

Total Heterotrophic Bacterial population in *Achyranthes aspera* L.

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ABSTRACT:

Clinical microbiologists have great interest in screening of medicinal plants for antimicrobial activities and phytochemicals as potential new therapeutics. *In vitro* antibacterial assay to find the efficacy of *Achyranthes aspera* L. (stem) using various extracts like hexane, butanol, ethanol, chloroform and water was carried out and the result exhibited a broad spectrum of antibacterial potential. When compared the antibacterial efficiency of various extracts of *A.aspera* the gram negative bacteria like *Pseudomonas aeruginosa* and *Klebsiella sp.*, were found to inhibit more than the gram positive bacteria like *Micrococcus sp.*, and *Staphylococcus aureus* . Among the various extracts, the minimum inhibitory concentration (MIC) of *A.aspera* against bacterial pathogens, the ethanol extract showed highest activity than all other solvent extracts. The MIC value was found to be in the ranges from 25-30, 35-40, 45-50, 30-35 µg/ml against *Staphylococcus aureus*, *Micrococcus sp.*, *Klebsiella sp.*, and *Pseudomonas aeruginosa* respectively. In ethanol extract of *A.aspera*, the MBC value was highest in *Klebsiella sp.*, 80µg/ml and lowest in *Staphylococcus aureus* 50 µg/ml. In mice exposed to alloxan and ethanol extract of *A.aspera* significant reduction in their number compared to normal mice and the value was 34 ± 2 CFU/g, 52 ± 3 CFU/g and 144 ± 10 CFU/g in stomach, intestine and rectum respectively. The present study clearly indicate that the test plant (*A.aspera*) has high level of antimicrobial activity and control the bacterial population in an said area.

Keywords: *Achyranthes aspera* L. *Staphylococcus aureus*, *Micrococcus sp.*, *Klebsiella sp.*, and *Pseudomonas aeruginosa*, anti microbial activity, MIC ,MBC and THBP.

Introduction

Nature has been of material agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources (Cragg and Newman, 2001 and Alam *et al.*, 2009). Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. (Nascimento *et al.*, 2000). The use of plant extract for medical treatments is enjoying great popularity since 1990s when people realized that the effective life span of antibiotic is limited and over prescription and misuse of traditional antibiotics are causing microbial resistance (Cohen, 1992, Eisenberg *et al.*, 1993 and Nascimento *et al.*, 2000). The antimicrobial activities of plant extracts may reside in a variety of different components, including aldehyde and phenolic compounds. Naturally occurring combinations of these compounds can be synergistic and often results in crude extracts having greater antimicrobial activity than the purified individual constituents (Delaquis, 2002 and Alam *et al.*, 2009).

Numerous surveys on antimicrobial medicinal plants had been made in United States and in many countries throughout the world. Such study had demonstrated the wide occurrences of active compounds in higher plants (Hughes, 1952). Over the last 40 years, intensive efforts have been made to discover clinically used antibacterial and antifungal drugs (Sofowora, 1984, Valsaraj *et al.*, 1996, Ahamed *et al.*, 1998, Sardari *et al.*, 1998, Werner *et al.*, 1999, Kudi *et al.*, 1999, Perumalsamy *et al.*, 1999, Uma Devi *et al.*, 2007, Sharma and Smita Sharma, 2010 and Pandey *et al.*, 2010).

Plants remain the most common source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava *et al.*, 1996, Bibitha *et al.*, 2002 and Mahesh and Sathish, 2008). A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. Their usage as traditional health remedies is the most popular for 80% of world population in Asia, America and Africa and is reported to have minimal side effects (Maghrani *et al.*, 2005). The different parts used include root, stem, flower, fruit, twigs exudates and modified plant organs (Uniyal *et al.*, 2006). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of have not been adequately evaluated (Balandrin *et al.*, 1985 and Mahesh and Sathish, 2008). The search for antimicrobials of plant origin has been mainly stimulated by the fact that some of the major antibacterial agents have considerable drawbacks in terms of limited antimicrobial spectrum. Now-a-days multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease (Lakshmi Naidu *et al.*, 2006). To date, resistance in bacteria is most prevalent. For example, methicillin resistant *Staphylococcus aureus* (MRSA) has become a huge problem worldwide to treat nosocomial infections since 1990s (Lee *et al.*, 2007). Therefore, in the present investigation the antimicrobial activity of *A. aspera* was assessed against human bacterial pathogens.

