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Anti-Bacterial and Anti-Oxidant Activity of *Achyranthes Aspera* Leaf Extract and Its Effect on Gall Bladder Stones

Md. Jakir Hossain[1] , Laila Khaleda[1], A.M. Masudul Azad Chowdhury[1], Md. Arifuzzaman[2], Mohammad Al-Forkan[*1]

1. Department of Genetic Engineering and Biotechnology, University of Chittagong, Chittagong-4331, Bangladesh.
   [E-mail: alforkancu@hotmail.com; Tele: +88-01819383213]
2. Department of Biochemistry and Biotechnology, University of Science and Technology, Chittagong (USTC), Foy's Lake, Chittagong-4202, Bangladesh.

In recent years, bacterial infections are increasing due to emergence of antibiotic resistance. Oxidative stress and gall bladder stones also pose a great health threat to millions. This study attempted the assessment of anti-bacterial, anti-oxidant and anti-gall bladder stones activity of an important medicinal plant *Achyranthes aspera* methanolic leaf extract. Anti-bacterial activity was performed by disc diffusion method. Significant susceptibility was observed against gram positive bacteria than gram negative strains. Anti-oxidant activity was studied in terms of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging potential with ascorbic acid as standard. Highest DPPH scavenging activity for *A. aspera* and ascorbic acid was 59.21% and 92.41%, respectively. IC50 value was 472.93 µg/ml for *A. aspera* and 1.4965 µg/ml for ascorbic acid. Effects of *A. aspera* leaf extract on gall bladder stones were also investigated. 2.0 mg/ml extract concentration showed the highest amount of cholesterol release (39.69 mg/dl) and the highest amount of dry weight reduction (2.3 mg) from gall stones. Morphological changes like color change and fragility in gall stones were also observed at concentrations higher than 1.5 mg/ml.

Keyword: Medicinal Plant, *Achyranthes aspera,* Anti-bacterial, Anti-oxidant, Gall bladder stone, DPPH, IC50

1. Introduction

Bangladesh harbors a large number of medicinal plants but therapeutic potential of many of these important medicinal plants are yet to be revealed. Medicinal plants and their formulations are used enormously for treating a range of illness in ethnic medical practices as well as traditional system of medicine in India[1]. Since the beginning of world history, nearly all culture and civilization depended either fully or partially on plant derived medicines because these drugs are effective, low-cost, readily available and believed to be safer and less toxic[2]. For these reasons, herbal medicines and medicinal plants are not only meeting treatment needs in developing countries but also getting popular in developed world[3]. *Achyranthes aspera* is a perennial herb belonging to the family of Amaranthaceae. It grows throughout the tropical and warmer regions of the world[4]. It was reported as an invasive alien species in northern Bangladesh[5]. *Achyranthes aspera* was

reported to contain many phytochemicals like alkaloids, flavonoids, tannins, terpenoids, saponins, glycosides, steroids etc. Efficacy of *Achyranthes aspera* was also established by many scientific investigations as anti-microbial[6,7], hypoglycemic[8], cancer chemo-preventive[9], hepatoprotective[10], analgesic[11], anti-pyretic, anti-inflammatory and anti-arthritic[12], hypolipidemic[13], nephroprotective[14], diuretic[15] and immunomodulatory[16] etc.

Bacterial infections are great health concern worldwide. Emergence of antibiotic resistance and multidrug resistance in recent years is increasing the incidence of infections. Infectious diseases are the world's leading cause of premature deaths[17]. Therefore, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action. Medicinal plants represent a rich source of antimicrobial agents. In recent years, there is a growing interest to evaluate plants possessing antibacterial activity[18] and it has also been proved that various medicinal plants extracts possess bacteriostatic and bactericidal effects[19]. Natural compounds in plants such as tannins, terpenoids, alkaloids, and flavonoids have been found to have antimicrobial properties established through *in vitro* studies.

