Antibacterial Activity of Vacuum Liquid Chromatography (VLC) Isolated Fractions of Chloroform Extracts of Seeds of *Achyranthes Aspera*

[1]NOOR-UL-AMIN, [1]M. IMRAN QADIR*, [2]TAHIR JAVED KHAN, [1]GHULAM ABBAS, [3]BASHIR AHMAD, [3]KHALID HUSSAIN JANBAZ AND [4]MUHAMMAD ALI
[1]*College of Pharmacy, Government College University, Faisalabad, Pakistan.*
[2] *University College of Pharmacy, University of the Punjab, Lahore, Pakistan.*
[3]*Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan.*
[4]*Institute of Biotechnology, Bahauddin Zakariya University, Multan, Pakistan.*
mrimranqadir@hotmail.com*

(Received on 11th May 2011, accepted in revised form 6th February 2012)

Summary: Antibacterial activities of locally occurring weed *Achyranthes aspera* were studied. Three solvents (Hexane, Chloroform, and Ethanol) were used successively for the extraction of active principles from the seeds of this plant. The extracts were concentrated on vacuum rotary evaporator. The concentrated extracts were tested for their antibacterial activities after making their solution in gum acacia. The six bacterial strains used in the antibacterial studies were *Bacillus subtilis*, *Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeuroginosa*, *and Salmonella chloerasuis*. Antibacterial activities of the extracts were compared with streptomycin and ampicillin in terms of zones of inhibition. Chloroform and ethanol extracts demonstrated antibacterial activity. Hexane extract did not demonstrate antibacterial activity. Chloroform extract was more potent than alcohol extract in terms of antibacterial activity. An attempt was made to identify the nature of compound by isolation through vacuum liquid chromatography (VLC). The fractions isolated by VLC were subjected to thin layer chromatography (TLC). TLC showed the presence of alkaloids and terpenoids. The active fractions were tested for their antibacterial activity. One of the fractions exhibited antibacterial activity.

Key Words: *Achyranthes aspera,* Vacuum liquid chromatography, Isolated fractions, Antibacterial activity.

Introduction

Infectious diseases are important health hazard all over the world both in developing and developed countries. Several antibiotics are employed in the treatment of infectious and communicable diseases. The problem of resistance has decreased the value of existing antimicrobial drugs. This problem of resistance has been tried to be overcome by increasing the drug delivery to the target site by the use of polymers [1,2] or through nanotechnology [3,4], synthesis of new antimicrobial drugs, either by the use of proteomics [5-7], or syntheis of drugs from lactic acid bacteria [8] or marine microorganisms [9]. However, now a day, the trend is being changed to the use of herbal products or extracts to control the diseases in human beings [10].

Medicinal plants are providing an effective local aid to health care and disease free life and they contain physiologically active principles that over the years have been exploited in traditional medicines for the treatment of various ailments. These medicinal plants are used in the treatment of diseases either alone or in combination with other plants [11]. Moreover, pharmacological properties of medicinal plants may be used as lead compound in developing novel therapeutic agents.

Medicinal plants thus form an important component of traditional medicines [12]. Medicinal plants used in the traditional medicines should therefore be studied for the safety and efficacy in light of modern scientific investigation. About 74% of pharmacologically active plant derived component were discovered after following up on the ethno medical use of plants. South Asian regions including India, Pakistan and Bangladesh are very rich in medicinal plants, but relatively little chemical work has been done on medicinal plants from these regions. The application of herbal drugs is gaining momentum at very fast speed. A number of herbal drugs are traded into the market at national and international level.

Achyranthes aspera, is an important herb of family Amaranthaceae. It is distributed throughout the tropical and subtropical regions including Pakistan, India, and Bangladesh. It is an erect annual herb which attains a maximum height of 1 m [13]. It is reported to contain alkaloids, flavanoids, saponins, steroids and terpenoids. It is used as a purgative,

*To whom all correspondence should be addressed.

diuretic, antiarthritic, antispasmodic, cardio-tonic, and expectorant [14]. This study has been carried out to determine the antibacterial activity of extract of seeds of *Achyhranthes aspera* and its isolated fractions.

