion assay against the test bacteria and fungi. The sterile discs were impregnated with MIC concentration of test compounds. The fluconazole (10 µg/disc) was used as positive reference standards. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. All the assays were carried out in triplicate.

Statistical Analysis

Statistical analyses were performed with the SPSS software packages. All values are expressed as mean ± SD of three parallel measurements.

Results

Antioxidant Activity

DPPH Free Radical Scavenging Activity

The methanol extract of the root of the candidate species were found to be effective in reducing the stable DPPH radical to yellow coloured diphenylpicryl hydrazine, indicating that methanol extract is active in DPPH radical scavenging (Fig. 1 A). Butylated Hydroxyanisole (BHA) tested as control had high scavenging activity. In this study, DPPH antioxidant capacity results were expressed by considering kinetic parameters by testing different initial concentrations (20–100 µg/ml) of the test samples. The highest activity was recorded by *A. tagala* with an IC 50 value at 60 µg/ml, followed by *A. krisagathra* at 80 µg/ml concentration (Fig. 1A). There was a statistically significant difference between the extracts and the control ($P < 0.05$).

Reducing Power Assay

Reducing power is based on the reduction of Fe^{3+} to Fe^{2+} ions in the presence of reductants present in the methanol extract. Reducing power assay is an important parameter used in evaluating antioxidant activities of natural compounds. It inhibits Lipid Peroxidation (LPO) by donating a hydrogen atom, resulting in termination of free radical chain reaction. The present study showed the relative reducing power of methanol extract of four candidate species (Fig.1 B). Highest activity was recorded by *A. bracteolata* having an IC 50 value of 40 µg/ml, followed by *A. krisagathra* (Fig. 1B).

NBT Superoxide Anion Radical Scavenging Activity

The scavenging of superoxide anion radicals by the tested methanol extracts is shown in Fig. 1 C. All the four methanol extracts showed relatively good superoxide anion radical-scavenging activity, among which, *A. bracteolata* showed maximum superoxide anion radical scavenging activity followed by *A. tagala* and *A. krisagathra* (Fig. 1C), which were almost equivalent to the activity of BHA, the standard antioxidant agent. There was a statistically significant difference between the extracts and the control ($P < 0.05$).

Anticancer Activity

MTT Assay

Cell viability assay of the methanol extract of four candidate species was determined by MTT assay after 72 hours of treatment. Dose-dependent growth inhibition in cell lines was observed when exposed to the methanol extract of four *Aristolochia* species in the range, 10–500 µg/ml. Methanol extract of *A. krisagathra* recorded significant activity and best activity was recorded against Cervical Cancer Cell lines (HeLa) and breast cancer cell lines (MDAM B-231) (IC_{50}–25 µg/ml) (Fig 2C). Methanol extract of *A. indica* also recorded significant activity and the best activity was recorded against MDAM B-231 (IC_{50}–25 µg/ml) and HeLa (Fig 2B), followed by the extract of *A. bracteolata* (Fig 2A). Out of four cell lines tested HeLa cells turns to be most sensitive to the methanol extracts of *Aristolochia* species.

Antibacterial Activity

The antibacterial activity of the four methanol extracts against the tested microorganisms is shown in the Table 1 & 2. The maximum antibacterial activity was found in the methanol extract of *A. indica*, which inhibited all of the tested bacteria in relatively low concentration below 125 µg/ml, except *Salmonella typhi* and *Staphylococcus simulans*. The extract of the Aristolochia tagala also manifested relatively good antibacterial activity which inhibited all of the tested bacteria. *A. bracteolata* and *A. krisagathra* extracts showed comparatively lower antibacterial activity. The result of disc diffusion assay was comparable with that of MIC result as shown in Table 1.

Antifungal Activity

The methanol extracts of Aristolochia species showed poor antifungal activity in general and the best activity was recorded by *A. bracteolata* against *T. rubrum* (16 µg/ml). The result of disc diffusion assay (Table 4) was comparable with that of MIC result (Table 3). In a negative control, DMSO had no inhibitory effect on the tested organisms.

Fig 1: Antioxidant activity of 3, 5-Dihydroxy-4-isopropystilbene in various in vitro assays. **[A]** DPPH free radical scavenging activity, **[B]** Reducing power assay, **[C]** NBT Superoxide anion radical scavenging activity. All the measurements were done in triplicates and the results are expressed as arithmetic mean ± standard error on the mean.

