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Antibacterial activity of alkaloids present in plant *Achyranthes aspera*

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Abstract
The aim of the study undertaken is to extract the alkaloids present in the herb *Achyranthes aspera*, and examine their antibacterial activity. *Achyranthes aspera* (Amaranthaceae) is an important medicinal herb found as a weed throughout India. Though almost all of its parts are used in traditional systems for the last few decades or so, extensive research work has been done to prove its biological activities and pharmacology of its extracts. The analysis of alkaloids was done using HPLC technique in which various peaks were shown at different retention time. Out of eight probable alkaloids four peaks were of *Achyranthine, Betaine, Betanin*, and *Isobetanin.* The antibacterial activity was evaluated using the agar well diffusion method. DMSO was used as a solvent to dissolve the extracts and Ampicillin as positive control. The antibacterial activities were evaluated against four species viz. *Bacillus subtillis*, *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Highest activity was shown against *Bacillus subtillis* at concentration 40mg/ml. The antibacterial activity against both the type of bacteria are due to the presence of broad spectrum antibiotic compound or metabolic toxins. Hence it can be used as a therapeutic drug to inhibit the bacterial pathogens.

Keywords: DMSO (Dimethyl Sulphoxide), agar well diffusion, ampicillin, *Achyranthes aspera*, HPLC, *betaine*, achyranthine, betanin, isobetanin

Introduction
The herbal products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to human and environment. Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today provide safety in contrast to the synthetics that are regarded as unsafe to human and environment (Mukherjee, 2013) [17]. One of the many plants which are being evaluated for their therapeutic efficacies is *Achyranthes aspera* which is commonly known as Latjeera (Hindi) & Rough Chaff tree (English). It is an erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides. Although it has many medicinal properties, it is particularly used spermicidal, antipyretic & as a cardiovascular agent.

Fig 1: *Achyranthes aspera*

The seeds contain chemical constituents like 10-tricosanone, 10-octacosanone & 4-tritriacontanone (Rastogi and Malhotra, 2004) [21]**.** Traditionally, the plant is used in asthma and cough. It is pungent, anti -phlegmatic, antiperiodic, diuretic, purgative and laxative, useful in oedema, dropsy and piles, boils and eruptions of skin *etc.* Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints (Patil and Sharma, 2013) [20]**.** The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and cutaneous diseases (Nadkarni, 2009) [18]. The plant is useful in liver complaints, rheumatism, scabies and other skin diseases. It also possesses tranquillizing properties.

The work undertaken is basically related with the extraction of alkaloids from the plants' powdered form. The term "Alkaloid" is linguistically derived from the Arabic word *al-qali,* the plant from which soda was first obtained- is nitrogenous compounds that constitute the pharmacologically active "basic principles" of predominantly, although not exclusively, flowering plants. Since the identification of the first alkaloid morphine, from the opium poppy, *Papaver somniferum*, nearly 10000 alkaloids have been isolated and their structures elucidated (Southon and Buckingham, 1989) [23]**.** Alkaloids generally exert pharmacological activity particularly in mammals such as humans. Even today many of our most commonly used drugs are alkaloids from natural sources.Alkaloids such as berberine are known to be anti-microbial. They inhibit estarases as well as DNA and RNA polymerases. Moreover, berberine inhibits cellular respiration and acts in DNA intercalation. Alkaloids are significant for the protection and survival of plants because they ensure their survival against micro-organisms (anti-bacterial and anti-fungal activities), insects and herbivores (feeding deterrents) and also against other plants by means of allelopathically active chemicals (Wink *et al*. 1998 and Wink *et al.* 2003) [28, 27]. *Betaine* ($C_5H_{11}NO_2$) was reported from the whole plant which is also a water soluble alkaloid (Danial, 2006) [7]**.** *Achyranthine* a water soluble alkaloid in the plant *Achyranthes aspera*, which possess pharmacological actions like dilation of the blood vessels, lowering of blood pressure, depression of heart and increase the rate and amplitude of respiration was reported (Goyal *et al*. 2007). *Betalains* occur only in the plants of the order Caryophyllales (Old name: Centrospermae), such as the family Amaranthaceae. Several betalains (16 red-violet betacyanins and 3 yellow betaxanthins) were reported to be isolated and identified in different portions (viz. stems, leaves, and inflorescences) from plants of the Amaranthaceae family. Betalains are attracting increasing attention because of their use for food coloring and their antioxidant and radical scavenging properties against certain oxidative stress-related disorders, anticancer, antiviral and antiparasitosis properties (Biswas *et al.* 2013) [3]

