ANTIMICROBIAL AND HEMOLYTIC ACTIVITY OF DIFFERENT EXTRACTS OF ARNEBIA BENTHAMII

[1]M. A. RATHER, [2]D. P. PANDEY, [3]R. P. SINGH, [4]Y. SINGH

[1,2,3,4]Department of Chemistry Govt. P. G. College, Uttarkashi-249 193, Uttarakhand India.
E-mail: [1]aslam.aslam2006@rediffmail.com

Abstract— Arnebia benthamii a herbaceous perennial herb belonging to the family Boraginaceae is a high-value medicinal plant that occurs in the alpine and subalpine Himalaya. The flowering shoots are used for making various products such as syrup and jam. Gule Kahzaban" a very costly medicine is isolated from it. It is principally used in several cases of chronic constipation, fever, cough, cold and wound healing. Arnebia benthamii plant is used in various diseases e.g. cardiac disorder, fungal infections, jaundice and urinary problems. The phytochemical screening of the` Arnebia benthamii plant extracts showed the presence of alkaloids, phenols, anthraquinones and flavonoids in aerial part and root part extracts of the plant and the root part extract showed the presence of terpenoids. The Chloroform extract of leaves showed maximum antibacterial action against *M. luteus* , *B. licheniformis* & antifungal activity against *A. flavus* and *A. niger*. The chloroform extract of leaves possess minimum hemolytic activity.

Keywords— *Arnebia Benthamii*, Antibacterial, Antifungal, Hemolytic Activity.

I. INTRODUCTION

A. benthamii (Synonym-*Macrotomia benthamii*) is a high-value medicinal plant that occurs in the alpine and subalpine Himalaya distributed in Jammu & Kashmir, Himachal Pradesh and Uttarakhand [1-3]. It is used as important herbal drugs in indigenous systems of medicine [4]. The root, which forms the actual drug, is considered to be anthelminic, antipyretic, antiseptic and claimed to be useful in the treatment of diseases of the eye, bronchitis, abdominal pain, itch and many more [5].

A. benthamii is a traditional medicinal herb of Ayurvedic and Unani systems of medicine [3, 4]. It is a major ingredient of the commercial drug available under the name "Gaozaban" which has antibacterial, antifungal, anti-inflammatory and wound healing properties [1, 6-8]. *A. benthamii* is used for treatment of diseases of the tongue, throat, cardiac and febrifuge [9, 10]. "Gule Kahzaban" a very costly medicine is isolated from it [3]. Various compounds such as flavonoids, quinones and terpenoids have been isolated from genus Arnebia which possess various biological activities.

II. MATERIAL AND METHODS

The plant material of *A. benthamii* was collected from Daggan Dhar (3748 m asl) Bhalessa, District Doda, Jammu & Kashmir, in July, 2010, The plant species were identified by Dr. Sumer Chand, Systematic Botany Division, FRI, Dehradun. The voucher specimen (Hr. no. 60) was deposited in the herbarium of Department of Botany, Govt. PG. College Uttarkashi, Uttarakhand.

III. PREPARATION OF EXTRACTS

The air-dried and powdered leaves of *Arnebia benthamii* (2.5 kg) were extracted with light petroleum ether ($60\text{-}80^0$). The petroleum free mass was extracted with 60% ethanol. The ethanol extract was concentrated under reduced pressure and partitioned with $CHCl_3$:H_2O (6:4) in a separatory funnel. The $CHCl_3$ layer was separated and concentrated under reduced pressure to give $CHCl_3$ extract (14.5g). The aqueous layer was concentrated under reduced pressure and the viscous liquid obtained was macerated successively with EtOAc and Acetone and the residue was dissolved with MeOH and filtered. Each layer was concentrated under reduced pressure yielded EtOAc extract (13.8g), acetone extract (12.5g) and methanol extract (11.6g). Few grams of each extract was subjected to series of purification as per pharmacological profiles.

The purified extracts i.e., Arnebia root Chloroform (ARCH), Arnebia root Ethyl Acetate (AREA), Arnebia root Acetone (ARAT), *Arnebia* root methanol (ARAL), *Arnebia* flower Chloroform (AFCH), *Arnebia* flower Ethyl Acetate (AFEA), *Arnebia* flower Acetone (AFAT), *Arnebia* flower methanol (AFAL), *Arnebia* leaf Chloroform (ALCH), *Arnebia* leaf Ethyl Acetate (ALEA), *Arnebia* leaf Acetone (ALAT) and *Arnebia* leaf methanol (ALAL), were subjected to biological studies and were tested for anti-bacterial, antifungal, and Hemolytic activity and those which were active were subjected to column chromatography to isolate bioactive compounds.

