Bioactivity of *Archislatifolia* using brine shrimp lethality, antioxidant & antimicrobial assays

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Abstract

The objective of the study was to evaluateprobable cytotoxic, antioxidant and antimicrobial activities of methanolic extract of *Archislatifolia's* leaves. The result showed that the extract was lethal against brine shrimps at higher concentration i.e. at 1000µg/ml. Survival rate was 8% at the aforementioned concentration while 48 and 70% survival rate was perceived at 500 and 100µg/ml correspondingly. The PFRSP (percent free radicals scavenging potential) of extract at 100, 250, 500 and 1000µg/ml was 41%, 52%, 68% and 81% individually and was matched to ascorbic acid that showed 58%, 69%, 85% and 88%. The IC_{50} value was 318µg/ml. The minimum inhibitory concentration (MIC) of numerousportions of extract was assessed for antibacterial action. Inhibitory values of *S. aureus*were: n-hexane fraction (5 µg/ml), methanolic fraction (2.5g/ml), ethyl acetate fraction (5 µg/ml) and butanol fraction (7.5 µg/ml) correspondingly. MIC for *E. coli* comprised methanolic fraction (5µg/ml). However, MIC for *K. pneumoniae* growth was 1µg/ml, in case of methanolic fraction, whereas 2.5µg/ml was for n-hexane fraction. Antifungal outcomesdisplayed that growth of *Aspergillusniger* inhibited by methanol was (54±0.91), n-hexane (46±0.88), Chloroform (50±0.56), Ethylacetate (44±0.56). Butanol and aqueous fractions didn't exhibited any inhibition. % inhibition against *A. flavus*was as under. Methanol (58±0.81), n-hexane (52±0.87),Chloroform (48±0.81), Ethylacetate (54±0.91).Similarly, against *A.Fumigatus,* Methanol (48±1.0), n-hexane (40±2.0), Chloroform (40±1.0), Ethylacetate (38±3.0), Butanol (28±1.0), Aqueous (34±0.08).

Keywords: Cytotoxicity, antioxidant, antimicrobial potential

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1. Introduction

The medicinal plants are proved to be an important tool in resolving the health care problemsin many countries of the world. Researchers are making great efforts to isolate compounds from the plants that have important medicinal values. A no of developing countries have implemented traditional medical practice as an essential part of their culture.Since prehistoric time, medicinal plant's extracts have been recommendedand incorporated, with virtually no alteration in the form or the approach they have been used, and with a robustconfidence in their practicality in diseases[1]. The plant's bioactive compounds study in achemical lab is every so oftenmired due to lack of anappropriate, modest and fast screening practice. A no ofprocesses for bioassay are applied using whole animals, isolated tissues or biochemical systems. These bioassays can be relativelycomplex and lavish. A practical technique for wide-ranging toxicity screening is, therefore, vital as aunderpinning stage in the study of bioactive compounds. A model animal that has been used for this purpose is the brine shrimp, *Artemiasalina*Leach[2]. Plants being ansarcastic source of bioactive secondary metabolites including alkaloids, tannins, saponins, terpenoids, flavonoids and other compounds, affirmed to have an *in-vitro* antifungal potential. Since the plant kingdom delivers a useful source of leading compounds of inimitable structure, an examination of species from the tropical areas has been considered. Therefore, the investigation work on compounds resulting from natural products has hurried in currentfew years due to their importance in drug discovery. A chain of molecules with antifungal potential against varied strains of fungi have been examined in plants, which are of prodigiousprominence to human. These molecules may be incorporated straight or considered as a forerunner for designing better molecules Researcher's interest in therapeutic activities of medicinal plants as antimicrobial has also increased during recent years. Many other plant groups have been stated in the search for new antioxidants [3,4,5] but generally, still it is needed to find out more information linking the antioxidant and antimicrobial activity of plant species.

The present study was conceded to assess cytotoxic, antioxidant and antimicrobial potential of *Archislatifolia*.

2. Materials and Methods

1.1. Collection and processing of plant material

KuramGarhi was the area from where*Archislatifolia*plant was collected.The identification was incorporated in Botany department of UST Bannu. Voucher specimen was deposited at the herbarium of the department. Washing and drying under shed was done successively. It was then grinded into powder mechanically by a local grinder machine.