Antioxidants are type of molecules that neutralize harmful free radicals, produced through a chain of reactions[20], that damage living cells, spoil foods, degrade materials such as rubber, gasoline, lubricating oil. Antioxidants terminate these chain reactions through the removal of free radical intermediates and inhibition of other oxidation reactions[21]. The use of antioxidants in pharmacology is intensively studied as oxidative stress might be an important part of many human diseases particularly stroke and neurodegenerative incidents[20]. Recently, there has been an increasing interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Many new plant species have been investigated in the search for novel antioxidants[22,23] but there is still a demand to find more information on the antioxidant potential of plant species.

Gall bladder is an important organ in human body exclusively employed in storing biles secreted from the liver and passes this bile in response to a fat rich diet. When this bile contains high level of cholesterol it becomes hardened, crystalline and doesn't move from gall bladder to other parts that is then termed as gall stones[24]. Gall bladder stones are mainly cholesterol stones, while pigment stones and mixed stones composed of bile pigments and bile salts are also seen[25]. Gallstones incidence is more common in western society[24]. An estimated twenty million Americans have gallbladder problems and approximately 750,000 of them will have their gallbladder removed each year[26]. About 80% of all gallstones are cholesterol stones that vary in color from light-yellow to dark-green or brown and are oval 2 to 3 cm in length, contain at least 80% cholesterol by weight (or 70%, according to the Japanese classification system) and remaining 20% is bilirubin[27]. Many believe that it is possible to treat gallstones with herbal products and they find it low cost, safe and painless. Medicinal plants with anti-oxidant, anti-inflammatory, anti-bacterial, diuretic and sedative properties are a good candidate for gall stone natural treatments.

In this study, *Achyranthes aspera* methanolic leaf extract were tested for anti-bacterial and anti-oxidant activity while efficacy of this plant extract against gall

bladder stones were also examined under *in vitro* conditions.

2. Materials and Methods:

2.1Collection of Plant and Preparation of Plant Extract

The fresh and healthy leaves of *Achyranthes aspera* were collected from Chittagong University Campus area. The plant was taxonomically classified and authenticated by standard taxonomical method. The fresh leaves of plant of *Achyranthes aspera* was washed with clean water immediately after collection. The collected leaves were chopped into small pieces, sun dried for about 5 days and grinded into coarse powder with a mechanical grinder and stored in an airtight container. 166 gm powder was macerated in 700ml 95% pure methanol (Sigma Chemicals Co., USA) for 5 days at room temperature 25±2^0 C with occasional stirring. After 5 days, methanol extract was filtered with Whatman No.1 filter paper. The extract was concentrated under reduced pressure below 50^{0}C through rotary vacuum evaporator[28]. The concentrated extract was collected in a Petri dish and allowed to air dry for complete evaporation of methanol. The whole process was repeated three times and finally, 14.77gm blackish-green colored, concentrated plant extract was obtained (yield 8.9% w/w) which was kept in refrigerator at 4^{0}C. The extract thus obtained is ready for subsequent therapeutic assessments.

$$\text{Yield calculation} = \frac{\text{Weight of particular extract}}{\text{Total amount of coarse powder}} \text{X} 100$$

2.2 Anti-Bacterial Activity of *A. Aspera* Leaf Methanol Extract

2.2.1 Bacterial strains used in the study

The microorganisms employed in the current study **(Table 1)** were procured from the Laboratory of Molecular Genetics, Dept. of Genetic Engineering and Biotechnology, University of Chittagong.

2.2.2 Preparation of discs

The discs of about 4 mm diameter were cut by punching machine from What man No.1 filter paper. The discs were taken in a Petri dish and sterilized by autoclave, dried in oven at 80^{0}C.

2.2.3 Preparation of Plant Extracts Solution

1.0 gm of *Achyranthes aspera* extract was accurately weighed and dissolved in 10 ml of DMSO to give solution of known concentration (100µg/µl). 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.

Table 1: List of Gram (+) ve and Gram (-) ve bacteria used for the study

Bacterial type	Test organism
Gram (+)ve	*Bacillus cereus*
	Bacillus subtilis
	Bacillus megaterium
	Staphylococcus sp.
Gram (-)ve	*Vibrio cholerae*
	Shigella sonnei
	Shigella dysenteriae
	E. coli
	Salmonella typhi
	Pseudomonas sp.