ANTIMICROBIAL ACTIVITY OF *A. BENTHAMII*

Source of Microorganisms

The organisms were obtained from IMTECH, Chandigarh. The organisms were stored on agar slant in McCartney bottles and kept in the refrigerator prior to subculture.

Evaluation of the Antibacterial Potential of Plant Extracts

Two gram negative and five gram positive bacteria viz. *E. coli*, *Salmonella typhi*, *Bacillus cereus*,

Micrococcous luteus, Bacillus pumilus, Bacillus licheniformis, and Streptococcus mutans were taken to evaluate antibacterial potential of the different extracts.

All the crude extracts were screened for the antimicrobial activity with the concentration of 100mg/ml. All the extracts were made to dissolve in 30% DMSO.

Preparation of Inoculums

Bacterial strains were preserved in nutrient agar at 4°C revived in nutrient broth (liquid medium) and incubated at 37±1°C overnight, and the suspension were checked to resembled with Mac Farland Constant.

The zone of inhibition decreases with increase in size of inoculums. Hence it is necessary to standardize the size of inoculum. A Mac Farland standard is compared with the turbidity of the inoculums. The standards approximately correspond to 1×10^6 organism /ml.

Assay Method

The antibacterial assay was performed by the well diffusion method using. The well diffusion assay was used to screen for antibacterial activity. A known amount of each plant extract was placed in separate small well. The wells were punctured on agar using sterile well puncher syringe, containing a confluent lawn of bacteria. The absence of bacterial growth around the well containing the extracts, indicate the plant extract containing antimicrobial activity against that particular bacterium [11, 12].

The Muller Hinton Agar media for antibacterial assay prepared with the aforesaid composition by dissolving accurately weight amount distilled water and adjusting the P^H by using 1N HCl and 1N NaOH solution. The media was then autoclaved at 121 ºC, 15 lbs for 15 minutes and poured quickly into sterile Petri dishes while hot to give a depth of 3-4 mm, under aseptic condition and allowed to cool and settle down. The bacterial culture (10µl) was introduced to the solid surface of agar media with the help of micropipette. Then spread across the surface of solid agar media by means of a sterile spreader and kept at room temperature for 15 min for absorption to take place. 100µl of extracts were poured in each well using micropipette. The Petri dish then incubated in BOD incubator for 24 hrs at temperature 37 ^{0}C. After incubation the degree of sensitivity was determined by measuring the zone of inhibition around the disc and compared with the zone of inhibition of standard antibiotic (Ampicillin). All the above procedure was carried out in an aseptic room under the laminar air flow in well aseptic condition with great care.

ANTIFUNGAL ACTIVITY OF *A. BENTHAMII*

Antifungal nactivity of the extracts was carried out against four pathogenic fungi namely *Aspergillus flavus, Aspergillus Niger, Nigrospora oryza* and *Fusarium graminearum.*

Assay Method

The antifungal assay was also performed by the well diffusion method. The known amount of each extract was placed separately in a small well. The well was punctured on agar growth medium containing a confluent lawn of fungus. The absence of fungus growth around the well containing the extracts, indicate that the plant extracts have antifungal activity against that particular fungus [11].

The assay media was autoclaved at 15 lbs for 15 minutes and was poured into sterile petri dishes while hot to give a depth of 3-4 mm, in sterile condition and allowed to cool and settle down. The fungal culture (0.1 ml) was introduced to the solid surface of Petri dishes with the help of cotton swap and was spread evenly on the surface of solid agar media by means of a swapper. The wells were punctured in the petri dishes using sterile well puncher syringe. 100µL of extracts were poured in each well using micropipette. The Petri dishes then kept in incubator at 25-27 $^{\circ}$C for 60-72 hrs.

After incubation the degree of sensitivity was determined by measuring the zone of inhibition around the disc and compared with the zone of inhibition of standard antifungal (Ketoconazole). All the above procedure was carried out in an aseptic room under the Leminar airflow in well sterile condition with great care.

