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Original Article

In Vitro Antibacterial Activity of a Compound Isolated from *Astilbe rivularis* Buch.–Ham. Ex D.Don Leaves

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ABSTRACT

Astilbe rivularis Buch. – Ham. Ex D. Don (*A. rivularis)*, one of the medicinal plants of North East Himalayan region of India, has several pharmacological activities including anti bacterial property. In the present study we have isolated a compound from *A. rivularis* leaves. In vitro anti bacterial property of the isolated compound was studied against four Gram - positive bacteriaviz. *Staphylococcus aureus, Bacillus megaterium, Bacillus subtilis*and *Streptococcus pyogenes* andfour Gram- negative bacteria like *Shigella dysenteriae, Pseudomonas aeruginosa , Escherichia coli,* and *Salmonella typhi.* Disc diffusion techniquewas used for in *vitro* antibacterial screening. Result showed that the isolated compound exerted anti bacterial activity against all bacteria used but the antibacterial activity was more in Gram - positive bacteria than in Gram - negative bacteria. Highest activity was noted against *Bacillus subtilis* and lowest activity was found for *Salmonella typhi.* The MIC (minimum inhibitory concentration) values of the compound isolated from the leaves of *A. rivularis* against the bacteria ranged from 4 – 64 microgram/mL. Results were comparable to that of standard antibiotic kanamycin suggesting thereby that the isolated compound had good in vitro anti bacterial activity against the tested bacteria, therefore, provides a scientific rationale for its use as anti bacterial agent in future.

Keywords:*A. rivularis*, Isolation of a compound, anti bacterial property, Disc diffusion technique, Zone of inhibition, Minimum inhibitory concentration

1. INTRODUCTION

Astilbe rivularis, Buch. Due to indiscriminate use of antibiotics in the treatment of infectious diseases, microorganisms developed resistance to many antibiotics[1].While *Streptococcus pneumoniae* was found resistant against penicillin, strains of *Streptococcus pyogenes* resistant to antibiotic macrolide have emerged [2].*Enterococcus faecalis* and *Enterococcus faecium* developed multidrug resistance[3]. Penicillin resistant

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Staphylococcus aureus was found in 1947.Methicillin-resistant *S. aureus* (MRSA) is now quite common in hospitals. Today, half of all *S. aureus* infections in the United States of America are resistant to penicillin, tetracycline, methicillin, vancomycin and erythromycin[4].

In the year 2004 multidrug resistant *Acinetobacter baumannii* (MRAB) with a few isolates resistant to all drugs tested were discovered [5].*Klebsiella pneumoniae* developed resistance even carbapenemtoo[6]. *Clostridium difficile* strains resistant to clindamycinas well as fluoroquinolone antibiotics, such as ciprofloxacin and levofloxacin were also reported [7,8].*Mycobacterium tuberculosis* was once sensitive to streptomycin developed resistance to streptomycin[9].Likewise *Neisseria gonorrhoeae*, once killed by penicillin developed resistance against penicillin[10].Strains of *N. gonorrhea* have been found to be resistant to third-generation antibiotic chephalosporins, fluoroquinolones, tetracyclines and aminoglycosides [11].*Pseudomonas aeruginosa*, strains of *Salmonella* and *Escherichia coli* also developed resistance against various antibiotics [12].

Antibiotic resistance is, therefore, a worldwide public health problem today and has created immense concern in treatment of infectious diseases [13]. To combat the situation continuous effort is going on for synthesis of new chemicals having antimicrobial activity [14]. Lot of chemical compounds are synthesized, their anti bacterial activity is established. Unfortunately, most of these compounds are potentially toxic and are not free from side effects on the host[15]. This has extended the research even in the field of medicinal plants for isolation of natural compounds which will be less toxic and a proper substitute of chemical antimicrobial agents [16].

Astilbe rivularis, Buch. – Ham. Ex D. Don (*A.rivularis*), family – Saxifragaceae, a tall herb stem and leaves are covered with hairs, is one of the medicinal plants of North Eastern Himalaya[17]. The plant, distributed at a range of 5000 – 9000 feet in Common Temperate of Himalaya and found on sloppy waste place has different names. In Lepcha it is called Pango and in Nepali the plant is known as Buriokahti[18]. Traditional use of the plant is in curing dysentery, headache, hemorrhages, diarrhoea, prolapse of uterus and to improve fertility [19]. The plant has anti microbial[20], anti viral[21], anti peptic ulcer[22] andanti oxidant activity[23].During phytochemical investigation comppounds like flavonoids, terpenoids and bergenin. 11-*O*-galloylbergenin, catechin, afzelechin, epiafzelechin and 2-(*β*-D-glucopyranosyloxy)-4-hydroxylbenzenacetonitrile were isolated from the plant [24].Methanol extract of *A. rivularis* of Nepal has highest anti bacterial activity against *Kliebsiellapneumonie*[25].Recently we confirmed in vitro anti bacterial activity of methanol extract of *A. rivularis* grown in North East Himalayan region of India and noted maximum anti bacterial activity during the months of July and August (paper is under communication). Aim of the present work was to isolate the anti bacterial compound present in *A. rivularis* leaves and to study its anti bacterial activity against number of Gram positive and Gram negative bacteria.