2.2.Preparationof plant Extract

100gm of powdered *Archislatifolia* was taken and was extracted in 1L commercial grade methanol (Merck Lab).It was shaked randomly for 3hrs on a shaker machine and was then kept for 7 days at room temperature under sterile conditions.Filtration was done after 7 days and the filtrate was further concentrated under reduced pressure on rotavap at 38^0 C.The concentrated methanolic crude extract was then applied to lyophilizer and converted into very fine powder form which was stored at 4^0 C in a Falcon tube

2.3. Preparation of Sample

Parent solution was made by dissolving 10mg of crude extract powder into10ml of methanol. Different sub-solutions were prepared by diluting the parent solution as 100µgm/ml, 250µgm/ml, 500µgm/ml and 1000µgm/ml for cytoxic and antioxidant assay.

2.4. Cytotoxic Brine Shrimp Assay

Standard procedure mentioned by Wasim Ahmed *et al*[6]was used forcytotoxic activity of methanolic crude extract of *Archislatifolia.*

2.8gm commercial sea salt (Sigma) was dissolved in 100ml of dH_2O with constant stirring for 2hr using magnetic bead. Brine shrimp's (also recognized as sea monkeys, are marine invertebrates about 1 mm in size) eggs were hatched in a shallow rectangular tray. A plastic partition with numerous 2mm holes were clamped in the tray to make 2 uneven sections. The eggs were spotted into the smaller section and were roofed with aluminum foil while the larger section was illumined. After 24 hrs, larvae were perceived in the lightened section.

1ml of all sub-solutions were put into cleaned test tubes and allowed to evaporate the methanol. Residues were re-dissolved in 3ml saline. A positive control was retained by pouring 3ml saline in a test tube. 10 shrimps were transported to each tube using Pasteur pipette, and artificial sea water was added to make 5ml. DMSO was used as a negative control. The nauplii can be tallied macroscopically in the stem of pipette against a lightened background. The vials were maintained under light at room temperature 25°C to 28°C. Survivors were counted with the aid of 3x

magnifying glass after 24h. In case, where control death occurred, the data was corrected using Abbott's formula [7]Abbot, 1925.

% Death = [(Test – Control) / Survivors of control] X 100

2.5. DPPH free radical hunting assay

The radical scavenging activity was assessed based on its ability to reduce the man-made DPPH radicals. This assay delivers info on the reactivity of extract with stable free radicals, which is representative of its capacity to forage free radicals self-reliantly from any enzymatic activity [8]. The DPPH assay was carried out according to the standard procedure of [9]Gyamfi*et al*, 1999, with little modifications. The fresh stock solution was prepared by dissolving .003gm 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 100mlmethylalcohal and then stored at 20°C. The running solution was gained by diluting parent solution with methanol to obtain an absorbance of about 0.826 at 517 nm using the spectrophotometer (BMS-UV 2601). A 900µl fraction of this solution was mixed with 100 µl of the plant extract at variable concentrations including 100, 250, 500, 1000µg/ml in respective solvent. The solutions in the test tubes were vertexed and incubated in the dark for 30 min at room temperature. The absorbance was taken at 517nm. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated as follow:

$$\%RSA = \left[\frac{(\text{Control absorbance-sample absorbance})}{(\text{Control absorbance})}\right] \times 100$$

As positive control, ascorbic acid was used. Each extract was assessed in duplicate and the results were expressed as mean values ± SD. The IC_{50} value indicates the concentration needed to inhibit a biological or biochemical function by half & was calculated via linear interpolation.

$IC50 = (50\%\text{-}Low_{Inh\%})/(High_{Inh\%}\text{-}Low_{Inh\%})x(High_{Conc}\text{-}Low_{Conc}) + Low_{Conc}$

2.6. Antimicrobial activity

2.6.1. Antibacterial assay

The antibacterial activity of the extract was conceded through the modified protocol of [10a]Bibi (2006) and [10b]Ahmad (2011).

2.6.2. Culture media

All microbes used in this study were cultured and grown using nutrient agar media while nutrient broth media was employed for inoculation, shaking, incubation and standardization of microbes.

2.6.3. Requirements

DMSO, Eextract of*Archislatifolia*, nutrient agar, nutrient broth, NaCl, dis:water, autoclave, laminar flow hood, flask, , petri plates, cork borer, incubator beakers, micropipette, tips.