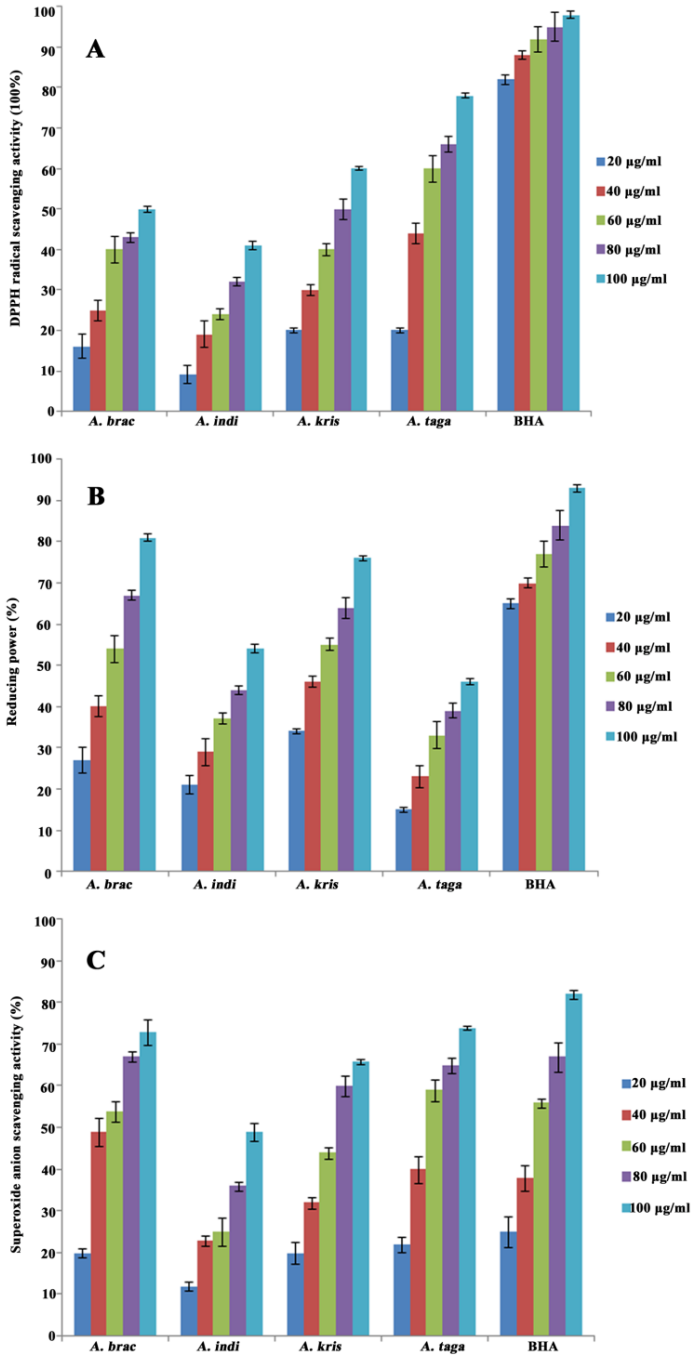
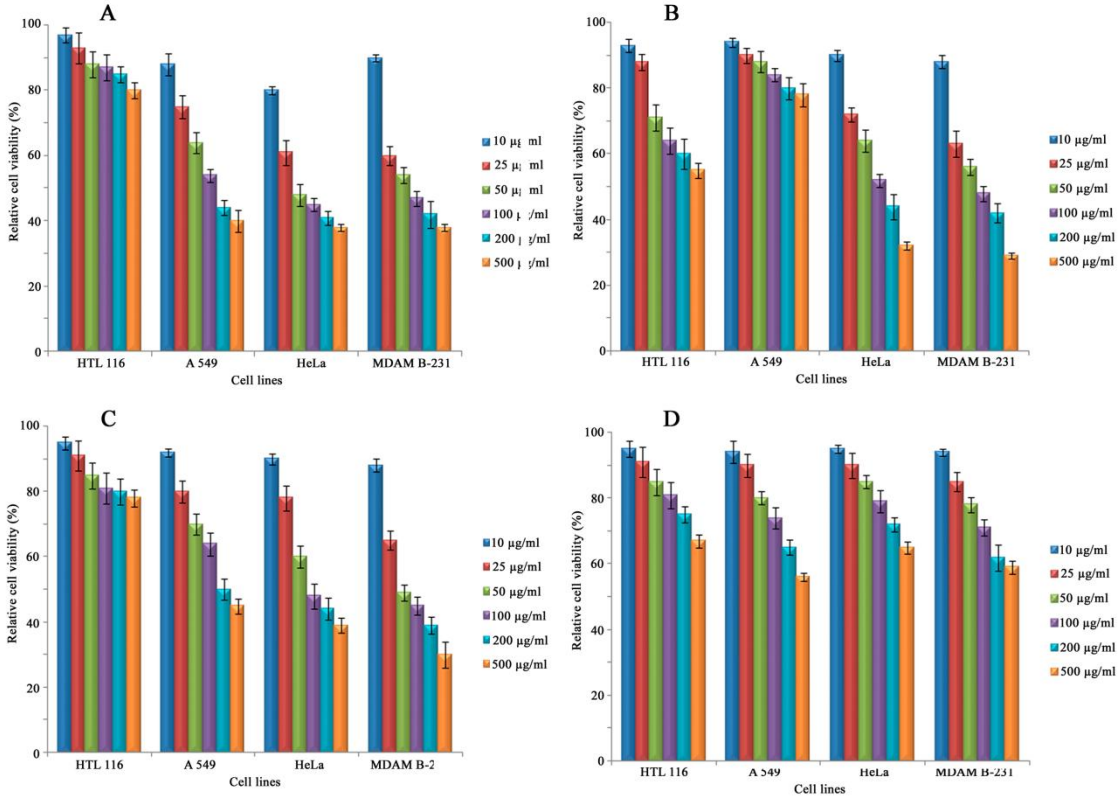