2.3 Standard and Media Used

Cefixime antibiotic disc (HiMedia Pvt. Ltd, Mumbai India) with concentration of 5µg/disc was used as standard in the present study. Mueller Hinton agar media no. 173 was purchased from Hi media Pvt. Ltd., Mumbai, India and the solvents used was analytical reagent (AR) grade and they were distilled before use.

2.4 Disc diffusion Method

The *in vitro* sensitivity of the bacteria to the test materials was done by disc diffusion method well established by Iennette (1985)[29]. It is essentially a quantitative and qualitative test indicating the sensitivity or resistance of the micro-organisms to the test materials and the extent of sensitivity as well.

2.5 Determination of Anti-oxidant activity of *A. aspera* leaf extract

2.5.1 Working procedures

The antioxidant activity of the plant extracts and the standard was assessed on the basis of the scavenging potential of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical by modified method[30]. Ascorbic acid (Sigma Aldrich chemical co. USA) was used as standard anti-oxidant. Seven different concentrations (10, 20, 40, 80, 160, 320 and 640 µg/ml respectively) of plant extract and ascorbic acid were used in this study. 100 mg of *A. aspera* leaf extract was dissolved in 20 ml of pure (95%) methanol to give final concentration of 5 mg/ml. Then further dilutions from this stock were also prepared in methanol for each concentration of extract tested. Ascorbic acid solution was prepared in the same way and same concentration that of the plant extrac. The diluted working solutions of the test extracts and ascorbic acid were prepared in methanol. 0.002% of DPPH was prepared in methanol. For each concentration, 1 ml of DPPH solution was mixed with 1 ml of sample solution. For standard, 1 ml of DPPH solution was mixed with 1 ml of ascorbic acid. For, negative control or blank, one ml of DPPH solution was mixed with 1 ml of methanol to see whether the solvent used has any anti-oxidant activity or not. These solution mixtures were kept in dark for 30 min and shaken vigorously, and optical density was measured at 517 nm using Cecil-Elect UV Spectrophotometer. For each concentration, the optical density of both the sample and standard was recorded against the blank and % inhibition of DPPH free radical was calculated using the formula given below[31]. All experiments were performed in triplicate.

$$\text{Percent (\%) inhibition of DPPH} = \frac{A - B}{A} X100$$

Where A = optical density of the blank and B = optical density of the sample.

Then, log C for each concentration of plant extract and ascorbic acid was calculated and plotted in the graph against corresponding % of DPPH scavenging activity. Then, IC50 (Inhibitory concentration at which 50% DPPH are neutralized) value for both plant extract and ascorbic acid was calculated by regression analysis and using statistical software "Biostat 2009".

2.6 Assessment of *A. Aspera* Leaf Extract Activity on Gall Bladder Stones

2.6.1 Working protocol

The gall bladder stones were dried at 45^0C in an oven and dry weight of the stones was accurately measured in an air tight 4-digit electronic balance. Then cholesterol content in plant extract (blank) was estimated at all ten concentrations before treating stones. For this purpose, 0.1 ml from each extract concentration was added to 9.9 ml of the $FeCl_3$-CH_3COOH solution into a glass stoppered centrifuge tube. Then it was mixed well and centrifuged at 4000 rpm for 15 minutes. After centrifugation, it was stood for 10 minutes for any proteins to flocculate (if any). Then from the supernatant 5 ml of clear solution was taken in a test tube. For the standard, 5 ml of cholesterol solution was taken in another test tube. As blank 5 ml of the $FeCl_3$-CH_3COOH solution was taken in a separate test tube. Then 3 ml of concentrated H_2SO_4 were added to all test tubes, mixed by shaking