2. METHODOLOGY

2.1 Collection of plant materials

A. rivularis leaves were collected from the medicinal plants garden of the University of North Bengal, Siliguri ($26^0 41' 30.9984''$ N, $88^0 27' 4.5756''$ E, elevation, 410 ft), Dist. Darjeeling, West Bengal. Leaves were collected in between 9 and 10 AM during the months of July and August as we have noted earlier that this is the time when the plant has maximum anti bacterial activity (paper is under communication). Leaves were authenticated by the experts of the department of Botany of the said University. A voucher specimen was kept in the department of department of Biochemistry, North Bengal Medical College, Siliguri, West Bengal, India for future references. Leaves were thoroughly washed, shade dried and powdered. The powder was used for isolation work.

Fig 1:*Astilbe rivularis*Buch. – Ham. Ex D. Don

2.2 Isolation work

This was done by the following scheme. Principles ofstandard isolation procedures of chemical compounds from plant sources were followed[26-29]

2.3 Chemicals

Chemicals required for the study were purchased from Loba Chem. Lab, Himedia Lab, India and from Merck, Germany

2.4 Bacteria

Four Gram - positive bacteria *viz.Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Streptococcus pyogenes* and four Gram-negative bacteria *viz.Pseudomonas aeruginosa*,*Shigelladysenteriae*, *Escherichia coli*and *Salmonella typhi* were employed to determine antibacterial activity and minimum inhibitory concentration. All these bacteria were collected from the department of Microbiology, North Bengal Medical College Hospital, Siliguri, West Bengal.

2.5 Media

Nutrient agar media (Difco laboratories) pH 7.2 and nutrient broth media (Difco laboratories) pH 6.8 were used for antibacterial screening and minimum inhibitory concentration determination.

2.6 Antibacterial screening

In vitro antibacterial screening was carried out by disc diffusion method[30]. According to this method, 20 ml quantities of nutrient agar were placed in a petri dish with 0.1 ml of 10^{-2} dilution of bacterial culture of 20 hours old. Filter paper discs (6 mm diameter) impregnated with 40 µg per disc concentration of the compound isolated from *A. rivularis* leaves were placed on test bacteria-seeded plates. Blank disc impregnated with water was used as negative control. Zone of inhibition was recorded after 18 hours of incubation. Diameters of zone of inhibition produced by the solution prepared from *A. rivularis* were compared with that of standard antibiotic kanamycin 40 µg per disc, Each sample was used for five times for the determination of anti bacterial activity.

2.7 Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration is defined as the lowest concentration of antibiotic completely inhibiting visible growth of bacteria after 18 – 24 hours of incubation at 37 ^{0}C. This was done by the method of Mosaddik and Haque[31]. According to this method, compound isolated from *A. rivularis* (1.0 mg) was dissolved in 2 ml nutrient broth media to obtain a stock solution of concentration 500 µg/ml. 3 drops of Tween 80 was added in nutrient broth to facilitate dissolution. Serial dilution technique was followed to obtain 250 µg/ml concentration of the compound. One drop (0.02ml) of prepared suspensions of organism (10^6organism/ml) was added to each broth dilution. These dilutions were then incubated for 20 hours at 37^{0}C. Growth of bacteria was examined by noting turbidity of the solution. The nutrient broth media with 3 drops of Tween 80 was used as negative control while kanamycin was used as positive control.

2.8 Statistical analysis

The values were expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) 20th versions. Differences between means were tested employing Duncan's multiple comparison test and significance was set at $p < 0.05$.

2.9Diagrammatic scheme for isolation of a compound from leaves of *A. rivularis*.

Powdered leaves of*A. rivularis*(50 g)

↓

SOLVENT EXTRACTION

Extracted with 500 ml of methanol in soxhlet at 37^{0}C for 15 minutes. It was then centrifuged. Supernatant collected and evaporated to dryness.

Active brown mass

↓

ACID REFLUX

Refluxed with 50 ml of 1(N) HCl for 10 min on a water bath at 100 ^{0}C. It was then cooled and centrifuged. Supernatant was evaporated to dryness.