Many efforts have been done to discover new antimicrobial compounds from various kinds of sources such as soil, micro organisms, animals and plants. One of such resources is folk medicine and systematic screening of them may result in the discovery of novel effective compounds (Janovska *et al.*, 2003). There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world (Chung *et al.*, 2004, Nair *et al.*, 2004 and De Boer *et al.*, 2005). Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species are used in herbal medicine (Essawi *et al.*, 2005).

Numerous aliphatic compounds have been reported from the seeds and the shoots of *A.aspera*. In light of the above, the present study was carried out to test the antimicrobial efficacy of the leaves extract of *Achyranthes aspera* Linn. with reference to microbes and the extraction of secondary metabolites was well documented by Dheeba *et al.*, 2010.

Antimicrobial activity of various extracts of *A. aspera* leaves against human bacterial pathogens were reported by several workers (Sharma *et al.*, 2006; Saravanan *et al.*, 2008; Manjula *et al.*, 2009, Prasad *et al.*, 2009; Khan *et al.*, 2010 and Saurabh *et al.*, 2011). In the present investigation *A. aspera* was extracted with a low polar to high polar organic solvents and against human bacterial pathogens.

Materials and Methods;

MIC and MBC test:

Minimum Inhibitory Concentration (MIC) of the extracts was determined from the culture plates that had the lowest concentrations and prevented the growth of bacterial strain. Minimum Bactericidal Concentration (MBC) was determined by using the method of Samy and Ignacimuthu (2001). The leaf extracts of *A.aspera* were diluted to obtain concentration ranging from 10 µg -100 µg /ml. The test tube containing 3ml of Muller Hinton broth and 0.1 ml bacterial suspensions and 0.1 ml plant extract were incubated at 37°C for 24h. Bacterial turbidity was measured at 650 nm to determine bacterial inhibition. Streptomycin at 20 and 40µg /ml was used as a reference for determination of minimum inhibitory and bactericidal concentrations respectively. The tubes containing only the growth medium were used as control. The minimum bactericidal concentration that showed the reduction of the bacterial colony as measured from the turbidity of the culture by optical density value.

Total bacterial count of each bacterial species was estimated by counting the number of bacteria in each test tube incorporated with different concentrations of plant extracts and control. The average of three counting was taken as the total number of colony forming bacterial suspensions. All determinations were made in triplicate of extracts.

Total Microbial population study:

The total heterotrophic bacterial population was enumerated by pour plate technique using nutrient agar medium. The stomach, intestine and rectum samples were homogenized individually using a known volume of sterilized distilled water to make serial dilutions. After serial dilution with precaution, one ml of aliquots of appropriate dilutions of the sample was pipetted out into sterile petridishes and 15 to 20 ml of sterile nutrient agar medium were poured. The medium and the inoculums were thoroughly mixed using turn table and the medium was allowed to solidify. Duplicate plates were also maintained. The numbers of bacterial colonies were counted after 48 hrs of incubation. The bacterial populations were expressed as number of colony forming units (CFU) per gram samples analyzed.

Representative of morphologically dissimilar well isolated colonies were selected at random from the nutrient agar plates of stomach, intestine and rectum samples. The selected colonies were sub cultured to check purity after noting morphology and pigmentation of colony. Then the pure bacterial strains were again sub cultured in nutrient agar slants. The slant cultures were stored at 4° C in refrigerator and periodical sub culturing was done to maintain the viability of the bacterial strains. The bacterial cultures were identified up to generic level by employing the scheme of Simidu and Aiso (1962).