Several works have been carried out to evaluate the plant's antimicrobial (Kumar, 2014) [14] and antifungal potential (Mishra *et al.* 1992) [16]. The plant has been reported as a potent antibacterial agent. Antibacterial activity of ethyl acetate extract of the stem, leaf extracts, ethanol and methanol extracts of the leaf and stem, ethanolic extract of the leaves and stem, aqueous flower extract have been reported to show anti-microbial property. Both antibacterial and antifungal activity of petroleum ether, chloroform and methanol extracts of dried leaves has been reported. The plant was found to have antibacterial property against hospital origin gram positive bacteria. It is used as herbal antimicrobial finish for cotton fabric in healthcare textiles (Thilagavathi and Kannaian, 2008) [25]. It was reported that antibacterial activity of the plant could be due to tannins, saponins, flavonoids (Kumar *et al.* 2010) [13]. There are scanty reports on its antibacterial activities of this plant. In order to demonstrate the antibacterial efficacy, test were conducted against human pathogenic bacteria including those responsible for causing inflammation. The ethanol, acetone and ethyl acetate root extracts display varying degree of antibacterial activities against the tested bacterial strains. The ethanol root extract is found to be the most effective against *Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, and Salmonella abony*strains. Acetone extract indicate the activity against *Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and Pseudomonas aeruginosa* strains. Ethyl acetate extract has noticed activity against S*almonella typhimurium, Escherichia coli, Bacillus subtilis, and Staphylococcus aureus* strains. Results were demonstrated using *Achyranthes bidentata* extracts on *Bacillus subtilis, Staphylococcus aureus, Escherchia coli, Pseudomonas Aeruginosa* and others in *Achyranthes aspera* which proved its antibacterial and antifungal activity both in crude extract as well as by isolated compounds. Chloroform and methanol root and shoot extracts of *Achyranthes aspera* showed good amount of antibacterial activity against *Klebsiella species* (Kaur and Thakur, 2005) [12].

There have been various methods developed to study, identify and characterize alkaloids. High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries. HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. Non-polar compounds will be more retained (i.e. have longer retention times) than a polar compound.

Method and materials

3.1: Sample collection: The sample i.e. the powdered form of whole plant of *Achyranthes aspera (2kg*) was collected from the Baidyanath Ayurvedic Centre, Allahabad.

3.2: Extraction of alkaloid: The dried plant was weighed and extracted in Soxhlet extractor with ethanol at 50° C. The extraction was monitored continuously at each stage and the composition of extract was confirmed by the colour. Solvent was taken from thimble and evaporated to check the absence of residue. The extract was filtered through What man filter paper no. 1 and evaporated to dryness at 40 °C with aid of distillation. Extraction of alkaloids was carried out by following a reported method of Berkov *et al*. (2005) [2]. The obtained extract (0.5 g i.e. 2.5% w/w) was dissolved in 200 ml hydrochloric acid (1 M) and shaken vigorously on a vortex mixer. After this, it was extracted twice with n-hexane (15×2mL) in a separating funnel in order to separate out fatty or non-polar contents. The remaining aqueous portion was

made basic (ca. pH 10) with ammonium hydroxide solution (10%). Finally, this basic solution was extracted three times with chloroform (15×3mL) and organic phase was washed twice with distilled water (20×2mL) and then dried in anhydrous sodium sulfate (5 g). The alkaloid fraction (0.05 g i.e. 0.25% w/w) was obtained by evaporating the chloroform that was dissolved in methanol (10 mL). The presence of alkaloids was confirmed by Dragendroff's reagent (prepared as in appendix) that showed a brownish-red precipitate, which is a sign of the presence of alkaloid (Parekh and Chanda, 2007) [19] After this it will be subjected to distillation in order to evaporate the chloroform, followed by concentration with water bath (Laghari *et al.* 2014) [15]

3.3 HPLC technique for validation of alkaloids

3.3.1 Sample and standard preparation

6 mg of the sample was added in a 1.5 ml centrifuge tube was mixed with 500 µl of methanol: water (1:1). Then the solution was homogenized and ultrasonicated for 20 min at room temperature. The mixture was then centrifuged for 15 min at 4000 g. After removal of the supernatant, it was dried under the nitrogen gas. For HPLC injection samples were redissolved in 100 µl of methanol: water (1:1). Subsequently, 20 µL of the sample was injected into the HPLC system for estimation of alkaloid. Standard solutions for construction of the calibration curves were made by adding 500 µL of methanol: water (1:1), following the preparation method described above.