2.6.4. Preparation of samples

A volume of 10ml of stock solution having a concentration of 15mg/ml was prepared in DMSO (dimethyl sulfoxide). Further dilutions of 12.5,10, 7.5, 5, 2.5 and 1mg/ml were prepared to govern the antibacterial activity of extract at altered concentrations. Solution of Roxithromycin and Cefixime-USP, 2mg/ml in DMSO, were used as positive control while pure DMSO was used as negative control.

2.6.5. Microorganisms/Preparation of inoculums

Three strains of bacteria namely*E-Coli, Staph aureus* and *Kpneumoniae*wereincorporated in this assay. 24hr old culture innutrient broth (MERCK) of selected bacterial strain wasmixed with physiological saline (0.9% NaCl w/v.) andturbidity was remedied by adding sterile physiological saline until a McFarland 0.5 $BaSO^4$ turbidity standard (CFU) per ml density was obtained. These inoculums were used for sowing the nutrient agar.

2.6.6. Assay procedure (agar diffusion method)

2g of nutrient agar was dissolved in 100ml of distilled water (pH 7.0) in flask and autoclaved at 121℃ and 20lbs pressure for 20min and was placed to cool up to 45℃. Then appropriate quantity of this agar medium (20ml) was put in to the autoclaved petri plates of 10cm and placed in laminar flow for solidification. A loop of bacterium streaked on the surface of agar media of Petri plates carefully. With the help of a sterile cork-borer, wells measuring 0.7cm in diameter were bored. Seventy microliter of crude extract solution was transferred aseptically into the wells; similarly 70µl of streptomycin was put in its respective well.

70µl of DMSO was also put in a well for negative control. Finally, all the petri plates were jam-packed in the laminar flow and incubated at 37℃ f or 24 hrs. After 24 hrs of incubation, the zone of inhibition of all the extracts and controls were measured in mm and compared with the control.

2.6.7. MIC determination

The MIC of the plant extract against sensitive pathogens was determined & measured by making serial dilutions of the plant extract to obtain 20, 10, 5, 2.5, 1.25 and 0.625mg/ml. These

serial dilutions were added into the marked wells and incubated for 24hr at 37°C. The sensitivity of each organism was determined by checking the growth. The least concentration (plant extract) with minimum inhibitory effect was taken as MIC of that extract against organism.

2.7. Antifungal activity

To check the antifungal activity, protocol of [11] Atta-ur-Rahman 1991, was used with some alteration.

2.7.1. Requirements

Extract of*Archislatifolia,* incubator, autoclave, SDA,DMSO, micropipette, tips, flask, beakers, ruler, test tubes, electronic digital balance, sterile safety hood, terbinafine, dis: water.

2.7.2. Preparation of samples

The parent solution of 12mg/ml (crude extract) was prepared in DMSO from which further 1ml solution of 200µg/ml was prepared with DMSO as a diluting solvent. Stock solution of 12mg/ml of terbinafine (positive control/antifungal agent) was prepared in the DMSO and made a final concentration of 200µg/ml as a diluting solvent. 1ml unadulterated DMSO was used as a negative control.

2.7.3. Assay procedure

The clinical fungal test organisms used for study are *Aspergillusflavus, Asp.niger* and *Asp.fumigatus*. The growth media used for fungus in this bioassay was Sabourad dextrose agar (SDA). 9.75g of the powdered SDA (Merck) was dissolved in 150ml dist. water. It was homogenized. The media, the tubes and micropipette tips were sterilized by treating them in an autoclave. The tubes were cooled at 50^0C, test samples were loaded in to the non-solidified SDA in biological safety cabinet. Each sample was loaded in test tubes for the given fungal cultures and for negative control. Tubes were arranged with their corresponding samples in slanting positions on the table at room temperature for 24 hours to form slants. Next day slants were checked for their sterility and each sample tube was inoculated with 4mm diameter of fungus removed from seven day old cultures of fungus. The tubes were incubated in fungal incubators at 27^0C-29^0C for 3-7 days. The following formula is used to calculate inhibition of fungal growth.

% inhibition = [(100 – Linear growth in test sample / Linear growth in control)]× 100

(Linear growth measured in mm).