Results and discussion:

MIC and MBC method:

In vitro antibacterial assay to find the efficacy of *A. aspera* using various extracts like hexane, butanol, ethanol, chloroform and water was carried out and the result exhibited a broad spectrum of antibacterial potential. When compared the antibacterial efficiency of various extracts of *A.aspera* the gram negative bacteria like *Pseudomonas aeruginosa* and *Klebsiella sp.*, were found to inhibit more than the gram positive bacteria like *Micrococcus sp.*, and *Staphylococcus aureus*. Similar antibacterial activity against gram positive and gram negative bacteria was observed by various workers (Thomas and Mccubbin 2003 and Nair *et al.*, 2004) in the same and different plants.

Minimum Inhibitory Concentration (MIC):

The minimum inhibitory concentration of various extracts of *A.aspera* against bacterial pathogen was given in Table 2.2.

Table 2.2. The Minimum Inhibitory Concentration of various extracts of *A.aspera* (µg/ml) against human pathogenic bacterial organisms.

	Hexane	Butanol	Ethanol	Chloroform	Water
<i>Staphylococcus aureus</i>	35-40	20-25	35-40	35-40	30-35
<i>Micrococcus sp.</i> ,	30-35	35-40	55-60	45-50	40-45
<i>Klebsiella sp.</i> ,	40-45	20-25	55-60	40-45	35-40
<i>Pseudomonas aeruginosa</i>	55-60	30-35	40-45	55-60	40-45

In the present study the MIC of hexane extract of *A.aspera* showed the highest effect in *Pseudomonas aeruginosa* and *Klebsiella sp.*, around 55-60 and 40-45 µg/ml and the lowest effect in *Staphylococcus aureus* and *Micrococcus sp.*, around 35-40 and 30-35 µg/ml. The findings of highest MIC in *Pseudomonas aeruginosa* and the lowest MIC in *Staphylococcus aureus* with *Acacia nilotica* reported by Raghavendra *et al.* (2006) coincided the results of the present study.

Among the various extracts, the minimal inhibitory concentration of *A.aspera* against bacterial pathogens, the ethanol extract showed highest activity than all other solvent extracts. The MIC value was found to be in the ranges from 35-40, 55-60, 55-60, 40-45 µg/ml against *Staphylococcus aureus*, *Micrococcus sp.*, *Klebsiella sp.*, and *Pseudomonas aeruginosa* respectively. However, the MIC of butanol extracts showed the highest effect in *Micrococcus sp.*, and *Klebsiella sp.*, around 30-40 µg/ml and

the lowest MIC in *Staphylococcus aureus* and *Pseudomonas aeruginosa* around 20-25 µg/ml. Similar observations due to different plants were reported by various workers (Akunyili *et al.*, 1993 and Nascimento *et al.*, 2000).

Regarding the minimal inhibitory concentration of chloroform extract of *A.aspera*, the highest effect was seen in *Pseudomonas aeruginosa* (55-60 µg/ml) and lowest effect was found to occur in *Staphylococcus aureus* (35-40 µg/ml) (Table 2.2). The highest MIC effect was seen in *Pseudomonas aeruginosa* and the least activity in *Staphylococcus aureus* due to ethanol and aqueous extract of *Tamarindus indica* reported by Doughari, 2006 corroborated the result of the present study.

The MIC activity of water extract the of *A.aspera* highest effect in *Micrococcus sp.*, and *Pseudomonas aeruginosa* (40-45 µg/ml) and least effect in *Staphylococcus aureus* (30-35 µg/ml) were noticed. Similar findings due to leaf extract of *A.aspera* were reported by Mohan *et al.*, 2008 and Alam, 2009.

Minimum Bactericidal Concentration (MBC):

The minimum bactericidal concentration of the various extracts of *A.aspera* for the four bacterial pathogen was enumerated (Table 2.3).

Table 2.3. The minimum bactericidal concentration of various extracts of *A.aspera* (µg/ml) against human bacterial pathogens.

	Hexane	Butanol	Ethanol	Choloroform	Water
<i>Staphylococcus aureus</i>	65	45	60	50	60
<i>Micrococcus Sp.</i> ,	65	50	80	60	70
<i>Klebsiella Sp.</i> ,	80	45	90	75	65
<i>Pseudomonas aeruginosa</i>	90	55	85	80	70

The minimum bactericidal concentration of the various extracts of *A.aspera* was found to have an appreciable activity as that of standard antibiotic, streptomycin. Regarding the MBC value of hexane extract of *A.aspera* the highest activity was seen against *Pseudomonas aeruginosa* (90 µg/ml) than to other all tested pathogens. The least MBC value was observed in *Staphylococcus aureus* and *Micrococcus sp.*, (65 µg/ml).