3.3.2: Chromatographic conditions: The analysis was performed using In house method. Solvent used was Methanol solvent system. The HPLC system consisted of a reversed-phase C18 column (150 X 4.5 mm, i.d. 5 µm, Luna, Phenomenex) with integrated pre-column guard in isocratic mode with two pumps (LC-10ATVP, Shimadzu, Kyota, Japan), system controller (SCL-10AVP) and an UV detector (SPD-10AVP) with a variable wavelength (190-370 nm) range, an injector with a 20µl sample loop was used for the analysis. The system was operated with Shimadzu Class VP series software. The mobile phase used was water: acetonitrile (70:30), pH 3.0 adjusted with orthophosphoric acid at the flow rate of 1 mL/min. HPLC-UV detector at wavelength 280 nm and run time for assay was 30 min. *Achyranthine, Betaine* standard and samples were dissolved in 100 µL of water-methanol (1:1). The diluted *Achyranthine* and *Betaine* standard solution and samples were injected into 20 µL loop of the HPLC system with the help of a Hamilton microliter syringe. The different concentrations of *Achyranthine* standards were tested individually to record detection limit, retention time and peak area under isocratic conditions. This was repeated five times with each concentration to verify the concurrence of the retention time and to set up concentration vs. peak area curve. The response was linear with the concentration ranges used (1 pg/mL to 10 +ng/mL).

3.4 Antibacterial Activity

3.4.1 Requirements: Nutrient agar, sterile plates, swabs and borer

3.4.2 Principle: The antimicrobial present in the plant extract were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zone of inhibition will be uniformily circular due to confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

3.4.3: Collection of test organisms: The following pathogenic bacteria strain were obtained from the" Microbial Culture Collection Bank", Department of Microbiology and Fermentation Technology, Sam Higginbottom Institute of Agriculture, Technology & Sciences

- *Bacillus subtilis*-0008 (gram positive)
- *Staphylococcus aureus*-0171(gram positive)
- *Pseudomonas aeruginosa*-0034 (gram negative)
- *Escherichia coli*-0018 (gram negative)

3.4.4 Solvent for dissolving the extract

Dimethyl sulphoxide (DMSO) which is a colourless hygroscopic liquid with boiling point of 189 °C was used for dissolving the plant extract in order to check anti-bacterial ativity. It is important polar aprotic solvent that dissolves both polar and non-polar compounds and is miscible in a wide range of organic solvent as well as water 1 ml of DMSO was added to all the concentrations of plant extract. DMSO was chosen as solvent because, in addition to that it can be used in dissolving the crude extract completely and it has no inhibitory effect on the cultures.

3.4.5 Media preparation of culture

Antibacterial activity of the crude extracts was determined by the agar well diffusion method (Kumar *et al.* 2010) [13]**.** The first step towards the evaluation of anti- bacterial activity was the preparation of broth. The broth is that medium in which bacteria are grown (Karou *et al*. 2005) [11]. In the preparation of Broth pH was checked which was found to be 7 (Saraf and Samant, 2014) [24] Agar was prepared and autoclaved. Plates and test tubes were sterilised. With a sterile wire loop four to 10 cfu/ ml from each bacteria were touched and transferred to 10 ml nutrient broth, the tubes were incubated for 24 hours at 37 ºC, a volume of 0.1 ml of the suspension was transferred to broth. The bacterial inoculum was uniformly spread using sterile cotton swab on a sterile Petri dish Muller Hinton agar to each of the 4 wells (4 mm diameter holes cut in the agar gel). The systems were incubated for 24 h at 37 ºC under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition zone of the bacterial growth was measured in mm (Cleidson *et al*. 2007) [6].

Results and Discussion

In the present study entitled "Antibacterial activity of alkaloids present in plant *Achyranthes aspera*" the following facts were elucidated.