2. **Results and Discussion**

3.1. Cytotoxic screening

Cytotoxic effect of the plant extract was screened against brine shrimps growth under controlled condition using normal control. After complete hatching, shrimps were transported into glass test tubes that contained sea salt and extract of numerous concentration of the plant. After 24 hrs, the effects of extract of different concentration was noted and found that the brine shrimp survival is inversely proportional to the concentration of the plant extract. From figure 1, it is obvious that at 1000µg/ml, 92% deaths occurred. Similarly, at 500 and 100µg/ml, 52% and 30% deaths occurred, respectively. Our results showed that the brine shrimp survival is inversely proportional to the concentration of the extract used. Kanegusuku*et al*. [12] reported organic fraction of Rubusimperialis(C.) which showed more cytotoxicity. Zaidi*et al*. 2006, [13] studied that methanolic fraction of Arceuthobium oxycedripossessed100% lethality for brine shrimps at high dose which are in accordance with our results. The results of present study suggest that methanolic fraction possess some bioactive constituents having anticancer activities that can be the point of interest for new drugs possessing anticancer and protective role against different pathogens.

3.2. Antioxidant activity

Momentousforaging potential of the etract was perceived atnumerous concentration of *Archislatifolia*extract, in an increasing order. Parallel result was offered atnumerous concentration of ascorbic acid used as a reference. The %RSA of DPPH at 100, 250, 500 and 1000µg/ml was 41%, 52%, 68% and 81% respectively and was associated to ascorbic acid (58%, 69%, 85% and 88%). The IC50 value of the plant's extract was 318/µg/ml.

Our result displays resemblance with the research of [14] Hagerman *et al*. 1998 and [15]Fallehe*t al*. 2008, who described that medicinal plants categoricallyhunt free radicals.

The antioxidant potential of *Archislatifolia*could be attributed due to the presence of plant bioactive phenolic and polyphenolic compounds which significantly decrease the free radicals which cause oxidative stress. Oxidation is an essential progression of living things for energy production; however during normal metabolism, oxygen utilization, through many enzymatic systems, produces reactive free radicals (RFR). In small amounts, these ROS are advantageous in signal transduction and growth regulation. However, hefty amount of ROS produced oxidative stress, assault many molecules such as protein, DNA and lipids [16]. DPPH free radical scavenging procedure has been widely in practice for studying antioxidant potential. Figure 2 shows the % scavenging activity of *Archislatifolia*for free radicals of DPPH. The results acquired by [17]Duenas*et al*. 2006 and [18]Kilani *et al*. 2008 also support our findings. The data of this work suggests that the methanolic extract of *Archislatifolia*has capacity to act as antioxidant agent.

3.3. ***Antibacterial assay***

The MIC of innumerable fractions of *Archislatifolia*was assessed for antibacterial activity (Table 1). Gram negative bacteria such as *S. aureus*was inhibited by MIC value of n-hexane fraction (5µg/ml), methanolic fraction (2.5g/ml), ethyl acetate fraction (5µg/ml), butanol fraction (7.5µg/ml) respectively. MIC for *E. coli* comprised methanolic fraction (5µg/ml). However, MIC for *K. pneumoniae* growth was 1µg/ml in case of methanolic fraction whereas 2.5µg/ml for n-hexane fraction. Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization (WHO) estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extract obtained from *Salix nigra*shows strong activity against most of the tested bacterial and fungal strains.

Antibacterial activity exhibited by titled plant is labeled in Table I.

3.4. Antifungal assay

In this present investigation of antifungal assay, all the extracts of *Archislatifolia*were evaluated against fungal strains as shown in Table 2. Growth of *Aspergillusniger* inhibited by methanolic fraction was (54±0.91%), hexane fraction (46±0.88%), Chl. fraction (50±0.56%), Ethy (44±0.56%). But.and aq. fractions didn't show any inhibition.. In case of *A. flavus,* % inhibition was as under. Meth. (58±0.81), Hex. (52±0.87), Chl. (48±0.81), Ethy.(54±0.91). Similarly, against *A.Fumigatus,* the results were: Meth (48±1.0), n-hex (40±2.0), Chl (40±1.0), Ethy (38±3.0), But (28±1.0), Aqu (34±0.08).

4.Conclusion

The current study shows the potential of *Archislatifolia*as a potent cytotoxic, antioxidant and antimicrobial agent. Interestingly, the antioxidant activity of extracts positively correlated with their phenolic contents, and the antibacterial activity positively correlated with their flavonoid contents, respectively. Further studies should be carried out to investigate the individual components, in-vivo antioxidant and other relevant biological activities.