carefully. After 30 minutes, the optical density (O.D) was recorded in UV spectrophotometer for all the extract concentrations and standard against the blank at 560 nm. There were two test tubes for each sample concentrations and standard. Afterwards, 5 ml of each extract concentration was taken in a test tube and pre-weighed stones were put in the sample. As blank, 5 ml of distilled water was taken in a separate test tube. There were 3 test tubes for each concentration and one stone in each tube. Then, these tubes with stones were incubated at 37^0C for 7 days. After incubation, the stones were picked up, dried and again dry weight were taken. Difference in this dry weight and dry weight before treatment were calculated to see weight reduction due to extract effect on stones. Then, the cholesterol content of the extracts after treating stones was again measured in the same procedure as described earlier for measuring cholesterol level in plant extract before treating stones. From the difference in the cholesterol content after and before treatment were calculated to see the amount of cholesterol released from the stones due to effect of the extract.

3. Results and Discussions

3.1 Antibacterial assay of *Achyranthes aspera* leaf methanol extract

Antibacterial activity of *Achyranthes aspera* leaf methanol extract was studied against four Gram positive and six Gram negative bacteria by disc diffusion method and compared with the standard antibiotic disc of Cefixime (5µg/disc). DMSO 2mg/disc was used as negative control. Antibacterial activity of *Achyranthes aspera* leaf methanol extract was tested at three different concentrations such as 1 mg/disc, 2 mg/disc and 3 mg/disc. All three concentrations produced zone of inhibition and thus showed different degree of antibacterial activity against all ten strains of the bacteria. It was observed that gram positive bacteria showed slightly greater susceptibility than gram negative bacteria to the plant extract. A dose dependent antibacterial activity was also found. With the increase in extract concentration, the zone of inhibition was also increased. However, the highest zone of inhibition was observed in 3 mg/disc extract for all the strains. For 3 mg/disc, zone of inhibition was the highest (13.5 mm) in *Bacillus cereus* and *Bacillus megaterium* and the lowest (7.5 mm) in *Pseudomonas sp*. For 2 mg/disc, zone of inhibition was highest (10.5 mm) in *Bacillus cereus* and the lowest (6.5 mm) in *Salmonella typhae*. For 1 mg/disc, zone of inhibition was the highest (8.5 mm) in *Bacillus megaterium* and the lowest (5.0 mm) in three strains namely, *Staphylococcus aureus, E. coli* and *Salmonella typhae*. Disc with DMSO showed no zone of inhibition at all. Standard antibiotic disc of cefixime also exhibited no susceptibility to the strains tested. **(Table 2)**. An inhibition zone of 10mm or greater was considered to indicate good antibacterial activities[32]. Even low concentration of plant extract (1 mg/ml) also inhibited bacterial growth in *Bacillus megaterium* and *Bacillus cereus* (8.5 and 7.5 mm respectively). This result is proved to be better in compared to previous study of[32,33,34]. Antibacterial study in other species of this genera also showed poor response and required high dose[35,36,37].

Table 2: *In vitro* antibacterial activity of *A. aspera* leaf methanol extract

Type of organism	Bacterial strain	Diameter of zone of inhibition (mm)		
		Different conc. of plant extract	Cefixime (standard)	DMSO (Negative control)

		1mg/disc	2mg/disc	3mg/disc	5µg/disc	2mg/disc
Gram (+)ve	*Bacillus cereus*	7.5	10.5	13.5	0	0
	Bacillus subtilis	6.5	8.5	9.0	0	0
	Bacillus megaterium	8.5	9.0	13.5	0	0
	Staphylococcus aureus	5.0	7.0	8.5	0	0
Gram (-) ve	*Vibrio cholerae*	6.5	8.5	10.0	0	0
	Shigella sonnei	6.0	8.0	9.5	0	0
	Shigella dysenteriae	6.0	8.0	9.5	0	0
	E. coli	5.0	8.0	8.5	0	0
	Salmonella typhae	5.0	6.5	8.5	0	0
	Pseudomonas sp.	6.0	7.0	7.5	0	0