Fig 2: **[A]** MTT assay of methanol extract of *Aristolochia* species against human cancer cell lines. **[A]** *A. bracteolata*, **[B]** *A. indica*, **[C]** *A. krisagathra*, **[D]** *A. tagala*.



Tables

Table1. MIC of methanol extract of *Aristolochia* species against bacteria

Test bacteria	MIC (µg/ml)				
	A.brac	*A.indi*	*A. kris*	*A.taga*	ciprofloxacin
B. subtilis	1000	32	250	125	2
S. aureus	500	32	500	125	2
E. coli	-	64	500	125	2
P. aeruginosa	-	125	-	500	4
S. epidermis	-	16	-	64	4
P. mirabilis	-	64	-	125	8
V. cholerae	-	125	-	500	4
K. pneumonia	-	32	-	2000	4
S. simulans	-	500	-	1000	8
S. typhi	-	250	-	500	1

Values represent mean of three replications. - no MIC up to 2000 µg/ml

Table2. Disc diffusion assay of methanol extract of *Aristolochia* species against bacteria

Test bacteria	Diameter of zone of inhibition (mm)				
	A.brac	*A.indi*	*A. kris*	*A.taga*	ciprofloxacin
B. subtilis	8±0	18±0.52	13±0.52	13±0.52	30±1
S. aureus	7±0.52	19±0	12±1.72	12±1	30±0
E. coli	-	14±1.72	14±0	15±0.77	31±1.15
P. aeruginosa	-	12±1.52	-	11±0.52	28±1
S. epidermis	-	21±0.52	-	14±0	28±0.72
P. mirabilis	-	16±1	-	16±1.52	26±0
V. cholerae	-	14±1	-	12±0.52	24±0.72
K. pneumonia	-	16±0.72	-	11±0	27±1
S. simulans	-	12±0.52	-	10±0.72	29±0
S. typhi	-	13±1	-	9±1.15	33±1.15

Values represent mean of three replications. – no zone of inhibition

Table3. MIC of methanol extract of *Aristolochia* species against fungi

Test bacteria	MIC (µg/ml)				
	A.brac	*A.indi*	*A. kris*	*A.taga*	fluconozole
A. flavus	-	-	-	-	1
C. tropicalis	32	32	64	125	0.5
C. albicans	64	64	250	250	1
T. rubrum	16	32	64	34	1
F. oxysporum	-	-	-	-	8

Values represent mean of three replications. - no MIC up to 2000 µg/ml

Table4. Disc diffusion assay of methanol extract of *Aristolochia* species against fungi

Test bacteria	Diameter of zone of inhibition (mm)				
	A.brac	*A.indi*	*A. kris*	*A.taga*	fluconozole
A. flavus	-	-	-	-	22±1
C. tropicalis	12±1	16±0	15±1.15	13±2.21	24±0.77
C. albicans	15±1.15	18±1.72	13±1.72	10±0.72	20±0
T. rubrum	20±1.72	17±0	21±0	17±0.52	24±1.72
F. oxysporum	-	-	-	-	21±1.52

Values represent mean of three replications. – no zone of inhibition

Discussion

Plants and plant products are magnificent sources of phytochemicals and have been found to hold an array of biological activities including antioxidant potential [19]. Plants synthesize antioxidant compounds, mostly flavonoids and polyphenols, which have been reported to protect the human body from various diseases by neutralizing ROS. Recently, phenolic compounds have received increasing significance among various phytochemicals, due to their wide distribution in the plant kingdom and for their biological activities viz., anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities [20-22]. Antioxidants can be either used as dietary, food supplement or as a drug by humans [23]. Several studies have revealed that the increased dietary intake of natural antioxidants such as flavonoids and other phenolic compounds, almost present in plants, exhibit potential protective effects against many degenerative diseases [24-26].