The butanol extract of *A.aspera* the MBC value was the highest in *Pseudomonas aeruginosa* (55µg/ml) and the lowest value in *Staphylococcus aureus* (45µg/ml). The chloroform extract of *A.aspera* the MBC value was seen higher in *Pseudomonas aeruginosa* (80 µg/ml) and the value was 50 µg/ml in *Staphylococcus aureus* (Table 2.3). The result of the present study coincided with the report of Sabahat Saeed and Parveen Tariq 2005 and they reported the leaves of *M.piperata* exhibited highest antibacterial activity against *Pseudomonas aeruginosa*. In ethanol extract of *A.aspera*, the MBC value was highest in *Klebsiella sp.*, 90µg/ml and lowest in *Staphylococcus aureus* 60 µg/ml. The present investigation confirmed the previous woks carried out by Ellof, 1998 , Nascimento, 2000 and Doughari,2006.

The water extract of *A.aspera* exhibited highest activity in *Micrococcus sp.*,and *Pseudomonas aeruginosa* (70 µg/ml) and least activity in *Staphylococcus aureus* (60 µg/ml). Similar results were observed in various medicinal plants including *A.aspera* by Perumalsamy *et al.*, 2010.

From the overall observations of the present investigation, among the various extracts, the ethanol extract of *A.aspera* was found to have the highest activity (MIC and MBC) than all other extracts for the tested pathogens. It is interesting to note that regarding minimum inhibitory concentration value of the various extracts of *A.aspera* against tested pathogens, the ethanolic extract was found to inhibit good and that bacteria only at the concentration of 55-60 ug/ml and thereby it wouldn't affect the beneficial microflora of intestine. Therefore, it may be recommended for the preparation of plant based drugs against human bacterial pathogens.

Total heterotrophic Bacterial Population (THBP)

Total heterotrophic bacterial population in mice due to individual effect of alloxan and alloxan in combination with various extracts of *A.aspera* was enumerated and recorded in Table 2.4.

In mice maintained as control, THBP population was found to be 96 ± 12 CFU/g in intestine, 103 ± 12 CFU/g in stomach and was 190 ± 14 CFU/g in rectum.

In alloxanised mice the THBP was found to decrease than control and was 54 ± 3 CFU/g, 59 ± 2 CFU/g, 142 ± 8 CFU/g in stomach, intestine and rectum respectively. (Table 2.4.)

In mice exposed to alloxan and hexane extract of *A.aspera*, decreased level of THBP than the control was exhibited and the values were 68 ± 4 CFU/g, 62 ± 2 CFU/g, 146 ± 12 CFU/g in stomach, intestine and rectum respectively. (Table 2.4.)

In alloxanised mice after administering with butanol extract of *A.aspera*, THBP in stomach was found to be increased than control, while it was decreased in intestine and rectum. The bacterial density was found to 97 ± 6 CFU/g, 98 ± 4 CFU/g and 174 ± 0 CFU/g in stomach, intestine and rectum respectively.

In mice exposed to alloxan and ethanol extract of *A.aspera* significant reduction in their number compared to normal mice and the value was 44 ± 2 CFU/g, 72 ± 3 CFU/g and 164 ± 10 CFU/g in stomach, intestine and rectum respectively.

In chloroform extract of *A.aspera* and alloxan treated mice, decreased THBP population was observed than control and was 86 ± 4 CFU/g, 92 ± 4 CFU/g and 148 ± 12 CFU/g in the respective three regions.

Mice exposed to water extract of *A.aspera* in combination with alloxan a increased THBP was seen than in mice maintained as control and was 99 ± 7 CFU/g, 100 ± 12 CFU/g and 196 ± 20 CFU/g in stomach, intestine and rectum respectively.

Table 2.4. Total heterotrophic bacterial population in the alimentary canal of mice maintained as control and the effect of various extracts of *A.aspera* on alloxanised mice.