5. **Reference**

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Figures and Legends

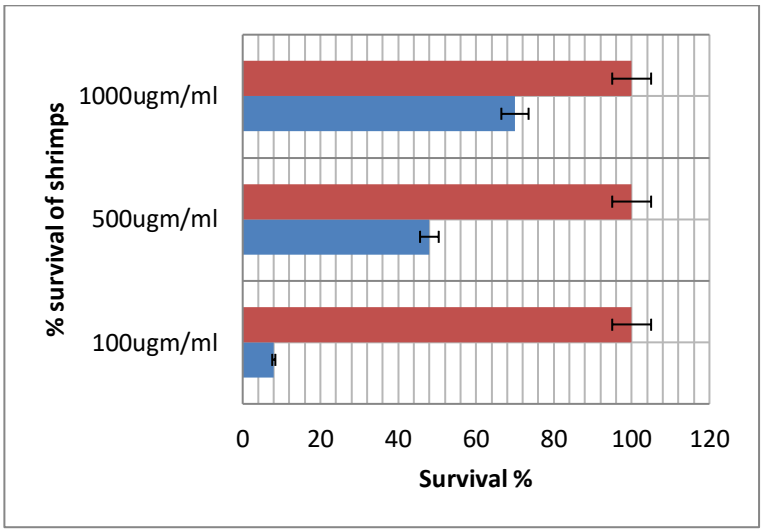

Figure 1: Brine shrimps survival rate against extract concentration

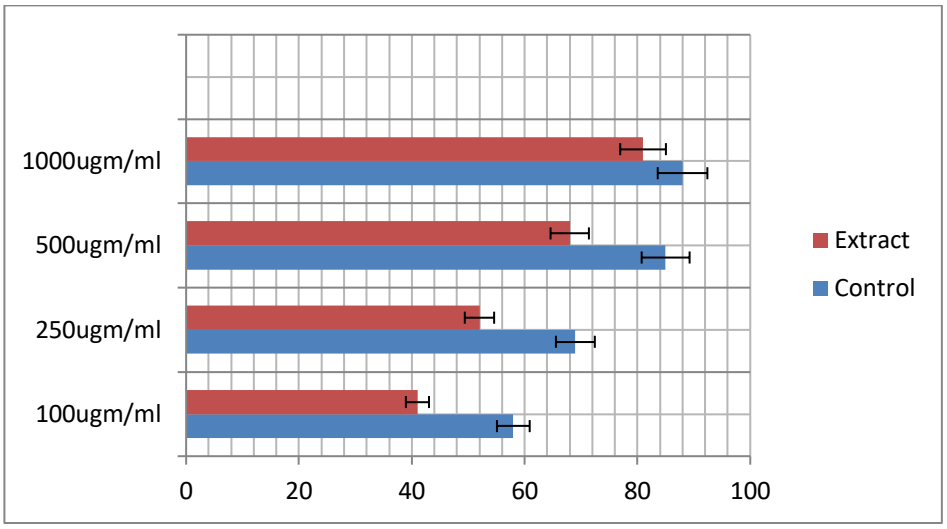

Figure 2: %DPPH free radicals scavanging of extract concentration

Table 1: Antibacterial activity exhibited by *Archislatifolia*extracts

Group	*E-coli*		*S.aureus*		*K.pneumoniae*	
	Conc.	Zone (mm)	Conc.	Zone (mm)	Conc.	Zone (mm)
Cefix	1	28	1	24	1	16
Roxith	1	20	1	18	1	18
Meth	5	16	2.5	14	1	10
Hex	-	-	5	10	2.5	8
Chl	-	-	-	-	-	-
Ethy	-	-	5	12	-	-
But	-	-	7.5	12	-	-
Aqu	-	-	-	-	-	-

sTable 2: Antifungal results of *Archislatifolia*against various fungal strains

% Inhibition

Fungal Strain	Meth	Hex	Chl	Ethy	But	Aqu	Standard
A. falvus	58±0.81	52±0.87	48±0.81	54±0.91	_	_	*84.8 ± 2*
A. niger	54±0.91	46±0.88	50±0.56	44±0.56	_	_	86.2 ± 1
A. fumigatus	48±1.0	40±2.0	40±1.0	38±3.0	28±1.0	34±0.08	90.5 ± 1