4.1 Isolation of Alkaloids

The isolated alkaloids from the ethanloic extract of *Achyranthes aspera* was preliminary confirmed by Dragendroff's reagent and Mayer's reagent showing dark red precipitate and yellow precipitate respectively. After the preliminary conformation, the alkaloids present in the extract of *Achyranthes aspera* were confirmed by HPLC, as shown in Table 1

4.2 High Performance Liquid Chromatography (HPLC)

In this report, a method based on High Performance Liquid Chromatography was employed to determine the different alkaloids present in the plant extract. The retention time as shown in Table 1 were found to be of 8 probable alkaloids. Alkaloids numbered 3 and 6 were having retention time

values of 3.883/min and 3.000/min which when compared with the standards shown in Fig 2 & Fig 4 was found to be of *Achyranthine* and *Betaine*. As well as alkaloids represented by number 7 and 8 when compared with the results of Cai *et al*. (2005) [5] were found to be *Isobetanin* and *Betanin* having retention time 13.40/min and 12.645/min respectively as shown in Table 2

Table 1: Retention time of various probable alkaloids found in the ethanolic extract of *Achyranthes aspera* Detector A (280nm)

Alkaloids	Retention Time (Min.)	Area	Area %	Height	Height %
1	2.167	9198	2.553	700	5.906
2	2.558	13373	3.711	1046	8.825
3	2.683	12611	3.500	1078	9.095
4	3.000	29259	8.120	1569	13.237
5	3.317	22843	6.340	995	8.394
6	3.883	90907	25.229	1282	10.816
7	12.658	7730	2.145	347	2.928
8	13.400	174401	48.401	4836	40.800
Total		360322	100.000	11853	100.000