3.2 Determination of *in vitro* Anti-Oxidant Activity of *A. aspera* leaf Methanol Extract

The scavanging capability of DPPH was determined by the decrease in its absorbance at 517 nm and also by the degree of colour change from purple to yellow. Both ascorbic acid and *A. aspera* methanol extract showed dose dependent activity. Among the seven different concentrations tested in this study (10, 20, 40, 80, 160, 320 and 640 µg/ml) ascorbic acid showed 60.40, 69.80, 75.44, 80.18, 83.56, 87.30 and 92.41% DPPH free radical scavenging activity respectively, where 640µg/ml concentration produced highest scavenging activity (92.41%) . For all the seven concentrations, *A. aspera* methanolic leaf extract showed 17.86, 25.41, 32.59, 34.56, 35.79, 42.0 and 59.21% scavenging activity respectively, where the highest DPPH scavenging activity was 59.21% for *A. aspera* methanol extract at the concentration of 640µg/ml. It was observed that % of DPPH free radical scavenging activity linearly increased with the increase in concentrations for both ascorbic acid and *A. aspera* leaf extract. The anti-oxidant activity in terms of % of DPPH free radical scavenging activity or % of inhibition was plotted against log concentrations for both ascorbic acid and *A. aspera* leaf extract and from the graph IC50 (Inhibition concentration 50) value was calculated by linear regression analysis. IC50 value of ascorbic acid and *A. aspera* leaf methanol extract was found to be 1.4965 and 472.93 µg/ml, respectively (Fig.1 and 2). The graph in Fig. 3 shows comparison of anti-oxidant activity between ascorbic acid and *A. aspera* leaf extract.