Results and Discussion

One of the concentrated fractions exhibited antibacterial activity against the three strains of bacteria. The activity against the *Pseudomonas aeruginosa* is slightly more than that of *Escherichia coli* and *Bacillus subtilis*. The comparison of antibacterial effect of this fraction with streptomycin and ampicillin, their mean values (for six different agar plates), test statistics have been outlined in the following tables. Table-1 shows the mean values of zone of inhibition (mm) produced by the concentrated VLC isolated fraction and the reference antibiotics (streptomycin & ampicillin). This assay was performed in six different agar plates. Table 2 and 3 shows the standard deviation and calculation of T values with streptomycin and ampicillin respectively while the Table-4 shows the values of level of significance. Results with $P < 0.001$ were considered to be statistically significant.

From these results it can be concluded that some antibacterial component is present in the seeds of *Achyranthes aspera* which has been isolated by the VLC. Khan *et al*., [15] have demonstrated the activity of the plant. In addition to confirmation of the antibacterial activity of seeds of the plant, we demonstrated that TLC fraction containing alkaloids and terpenoids, isolated from VLC isolated fraction was the active component of the extract. Seeds of this plant are reported to contain saponins A and B which yield oleanolic acid as aglycone [16], while the carbohydrate component are D-glucose, L-rhamnose, D-glucuronic acid for saponin A; and saponin B is the β-D-galactopyranosyl ester of saponin A [17] and contains hexatriacontane, 10-octacosanone, 10-triacosanone, 4-triacontanone [18], some amino acids [19]. Therefore the antibacterial activity is due the presence of one of these components.

Table-1: Comparison of antibacterial activity of VLC isolated fraction of chloroform extract of seeds of *Achyranthes aspera* with streptomycin and ampicillin.

Drug/extract	Conc.	Zone of inhibition(mm)		
	(mg/ml)	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeuroginosa*
Streptomycin	01	23.68	23.36	24.00
Ampicillin	01	19.89	19.25	22.10
VLC isolated fraction	01	8.10	7.36	8.70

Mean values for six plates

Table-2: T values with streptomycin.

Drug/ extract	Zone of inhibition(mm)		
	Bacillus subtilis	*Escherichia coli*	*Pseudomonas aeuroginosa*
Streptomycin	23.68±0.98	23.36±1.30	24.00±0.61
VLC isolated fraction	8.10±0.80 t=29.96	7.36±1.59 t=19.04	8.70±2.06 t=17.58

Table 3: T values with ampicillin.

Drug/extract	Zone of inhibition(mm)		
	Bacillus subtilis	*Escherichia coli*	*Pseudomonas aeuroginosa*
Ampicillin	19.89±0.69	19.25±1.64	22.10±0.60
VLC isolated fraction	8.10±0.80t=27.42	7.36±1.59t=12.79	8.70±2.06 t=15.28

Table-4: Calculation of P values.

Drug/extract	Zone of inhibition(mm)		
	Bacillus subtilis	*Escherichia coli*	*Pseudomonas aeuroginosa*
Streptomycin	23.68±0.98	23.36±1.30	24.00±0.61
Ampicillin	19.89±0.69	19.25±1.64	22.10±0.60
VLC isolated fraction	8.10±0.80***c	7.36±1.59***c	8.70±2.06***c

The values with asterisks (Fisher and Yates, 1974) are significantly different from streptomycin (a=p<0.05, b=p<0.01, c=p<0.001) and ampicillin (*=p<0.05, **=p<0.01,***=p<0.001) according to the student "t" test.

Experimental

Extraction

Seeds of above mentioned plants were collected from the local market. Seeds were dried under the shade. The dried seeds were pulverized using pestle and mortar. Three solvents (Hexane, Chloroform, and Ethanol) were used successively for the extraction of active constituents from the dried and pulverized seeds. Soxhlet extraction apparatus was used for the purpose of extraction. The extracts were concentrated using the vacuum rotary evaporator stored at a temperature below 25^{o}C.