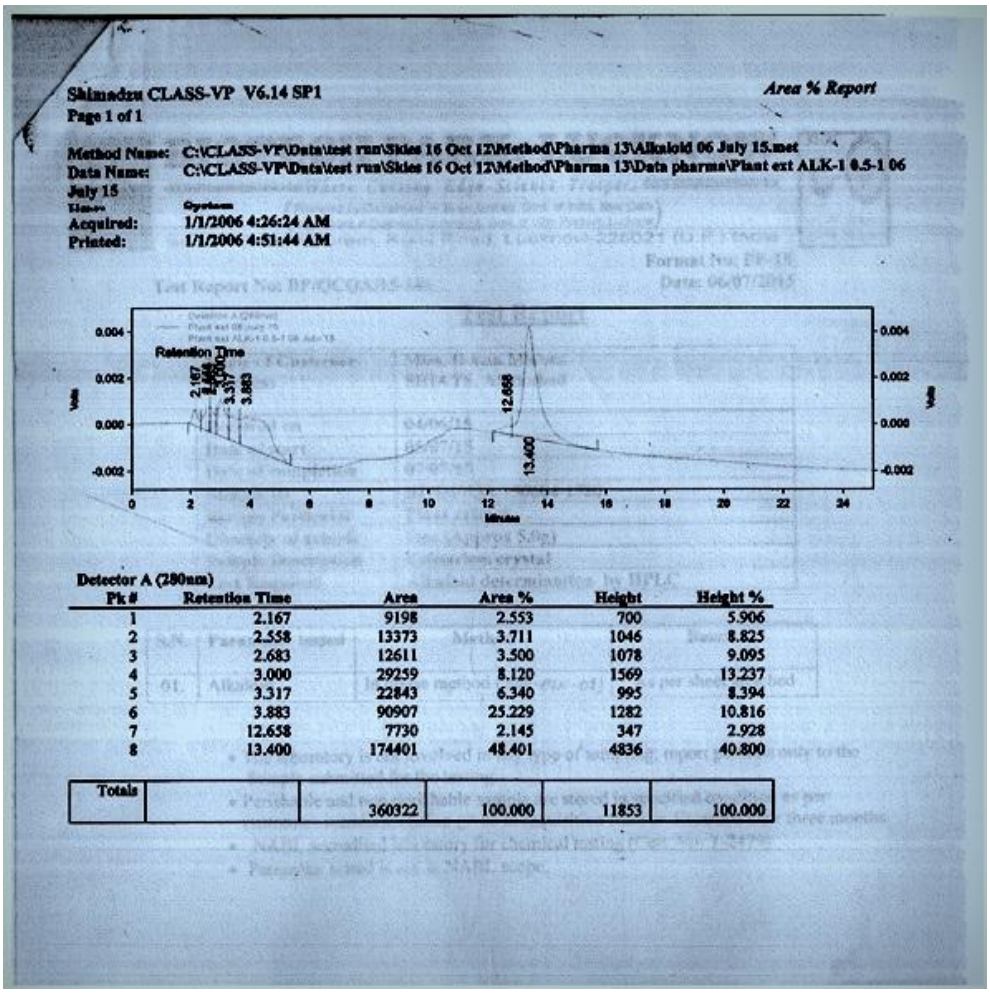


Fig 2: HPLC chromatogram of ethanol extract of *Achyranthes aspera*

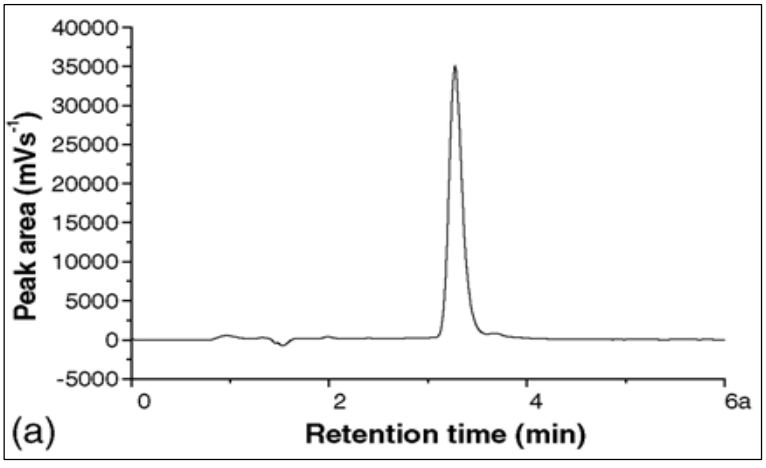


Fig 3: HPLC chromatogram of *Achyranthine* (Zimmer *et al*. 2006)

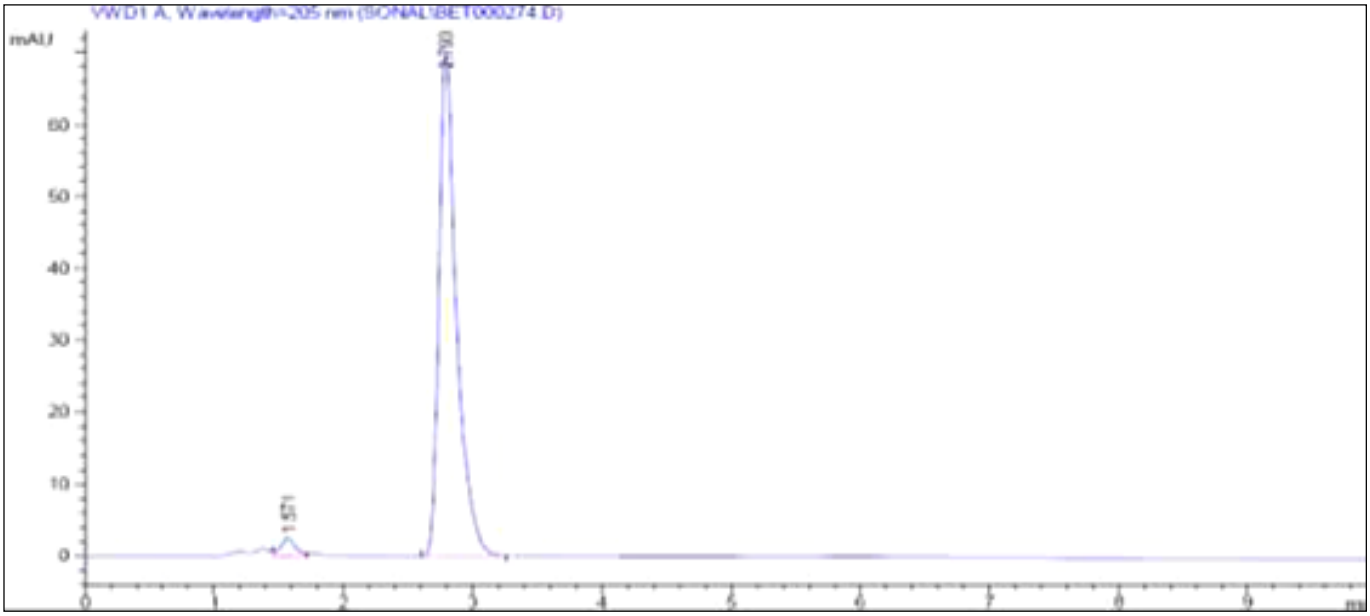


Fig 4: showing HPLC chromatogram of standard Betaine

4.3: Antibacterial activity

In vitro antibacterial assay was performed to assess the efficacy of alkaloids extracted from *A. aspera* to evaluate the inhibition of the growth of pathogenic bacteria organisms. Ampicillin was used as standard since it is a broad spectrum antibiotic.