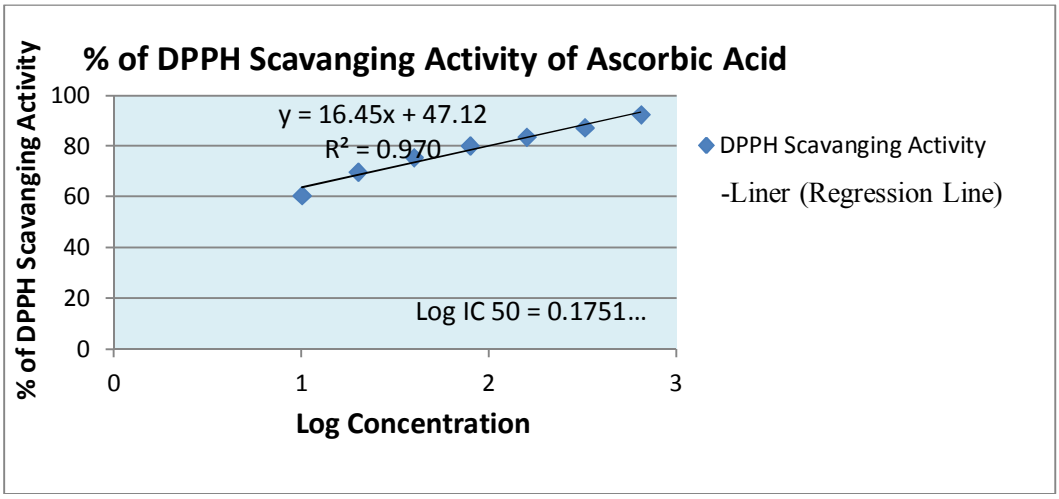

Fig. 1: Regression line for determining the IC50 value of Ascorbic acid

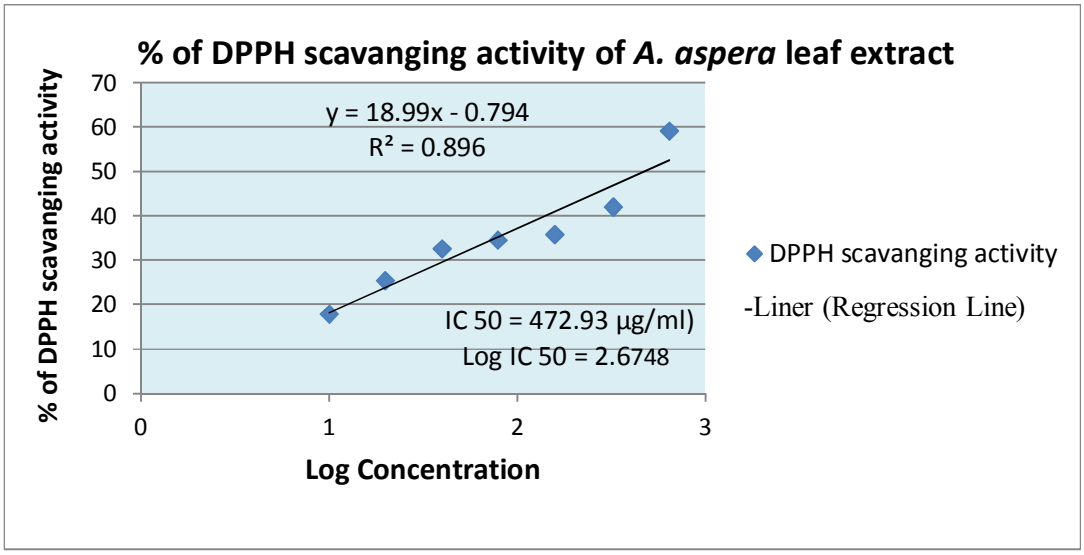

Fig. 2: Regression line for determining the IC50 value of *Achyranthes aspera* leaf methanol extract.

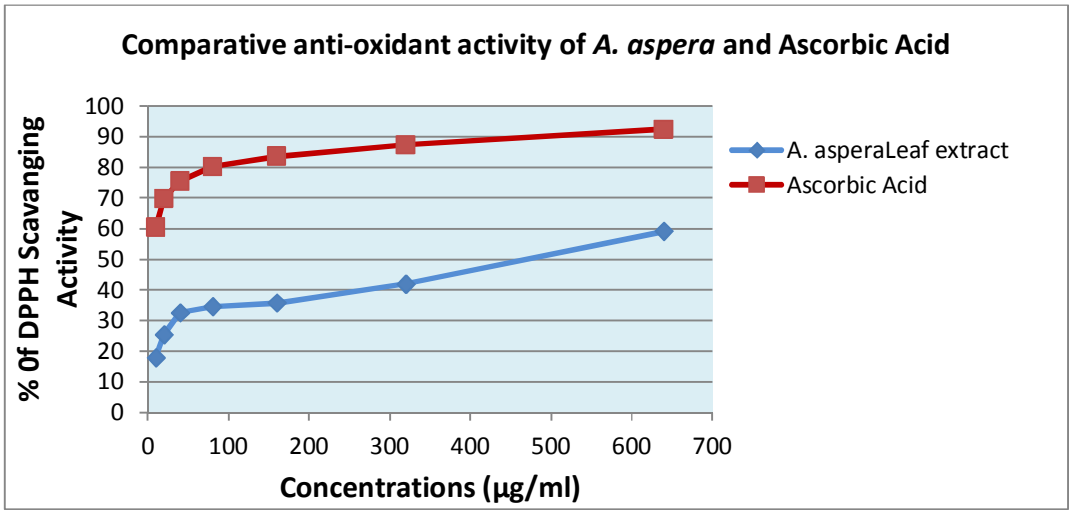

Fig.3: Comparison of anti-oxidant activity of *A. aspera* and Ascorbic acid

A good linearity was observed for regression line where R^2 value was 0.970 and 0.896 for ascorbic acid and *A. aspera* extract respectively (Fig. 1 and 2). The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm and also by the colour change from purple to yellow, which is induced by anti-oxidants[38]. The degree of discoloration indicates the scavenging potential of the antioxidant compound in the extracts. This result is in conformity with that previously obtained by other studies[33,39]. However, the chemical constituents present in the extract, which are responsible for anti-oxidant activity, need to be investigated. The phytochemical tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the crude methanolic extract. Several of such compounds are known to possess potent antioxidant activity[40]. The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins[41]. In *in vitro* condition, such information may be of potential value in the design of further studies to unravel novel strategies for disorders associated with free radicals-induced tissue damage[39].