S.No	Treatment	Bacterial density (CFU/g)		
		Stomach (10 ⁴)	Intestine (10 ⁵)	Rectum (10 ⁷)
1	Control	96 ± 12	103 ± 12	190 ± 14
2	Alloxanised mice	54 ± 3	59 ± 2	142 ± 8
3	Alloxan + Hexane	68 ± 4	62 ± 2	146 ± 12
4	Alloxan + Butanol	97 ± 6	98 ± 4	174 ± 10
5	Alloxan + Ethanol	44 ± 2	72 ± 3	164 ± 10
6	Alloxan + Chloroform	86 ± 4	92 ± 4	148 ± 12
7	Alloxan + Water	99 ± 7	100 ± 12	196 ± 20

Generic composition of bacterial strains

A total of 96 bacterial strains from *Micrococcus sp.*, *Klebsiella sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, *Bacillus sp.*, *Achromobacter sp.*, *Flavobacterium sp.*, *Proteus sp.*, *Aeromonas sp.* and *E.coli* were enumerated in mice maintained as control and in mice administering with various treatments (Table 2.5).

The number of 10 species of bacterial strains studied in the present study *Pseudomonas sp.*, *Bacillus sp.*, *Micrococcus sp.*, *Proteus sp.*, *Achromobacter sp.*, *Klebsiella sp.*, *Flavobacterium sp.*, *Staphylococcus sp.*, *Aeromonas sp.*, and *E.coli* varied in their numbers by different treatments. The order of strains viz., *Pseudomonas sp.*, *Bacillus sp.*, *Micrococcus sp.*, *Proteus sp.*, *Achromobacter sp.*, *Klebsiella sp.*, *Flavobacterium sp.*, *Staphylococcus sp.*, *Aeromonas sp.*, and *E.coli* in mice maintained as control was 17, 16, 14, 13, 9, 7, 7, 5, 4 and 4 respectively. After exposure to alloxan the values of the same strains observed in mice and the values were in the order of 20, 15, 12, 9, 9, 9, 8, 8, 4 and 2 respectively in *Pseudomonas sp.*, *Bacillus sp.*, *Proteus sp.*, *Micrococcus sp.*, *Staphylococcus sp.*, *Achromobacter sp.*, *Klebsiella sp.*, *Flavobacterium sp.*, *Aeromonas sp.*, and *E.coli*.

The various extracts of *A.aspera* in combination with alloxanised mice showed a varied number of bacterial strains. It is worthy to mention here that the ethanol extract of *A.aspera* treated alloxanised mice the beneficial bacterial strain population was slightly increased than all other treatments. More or less similar number of bacterial strains was observed due to ethanol extract of *A.aspera* treated alloxanised mice as in mice maintained as control (Table 2.5). It is obvious from the present study that the ethanol extract of *A.aspera* had a good bacterial activity as recorded by Tullanithi *et al.*, 2010 in *A.aspera*.

Table 2.5. Generic composition of bacterial strains isolated from mice exposed to alloxan and mice maintained as control.

S.No	Treatment	Bacterial genera										Total no. strains
		<i>Micro-coccus sp.</i>	<i>Strepto-coccus sp.</i>	<i>Staphylo-coccus sp.</i>	<i>Pseudo-monas sp.</i>	<i>Bacillus sp.</i>	<i>Achrom-obacter sp.</i>	<i>Flavo-bacterium</i>	<i>Prot-eus sp.</i>	<i>Aero-monas sp.</i>	<i>Escher-ichia sp.</i>	
1	Normal	14	7	5	17	16	9	7	13	4	4	96
2	Alloxan treated	9	8	9	20	15	9	8	12	4	2	96
3	Alloxan + Hexane	8	9	8	15	17	9	12	6	9	3	96
4	Alloxan + Butanol	8	8	9	18	20	8	10	8	5	2	96
5	Alloxan + Ethanol	5	7	12	16	10	12	9	10	8	7	96
6	Alloxan + Chloroform	8	6	10	19	14	10	8	12	3	6	96
7	Alloxan + Water	12	6	7	15	14	8	12	12	6	4	96

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