HEMOLYTIC ASSAY

Hemolytic effect of different extracts on human erythrocytes was evaluated by using washed erythrocytes (RBCs). Blood sample (O positive) was obtained from a healthy volunteer. The blood was used within 24h after bleeding and washed three times in nine volumes of sterile 0.85% NaCl saline solution [13]. After each washing, cells were centrifuged 800rpm for 5 min and the supernatant was discarded. The final pellet was diluted 1:9 (v/v) in sterile 0.85% NaCl saline solution and then in 1:24 (v/v) sterile Dulbecco's phosphate buffer saline (D-PBS), pH 7.0 containing 0.5 mM boric acid and 1 mM $CaCl_2$. Red cell suspensions (1 ml of final volume) were incubated with an aqueous solution of PTX standard, from 10^{-3} to 10^3ng/ml for 6 hours at 37 ºC [14].

After the incubation, the cell suspensions were centrifuged at 3000 rpm for 10 min and the supernatant was carefully collected. The hemolytic activity of the crude extract was tested under in vitro conditions in 96-well plates. The first well served as negative control containing only 100µl of 0.85% NaCl. 2nd well served as positive control containing 20 ml of 0.1% Triton X-100 in 0.85% saline. Remaining each well was added 100µg/ml, 250µg/ml and 500µg/ml of plant extract. Each well then received 100µl of a 2% suspension of human

erythrocytes in 0.85% saline containing 10 mM-$CaCl_2$. After 30-min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. The results are presented in table 3 and fig 3.1 as mean ± SEM (n=3).

IV. RESULT AND DISCUSSION

The results of antibacterial and antifungal activity of various extract against selected microorganisms are shown in Table 1 and 2, respectively. The results of antibacterial were comparable to 100µg/ml of Ampicillin against *E. coli, S. typhi, B. cereus, M. luteus, B. pumilus, B. licheniformis,* and *S. mutans*. The ALCH (**23±1.15**) showed maximum inhibitory action against *M. luteus.* Significant antibacterial activity at conc. of 100µg/ml against all the bacteria was shown by chloroform extract of leaves (ALCH). The ALEA (**18±1.53**) showed maximum inhibitory action against *B. licheniformis.* Least inhibitory action was shown mostly by all the extracts of roots.

The results of antifungal were comparable to 100µg/ml of Ketoconazole against *Aspergillus flavus, Aspergills niger, Fusarium graminearum* and *Nigrospora oryzae*. The ALCH showed maximum inhibitory action against *A. flavus* (**16±1.52**) and *A. niger* (**18±1.15**), AFAL showed maximum inhibitory action against *N. oryzae* (**17±2.08**) while ALEA showed maximum inhibitory action against *F. graminearum* (**17±1.15**). Least activity was observed from root extract. These observation leads to the conclusion that fractionation of ethanol extracts of leaves resulted in two most active fraction i.e. chloroform fraction (ALCH) and ethyl acetate fraction (ALEA).

Hemolytic activity on human erythrocytes of various extracts of roots, leaves and flowers of *Arnebia benthamii* was determined and the results are presented (Table 3. fig. 3.1). The total hemolysis was obtained using 20 ml of Triton X-100 (0.1%) and 1 h incubation. Hemolytic activity at three concentrations viz. 100 µg/ml, 250 µg/ml and 500 µg/ml were analyzed and it was found that at 100 µg/ml AFEA has maximum (**41.27 ± 0.35**) hemolytic activity while ALAL has least (**21.37 ± 0.29**). At 250 µg/ml and 500 µg/ml maximum activity was found in AFAL **(81.53 ± 0.34, 91.67 ± 0.28),** and minimum activity in ARCH **(35.74 ± 0.29, 48.96 ± 0.11)** respectively.

Hemolytic assays were performed because compounds possessing potent antioxidant and anticancer activity may not be useful in pharmacological preparations if they possess hemolytic effect.