3.3 Assessment of Gall Bladder Stones Treatment

Gall bladder stones collected from hospitals (operation theatre) were treated with ten different concentrations of *A. aspera* extract (0.0625, 0.125, 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0 mg/ml). Distilled water without extract was used as negative control. Stones were categorized as cholesterol stones (yellowish or brown color) and pigment stones (dark, blackish colored). First, the cholesterol content of the extract concentrations before treating stones were estimated. Very low level of cholesterol was found in the extract. It was observed that, with the increase in concentrations the amount of cholesterol was also found to be increased. The highest amount of cholesterol was obtained in 2.0 mg/ml concentration and lowest amount was obtained in 0.0625 mg/ml.

Then, amount of cholesterol in different concentrations of extract were again measured after treating gall bladder stones in extract for 7 days period at 37^0C. It was observed that, the amount of cholesterol was higher in all concentrations after treatment than that obtained before treatment which

indicates that some cholesterol must have been released in the extract from stones. From the difference between amount of cholesterol in extract before treatment and after treatment, the amount of cholesterol released was calculated for each concentration and presented in Fig. 4. With the increase in extract concentrations, the amount of cholesterol release also increased. The highest amount of cholesterol was released (39.69 mg/dl) from stone treated with 2.0 mg/ml extract concentration and lowest amount of cholesterol released (23.55mg/dl) was observed in 0.0625 mg/ml extract concentration whereas in the control or distilled water only 7.80 mg/dl cholesterol released.

The effect of the *A. aspera* extract on the dry weight of the stones before and after treatment was also examined. It was observed that the plant extract has negligible effect on the dry weight. Very minute amount of weight reduced after treatment. However, the amount of weight reduced was increased with increasing concentrations of extract. The maximum weight reduction was 2.3 mg observed in 2.0 mg/ml extract concentration while the control or distilled water reduced only 0.3 mg. This result of weight reduction is presented in column chart (Fig. 5). Morphological changes were also observed in stones after treating with plant extract. The color of cholesterol stones was changed from yellowish brown to white. Concentrations higher than 1mg/ml induced color change. No color change observed in dark or pigment stones. The stones were much harder before treatment and fragility was observed after treating with extract. Only the stones treated with extract concentrations of 1.50, 1.75 and 2.0 mg/ml showed fragility in a lesser extent.

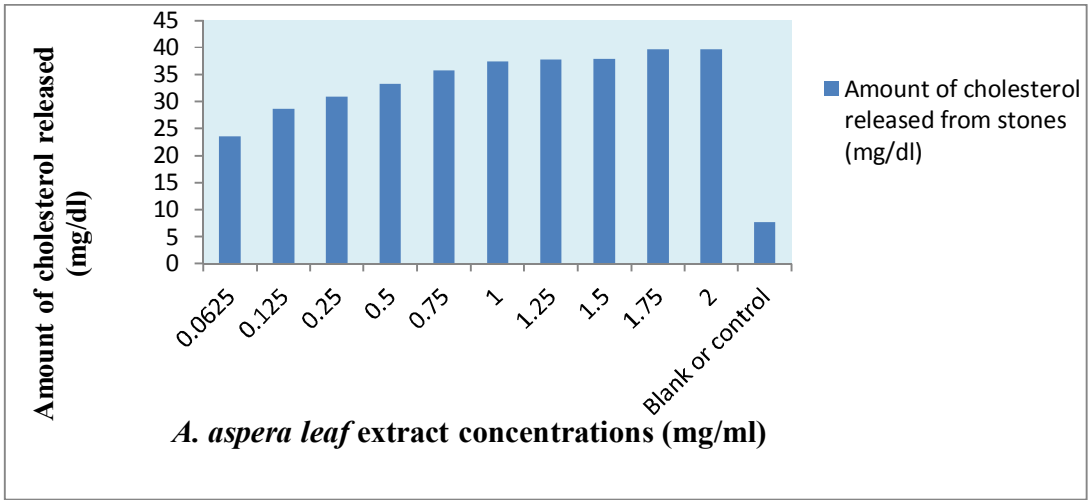

Fig.4: Column chart showing amount of cholesterol released from gall bladder stones after treating with A. aspera extract at different concentrations