In recent years, the search for phytochemicals possessing antioxidant, anticancer and antimicrobial activities have been on the rise due to their potential use in the therapy of various chronic and infectious diseases. Epidemiological and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular disease, cancer and aging [27]. Due to the risk of adverse effects encountered with the use of synthetic antibiotics, medicinal plants may offer an alternative source for antimicrobial agent with significant activity against pathogenic and infective microorganisms. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes [27]. Results of the present study confirmed the use of the selected *Aristolochia* species in traditional treatment systems as well as ethnomedicine. We found strong antioxidant, anticancer and antibacterial activities to the root extracts of the candidate species specifically in the superoxide anion radical scavenging activity**.**

The effect of antioxidants on DPPH radicals is thought to be due to their hydrogen donating ability [28]. Radical scavenging activities are very important to prevent the deleterious role of free radical in different diseases including cancer. DPPH free radical scavenging is an accepted mechanism by which antioxidants act to inhibit lipid peroxidation. This method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis. The results obtained in this investigation revealed that all the four Aristolochia species root extracts are free radical scavengers and able to react with the DPPH radical (Figure 1A), which might be attributed to their electron donating ability. Significant activity was recorded by *A. tagala*, followed by *A. krisagathra* (Fig. 1A).

The mutagenic capacity of free radicals is due to the direct interactions of hydroxyl radicals with DNA and therefore playing an important role in cancer formation [29]. Hydroxyl radicals can be generated by biochemical reaction. Superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently produce extremely reactive hydroxyl radicals in the presence of divalent metal ions, such as iron and copper. The results obtained in this study demonstrate that methanol extract of the root of four *Aristolochia* spp. had appreciable hydroxyl radical scavenging activity when compared with standard antioxidant BHA (Figure 1B) and could be served as anticancer agent by inhibiting the interaction of hydroxyl radical with DNA. The ability of the extracts to

quench hydroxyl radicals might directly be related to the prevention of lipid peroxidation [29]. Superoxide dismutase (SOD) is an enzymatic antioxidant that can scavenge superoxide anion radical (O_2 -) by catalyzing the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. This assay is based on the measurement of superoxide dismutase inhibition activity. The four extract recorded significant superoxide anion radical scavenging activity and can be comparable with that of the standard antioxidant agents (Fig. 1C).

Some species of *Aristolochia* stood out due to the potential cytotoxicity of cancer cell lines [30]. In our study, *Aristolochia* species exhibited significant anticancer activity (cytotoxicity) in cancer cells. Significant activity was recorded by A. krisagathra. Hence further objective is to identify a potential lead compound, which can be developed into a candidate anti-infective drug, in particular for treatment of infections by multidrug resistant pathogens like MRSA and VRSA.

The antimicrobial activity of the crude extract may be attributed to a specific compound or a combination of compounds. The knowledge about the chemical profile of the extract helps in explaining the observed activity and designing experiments for activity fractionation for isolation of the active principle. Alkaloids, flavonoids, coumarins, saponins and steroids are the compounds of plant origin known to have antibacterial activity. These compounds were detected in the *Aristolochia* species [31]. In the present study methanol extract of the root of *Aristolochia* species recorded significant antibacterial activity and our results were similar with that of the previous studies of A. indica [32-34]. The genus *Aristolochia* has revealed its potential as antimicrobial agent. *A. esperanzae* essential oil showed antibacterial activity against *S. aureus, E. coli, S. typhimurium, B. cereus, C. freundii* and *L. monocytogenes* [35]. Though antimicrobial activity of *A. indica* and *A. bracteolata* was studied by some researchers, the rest of the species remained uninvestigated so far. Moreover, comparative antimicrobial activity of root extract of four *Aristolochia* species was not reported and signifies the present study.

Conclusions

Strong antioxidant, antibacterial and anticancer properties were confirmed in the methanol extract of four *Aristolochia* species. These activities may be due to the occurrence of polyphenolic compounds such as flavonoids and tannins. The antioxidant superoxide anion radical scavenging activity was comparable to those of the standard drug BHA. *A. indica* and *A. tagala* recorded significant antibacterial activity against all the tested Gram positive and negative bacteria. Selective anticancer (cytotoxic) activity was detected in *A. krisagathra, A. indica* and *A. bracteolata* against HeLa, MDAM B 321 and A549. These findings provide scientific evidence to support its traditional ethnomedicinal uses and indicate a promising potential for the development of antioxidant, anticancer and antibacterial agents from these species. The present study also emphasize the relevance of detailed pharmacological investigations in order to reveal therapeutic value of these lesser known ethnomedicinal species.

Conflict of interest

The authors have declared no conflict of interests

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