Table 3 Hemolytic activity of different extracts obtained from various parts of *Arnebia benthamii*

CONC	100 µg/ml	250 µg/ml	500 µg/ml
ARCH	22.46 ± 0.28	**35.74 ± 0.29**	**48.96 ± 0.11**
AREA	32.00 ± 0.20	39.41 ± 0.38	54.81 ± 0.19
ARAT	40.75 ± 0.15	60.42 ± 0.31	79.98 ± 0.19
ARAL	31.68 ± 0.17	65.92 ± 0.24	91.54 ± 0.39
AFCH	31.79 ± 0.14	56.75 ± 0.19	72.82 ± 0.28
AFEA	**41.27 ± 0.35**	59.79 ± 0.61	61.12 ± 0.55
AFAT	22.44 ± 0.32	47.27 ± 0.27	53.69 ± 0.26
AFAL	21.92 ± 0.09	**81.53 ± 0.34**	**91.67 ± 0.28**
ALCH	25.45 ± 0.34	50.52 ± 0.51	50.78 ± 0.21
ALEA	30.51 ± 0.75	65.78 ± 0.21	87.64 ± 0.33
ALAT	32.56 ± 0.23	55.71 ± 0.35	82.72 ± 0.64
ALAL	**21.37 ± 0.29**	69.27 ± 0.27	76.48 ± 0.27

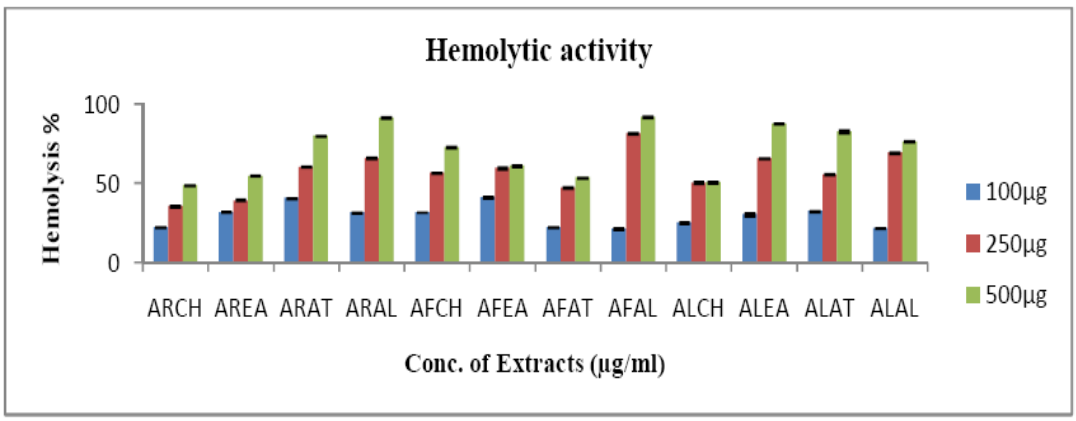

Fig. 3.1 Hemolytic activity of *A. benthamii* extracts.

Table 1: Effect of *A. benthamii* extract on zone of inhibition against selected bacterial species tested by well diffusion assay

Bacteria/ Extract	ARCH	AREA	ARAT	ARAL	AFCH	AFEA	AFAT	AFAL	ALCH	ALEA	ALAT	ALAL	STD
E. coli	6±0.58	8±0.58	10±1.15	4±0.58	11±1.53	11±1.53	12±0.58	10±0.58	21±1.53	17±1.15	11±1.15	6±0.58	18.0
S. typhi	8±1.52	8±1.73	8±1.00	7±0.58	8±1.53	10±1.53	9±1.53	10±1.15	16±2.08	12±1.53	12±1.53	8±1.53	15.0
B. cereus	5±1.15	7±1.15	10±1.15	7±1.73	11±1.0	7±1.53	9±0.58	9±0.58	17±2.08	16±1.53	11±0.58	8±0.58	17.0
M. luteus	4±0.57	8±0.58	7±0.57	9±1.15	10±1.53	14±0.58	10±1.15	10±1.15	12±1.15	13±1.15	8±0.58	9±1.15	18.0
B. pumilus	7±0.57	6±1.53	8±1.0	8±1.15	10±0.58	11±1.15	8±0.58	7±2.08	13±2.08	17±2.08	10±1.15	9±1.53	16.0
B. licheniformis	7±1.73	5±1.52	8±1.15	7±1.53	11±1.73	9±0.58	6±1.53	4±0.58	13±1.53	18±1.53	12±2.08	6±1.53	12.0
S. mutans	9±1.15	9±1.15	9±1.15	10±0.58	10±0.58	10±0.58	10±1.15	9±1.15	10±1.73	12±1.53	11±1.53	6±1.15	12.0