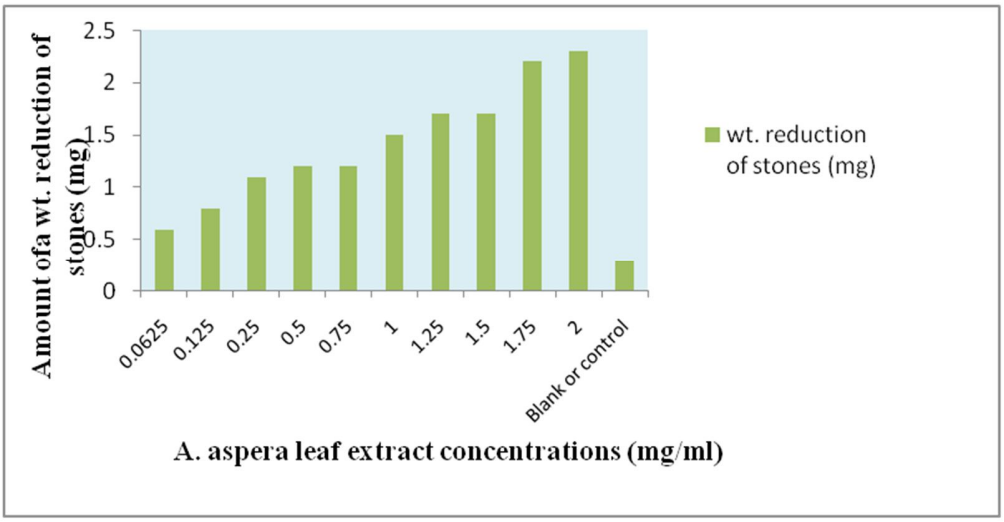

Fig.5: Column chart showing the weight reduction of gall bladder stones after treating with *A. aspera* extract at different concentrations.

Achyranthes aspera is the plant, which is commonly used in Ayurveda for the treatment of stomachache, digestive problems, piles, boils and skin erruptions[42]. *A. aspera* was reported to inhibit mineralization of urinary stones (calculi) like calcium oxalate, calcium carbonate and calcium phosphate[43]. Methanolic extracts were found to prevent lead induced nephrotoxicity in albino rats[14]. Efficacy of the roots of the plant was tested on nucleation of calcium oxalate crystal and growth *in vitro* and on oxalate induced injury in NRK-52E (rat renal tubular epithelial) cells[44]. As an approach to anti-lithiasis, inhibitory effect of hydroalcoholic extract of the plant on crystallization of calcium oxalate in synthetic urine was investigated[45]. This plant was reported to contain saponins. Saponins can emulsify cholesterol and fat present in the stones. Diuretic property of the plant also supposed to contribute in gall bladder stone treatment as it can increase the volume of bile thus preventing stone formation[15]. Antibacterial activity of the plant is also important in applying it for gall stone treatment to prevent infection. Anti-oxidant activity of the plant is also helpful in preventing oxidative stress to gall bladder[46]. Anti-inflammatory activity of *A. aspera* was reported earlier[47,12] supposed to reduce pain caused by cholicystitis. However, *in vitro* treatment of gall stones with medicinal plant extract was not previously attempted. No, established report was available in this regard. This protocol was a very preliminary approach which needs more *in vitro* and *in vivo* studies to reach a final conclusion.

4. Conclusion

This study observed an appreciable degree of anti-bacterial and anti-oxidant activity of *Achyranthes aspera* methanolic leaf extract as well as it's efficacy against gall bladder stones was also promising. Its effect on gall stones provided totally new findings and opened new window to think about the potentials of this plant. However, the identification, isolation and purification of active phytochemical constituents responsible for these therapeutic properties may lead to new drug development from this plant. The therapeutic activities tested in this study on *A. aspera* leaf extract had shed more light on pharmacological importance

of this plant and thus further *in vitro* and *in vivo* studies can help large number of people to utilize the natural medication from this plant.

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