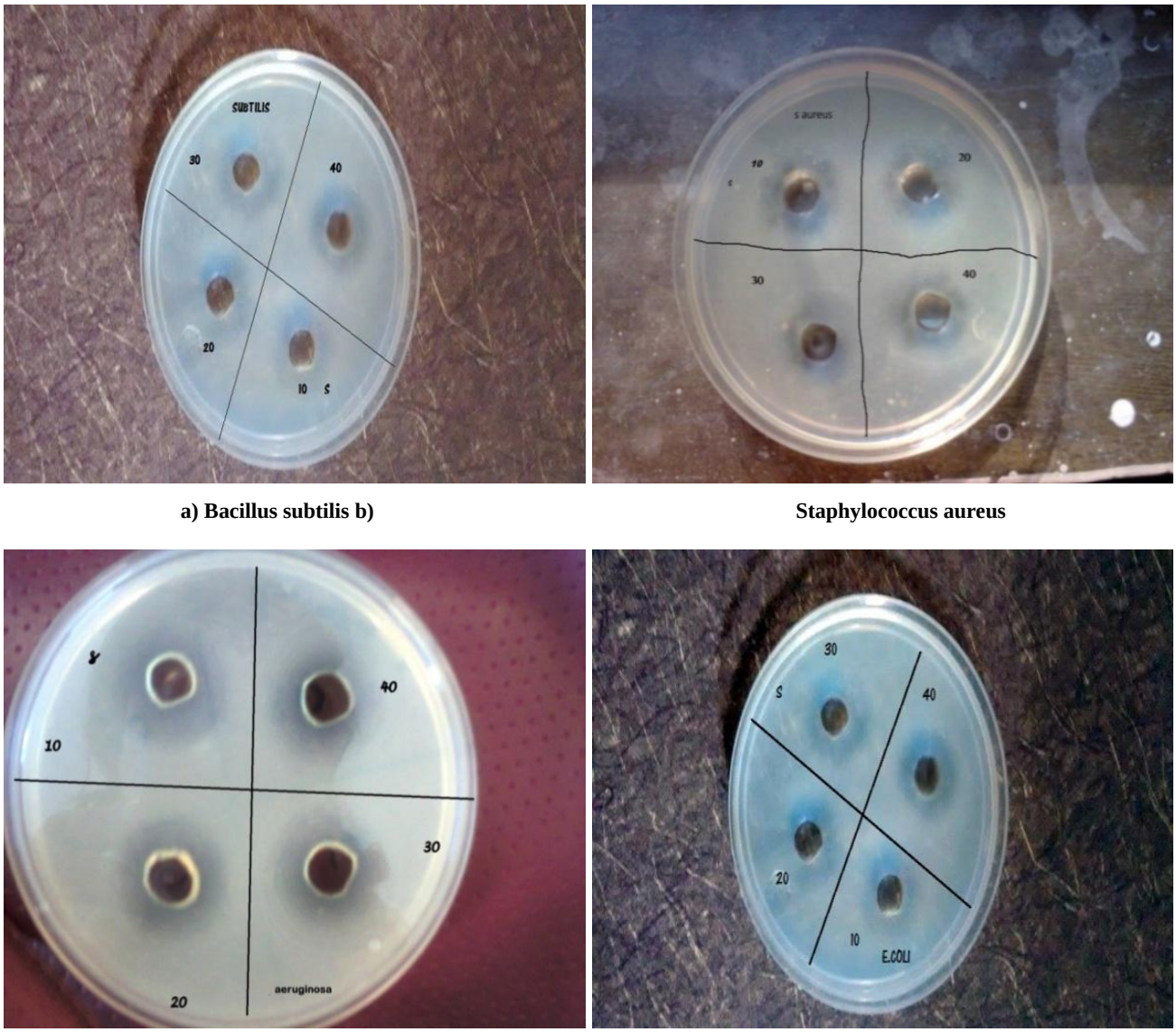
Alkaloids reported to be the most active ingredients to which the antimicrobial activities of many plant species are attributed. In the present study, alkaloids extracted from *Achyranthes aspera* showed inhibitory activity against strains used in experiment. Activity was expressed as mg/ml and the concentrations taken for checking the antibacterial activity of extracted alkaloids were 20, 30, and 40 mg/ml and simultaneously concentration of standard antibiotic (Ampicillin) was 20 mg/ml. Antibacterial activity was confirmed if the radius of zone of inhibition (mm) was greater than 4 mm (Hammer *et al*. 1999) [8]. Criteria taken for evaluation of the antibacterial activity was that, the zone of inhibition was considered as inactive if < 4.5mm; partially active if 4.5-6mm; active if 6.5-9mm and if greater than 9 mm then very active (Junior and Zanil, 2000) [10]. In the investigation the gram positive bacteria i.e *Bacillus subtillis* and *S. aureus* showed more sensitivity than gram negative ones as the zone of inhibition shown by gram positive bacteria were greater than gram negative bacteria. Size of Zone of inhibition formed by bacteria followed the following sequence- *Bacillus subtillis* > *Staphylococcus aureus* > *Pseudomonas aeruginosa* > *Escherichia* coli. Out of four microbial organisms tested the highest activity was shown by *Bacillus subtillis* with maximum zone of inhibition 18 mm at concentration 40 mg/ml. Between the two gram negative bacteria zone of inhibition of *Pseudomonas aeruginosa* was maximum i.e. 12 mm at concentration 40 mg/ml, which proves that alkaloid were very active at all concentrations.