Values are mean inhibition zone (mm) ± S.D of three replicates

Table 2: Effect of *A. benthamii* extract on zone of inhibition against selected fungal species tested by well diffusion assay

Fungus/ Extract	ARCH	AREA	ARAT	ARAL	AFCH	AFEA	AFAT	AFAL	ALCH	ALEA	ALAT	ALAL	STD.
A. niger	6±1.15	8±0.58	9±0.58	4±0.58	9±0.58	10±2.08	10±1.15	10±0.58	18±1.15	17±1.15	11±1.15	7±0.58	14.33
A. flavus	7±1.0	8±1.53	8±1.0	6±1.52	8±1.53	14±1.53	9±1.53	10±1.15	16±1.52	12±1.73	12±1.53	8±1.0	13.67
N. oryza	6±0.57	7±1.15	8±1.15	7±1.73	8±1.15	7±0.58	9±0.58	17±2.08	9±0.58	15±2.08	11±1.53	9±0.58	12.00
F. graminearum	4±0.57	7±0.58	7±0.58	8±0.58	9±1.53	10±1.53	8±0.58	7±2.08	12±1.53	17±1.15	8±0.58	9±1.15	15.33

Values are mean inhibition zone (mm) ± S.D of three replicates.

CONCLUSION

These observation leads to the conclusion that chloroform fraction (ALCH) is the most active fraction against microbes followed by ethyl acetate fraction (ALEA), ALAT and ALAL. The chloroform extract of leaves of *A. benthamii* had minimum hemolytic activity and high antimicrobial activity equivalent to standards. Therefore, it would certainly help to ascertain the potency of the chloroform extract of leaves of *A. benthamii* as a potential source of natural antimicrobial.

ACKNOWLEDGEMENT

The authors are thankful to the Principal and Head department of Chemistry Govt. P.G. College, Uttarkashi, Uttarakhand, India for providing necessary facilities for the present investigation.

REFRENCES

[1] N.S. Chauhan, (**1999**), *Medicinal and Aromatic Plants of Himachal Pradesh*. Gidwani, Indus publishing Co. New Delhi, pp. 96-98.

[2] T. Pullaiah, (**2006**), *Encyclopaedia of World Medicinal Plants, Volume 2*, Regency publication, New Delhi, pp. 211.

[3] M.K Kaul, (**1997**), *Medicinal Plants of Kashmir and Ladakh: Temperate and Cold Arid Himalaya*. Gidwani, Indus publishing Co. New Delhi, pp. 100-101.

[4] A. Srivastava, Y.H. Shukla, S. Kumar, *J. Med. Arom. Plant Sci.* **21**, 1131-1138 (**1999**).

[5] S. Khatoon, S. Mehrotra, U. Shome, B.N. Mehrotra, *Int. J. Pharmacog.* **31**, 269-277 (**1993**).

[6] S. Manjkhola, U. Dhar, *Current. Science.* **83**, 484-488 (**2002**).

[7] K.R. Kirtikar, B.D. Basu, *Indian Medicinal Plants*. Bishen Singh Mahendra Pal Singh, Dehradun, 3, 697 (**1984**).

[8] R.S. Sharma, V. Mishra, R. Singh, N. Seth, C.R. Babu, *Fitoterapia*. **79**(**7-8**), 589-591 (**2008**).

[9] K.A. Ganaie, S. Aslam, I.A. Nawchoo, *Journal of American Science*. **6**(**10**), 1133-1141 (**2010**).

[10] T. Pullaiah, (**2002**), *Medicinal Plants in Andhra Pradesh, India* vol. I, Regency publication, New Delhi, pp. 70-72.

[11] M.P. Panthi, R.P. Chaudhry, (**2006**), *Antibacterial activity of some selected folklore medicinal plants* from *west Nepal*. Scientific world. pp. 16-21.

[12] B. Bonev, J. Hooper, J. Parisot, *Journal of Antimicrobial Chemotherapy*. **61**, 1295-1301 (**2008**).

[13] S. Gleibs, D. Mebs, B. Werding, *Toxicon*. **33**, 1531-1537 (**1995**).

[14] D. Malagoli, *Invertebrate Survival Journal.* **4**, 92-94 (**2007**).

★★★