Bacteria Used for Antibacterial Studies:

Antibacterial studies were conducted upon six strains of bacteria which include *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 9341), *Escherichia coli* (ATCC 25922), *Pseudomonas aeuroginosa* (ATCC 27853) and *Salmonella choleraesuis* (ATCC 13312). Pure cultures were obtained from Schazoo laboratories, Lahore (Pakistan) and Schering pharmaceutical industries, Lahore (Pakistan) and from the microbiology laboratory, University College of Pharmacy, University of the Punjab, Lahore (Pakistan). Bacteria were maintained in nutrient agar media.

Assay of Antibacterial Activity

The hole-plate diffusion method was applied to test the antibacterial activity of crude extracts (Hexane, chloroform, and ethanol) of seeds of *Achyranthes aspera*. Nutrient agar media was prepared and autoclaved for about 15 minute at 121 °C at 15 pound pressure.

Prepared the fresh culture of each bacterium by incorporating one loop full of microorganism in separate slants and then kept in incubator at 30-35 °C for 24 hrs. Suspension of each bacterium was prepared by incorporating one loop full of fresh micro-organism in 10 ml of sterilized water.

Poured 1ml suspension of each bacterium in already sterilized Petri-dishes separately and then liquefied nutrient medium in the same Petri-dishes to give the depth of 8mm. The Petri-dishes were rotated for proper mixing of micro-organism in nutrient medium and left them to solidify.

The agar core was then removed from the set agar by a sterilized borer at six peripheral positions and numbered them as 1, 2, 3 and $1^{/}$, $2^{/}$, $3^{/}$. The holes were aseptically filled with reference antibiotic solutions(streptomycin and ampicillin 1mg/ml), different samples of extracts (5mg/ml, 50mg/ml, 100mg/ml) and gum acacia solution in such a manner that reference solution were filled in hole numbered 1 and 2 respectively and gum acacia in hole number 3. Plant extract were filled in three holes $1^{/}$, $2^{/}$, $3^{/}$ having the strength 5mg/ml, 50mg/ml, 100mg/ml respectively. The Petri-dishes were incubated at 35—38° C for 24hrs. Then zones of inhibition were observed and recorded. Chloroform and ethanol extracts demonstrated antibacterial activity but the range of antibacterial activity of chloroform extract was more as compared to ethanol extract.

Isolation of Active Constituents

The chloroform extract of seeds of *Achyranthes aspera* was further exploited in an attempt to isolate the active principle which exhibited the antibacterial activity. In the isolation procedure, different fractions were obtained by using vacuum liquid chromatography apparatus [20-23]. A sintered glass Buckner funnel attached to a vacuum line was packed with TLC grade silica gel. The silica gel was compressed under vacuum in order to achieve a uniform layer in order to get a better separation.

The greenish colored viscous chloroform extract was dissolved in a suitable volatile solvent (chloroform) and added to the same amount (200 mg) of silica gel in order to make a smooth paste. The solvent was evaporated to leave the dried extract adsorbed to the silica gel. The dried extract was then pulverized to get a uniform powder. This powder was transferred to the column again under vacuum to ensure a uniform layer.

Hexane and ethyl acetate were used as mobile phase in different ratios of increasing polarity from hexane to ethyl acetate. Each fraction was collected in a separate screw capped test tubes. The fractions were monitored by thin layer chromatography. The most active fractions having the similar thin layer chromatography profile were pooled together. The combined fractions were concentrated on vacuum rotary evaporator. The concentrated fractions were tested for their antibacterial effectiveness against three species of bacteria (*Pseudomonas aeuroginosa, Escherichia coli, and Bacillus subtilis*) by using disc diffusion assay in which 100 µl of each fraction was applied. Streptomycin and ampicillin were used as standard antibiotics in these studies. The assay procedure was repeated for the active fractions in six separate agar plates.

Conclusion

This study was undertaken to evaluate the antibacterial properties of the different extracts of seeds of *Achyranthes aspera*. Chloroform extract was subjected to VLC and further TLC for the isolation of active constituent . The active fractions were tested for their antibacterial effectiveness against three species of bacteria (*Bacillus subtilis, Pseudomonas aeuroginosa, Escherichia coli*). The *in vitro* antibacterial studies established the susceptibility of *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeuroginosa* to one of the isolated fraction. The activity of VLC isolated fraction appeared to be low in comparison with the reference antibiotics. But individual component may have greater activity. Saponins, hydrocarbons, amino acids are known to be the constituent of seeds of this plant. Therefore to explore the exact chemical nature of the compound, having antibacterial activities, further studies should be carried out so that activity could be related to a particular component. Further research on these molecules will provide the further evidence of therapeutic potential of this plant and perhaps the future development of antimicrobial product.