The highest activity of *Bacillus subtillis* can also be supported by the literature of Ismaeil *et al*. (2011) [9], which states that the Gram-positive bacteria were more sensitive than Gram-negative bacteria the reasons may be due to differences in cell envelop of both type of bacteria because the cell envelop of Gram-negative bacteria is more complex than Gram-positive bacteria therefore the Gram-negative bacteria was more resistance than Gram-positive bacteria (Ryan and Ray, 2004) [22]. In the present study Ampicillin was taken as the standard drug against pathogenic bacteria which showed least zone of inhibition for all bacteria as can be shown in below Table 2 when compared to the alkaloid extract. Thus it can be inferred that the alkaloids present in the sample can be further studied for antibacterial properties**.**

Table 2: Antibacterial activity of alkaloids of *Achyranthes aspera* and the standard Ampicillin shown against some bacterial strains by agar well diffusion method

Concentration (mg/ml)	Zone Of Inhibition of Bacteria (mm)*			
	Gram positive		Gram negative	
	B. subtillis	**S. *aureus***	***P. aeruginosa***	***E. coli***
20	15	12	8	5
30	16	14	10	7
40	18	16	12	9
Ampicillin (20mg/ml) Control	13	10	5	4

*Well size = 4mm



a) Bacillus subtilis b) **Staphylococcus aureus**

c) Pseudomonas aeruginosa **d) Escherichia coli**

Plates 1: Antibacterial activity of ethanolic extract of *Achyranthes aspera* against a) *Bacillus subtillis*; b) *S.aureus*; c) *P.aeruginosa* and d) *Escherichia coli*

Alkaloid extract of *Achyranthes aspera* contains potential antimicrobial components that may be of therapeutic use against various infectious diseases caused by gram positive bacteria and gram negative bacteria. Antibacterial activity of *Achyranthine* might be probably due to its intercalation with nucleic acid inhibiting the multiplication of cells as also evident in this study by its DNA and protein binding kinetics. Betanin does not allow the growth of microorganisms due to its antimicrobial activity and protect the food from spoilage (Sonja *et al.* 2011). As a strong anti-microbial agent, Betaine may be used in the treatment of AIDS, as it inhibits HIV-1 reverse transcriptase and also has uses in the treatment of infections, specifically eye infections and hepatitis (Aniszewski, 2007) [1] Betalains which is frequently found in beetroots have been reported to show antimicrobial activities (Brunet *et al*. 2011) [4]

The results of present study supports the traditional usage of the studied plants and suggests that some of the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. These traditional medicines are free of side effects unlike those of allopathy, the resources can be exploited against some multi drug resistant pathogens. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation.

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