References

1. S. H. Khalid, M. I. Qadir, A. Massud, M. Ali, M and H. Rasool, *Journal of Drug Delivery Science and Technology,* **9**, 413 (2009).
2. A. Hussain, S. H. Khalid, M. I. Qadir, A. Massud, M. Ali, I. U. Khan, M. Saleem, M. S. Iqbal, S. Asghar and H. Gul, *Journal of Drug Delivery Science and Technology,* **21**, 249 (2011).
3. S. Naz, M. I. Qadir, M. Ali, K. H. Janbaz, *Journal of Chemical Society of Pakistan*, **34**, 107 (2012).
4. O. Ehsan, M. I. Qadir, S. A. Malik, W. S. Abbasi and B. Ahmad, *Journal of Chemical Society of Pakistan*, (Ahead of Print) (2012).
5. M. I. Qadir, *Pakistan Journal of Pharmaceutical Sciences*, **24**, 593 (2011).
6. M. I. Qadir and S. A. Malik, *AIDS Research and Human Retroviruses,* **27**, 57 (2011).
7. M. I. Qadir and S. A. Malik, *Reviews in Medical Virology,* **20**, 23 (2010).
8. M. I. Masood, M. I. Qadir, J. H. Shirazi and I. U. Khan, *Critical Reviews in Microbiology*, **37**, 91 (2011).
9. F. Javed, M. I. Qadir MI, K. H. Janbaz and M. Ali, *Critical Reviews in Microbiology*, **37**, 245 (2011).
10. D. H. Hahm, M. J. Yeom, H. J. Lee and E. H. Shim, *Journal of Microbiology and Biotechnology,* **11**, 1061 (2001).
11. A. O. Adebayo, C. O. Adewumi and E. E. Essein, *International symposium on Medicinal Plants University of Ife, Nigeria,* pp. 152 (1983).
12. N. R. Fransworth, *Ciba foundation Symposium,* **185**, pp. 42 (1994).
13. K. R. Kirtikar and B. D. Basu, *Indian Medicinal Plants,* 2nd edition, Dehradum (2006).
14. S. K. Aggarwal, R. Singh, M. Haneef, S. S. Singh and S Kimar, *Journal of Medicinal Aromatic Plant Sciences,* **24**, 1024 (2003).
15. M. T. J. Khan, K. Ahmed, M. N. Alvi, Noor-ul-Amin, B. Mansoor, M. A. Saeed, F. Z. Khan and M. Jamshaid, *Pakistan Journal of Zoo*logy, **42**, 93 (2010).
16. G. S. Rawat and G. P. Singh, *Asian Journal of Chemistry,* **14**, 1059 (2002).
17. A. Banerji and M. S. Chadha, *Phytochemistry,* **9**, 1671 (1970).
18. M. Ali, *Oriental Journal of Chemistry*, **9**, 84 (1994).
19. L. Noora and N. H. Khan, *Dhaka University Studies, Part B,* **30**, 99 (1983).
20. S. W. Pelletier, H. P. Choski and H. K. Desai, *Journal of Natural Products,* **49**, 892 (1986).
21. R. J. Singh, *Journal of the Chemical Society of Pakistan*, **33**, 485 (2011).
22. A. Sharif, E. Ahmed, M. A. Munawar, S. Jabeen, M. U. A. Khan, R. Begum, A. Farrukh, M. Ashraf, S. Arshad and N. Afza, *Journal of the Chemical Society of Pakistan*, **33**, 578 (2011).
23. M. K. Khosa, S. A. S. Chatha, M. Nisar, K. M. Zia, Khalil-Ur-Rehman, M. A. Jamal and M. Yousaf, *Journal of the Chemical Society of Pakistan*, **33**, 421 (2011).