Active brown mass

↓

TREATMENT WITH ETHYL ACETATE

Extracted with 50 ml ethyl acetate on a rotary shaker for 15 min. It was then centrifuged. Supernatant was evaporated to dryness.

Active brown mass

↓

ALUMINA COLUMN CHROMATOGRAPHY

Extracted with 10 ml methanol for 5 min. It was then filtered. With filtrate alumina column chromatography was performed. Elution was done by methanol – ethyl acetate mixture (1:1 v/v).

Fourth band was found active

↓

SILICA GEL G CHROMATOGRAPHY

Eluent of active fourth band was evaporated to dryness. The dry masswas extracted with 15 ml methanol for 5 min. It was then filtered. Withfiltrate silica gel G column chromatography was done. Elution was done by chloroform : methanol mixture (1:1 v/v).

Second band was active

↓

POLYAMIDE COLUMN CHROMATOGRAPHY

Eluent of active secondband was evaporated to dryness. The dry mass wasextracted with 10 ml methanol for 5 min. It was thenfiltered and the filtrate was subjected tocolumn chromatography using polyamide as adsorbent. Elution was done by methanol, acetonemixture (1:1 v/v).

First band was found active

↓

CRYSTALLIZATION

Eluent of the active first band obtained from the above step was evaporated to dryness. Repeated crystallization was done from chloroform

ethyl acetate (40:60, v/v) mixture.

Crystals obtained (5.8 mg)

3. RESULTS

3.1 Isolation of compound

A compound was isolated from the leaves of *A. rivularis*.

3.2 Anti bacterial activity of the isolated compound

Results of disc diffusion method (Table – 1) indicatedthat the isolated compound from *A. rivularis*. leaves at 40 µg per disc concentrations exerted anti bacterial activity against all four Gram positive bacteria viz. *Bacillus subtilis, Bacillus megaterium,Staphylococcus aureus* and *Streptococcus pyogenes* and four Gram-negative bacteria *viz*. *Pseudomonas aeruginosa,Shigelladysenteriae, Escherichia coli* and *Salmonella typhi*. Activity was comparable to that of reference drug kanamycin in 40µg per disc concentration. Antibacterial activity of the isolated compound was more in Gram - positive bacteria than in Gram - negative bacteria. Highest activity was noted against *Bacillus subtilis* for Gram positive bacteria and *Escherichia coli* forGram-negative bacteria. Lowest anti bacterial activity of the isolated compound , however, was noted against *Streptococcus Pyogenes*and*Salmonella typhi*for Gram positive and Gram-negative bacteriarespectively.

Table – 2 shows results of minimum inhibitory concentration of the isolated compound from *A. rivularis* leaves and kanamycin against the said Gram positive and Gram negative bacteria . The MIC (Minimum Inhibitory Concentration) of the isolated compound ranged from 4 to 16 for Gram positive bacteria and 8 to 64 microgram/mL for Gram negative bacteria respectively. For kanamycin MIC value for Gram positive bacteria was found to vary between 2 and 8 and for Gram negative bacteria it came between 4 and 16.

Table 1: *In vitro* antibacterial activity of the isolated compound from *A. rivularis* leaves and kanamycin [Zone of inhibition (diameter in mm)] against Gram positive and Gram negative bacteria.

Bacteria	Strain	Compound isolated from*A. rivularis*leaves (40 µg per disc)	Kanamycin (40 µg per disc)
Gram – positive			
Bacillus subtilis	ATCC 19659	34 ± 0.3*	38 ± 0.5
Bacillus megaterium	NBMC 1122	30 ± 0.2	34 ± 0.6
Staphylococcus aureus	ATCC 25923	28± 0.3	30 ± 0.4
Streptococcus pyogenes	NBMC 1321	24 ± 0.2	28 ± 0.3
Gram – negative			
Escherichia coli	ATCC 25922	25 ± 0.3*	28 ± 0.7
Shigelladysenteriae	NBMC 1127	21 ± 0.2	24 ± 0.6
Pseudomonas aeruginosa	NBMC 1243	20 ± 0.5	23 ± 0.6
Salmonella typhi	MTCC 733	19 ± 0.4	22 ± 1.0

Data was in mean SEM (n = 5). Control was made with water. It had no zone of inhibition. So data has not been shown.* Significantp<0.01

4. DISCUSSION

Anti bacterial compounds have been isolated from medicinal plants.

Table 2 : Minimum inhibitory concentration of the isolated compound from*A. Rivularis* leaves and kanamycin against Gram positive and Gram negative bacteria

Bacteria	MIC values of the compound isolated from*A. rivularis* leaves (microgram/mL)	MIC values of kanamycin (microgram/mL)
Gram – positive		
Bacillus subtilis	4	2
Bacillus megaterium	8	4
Staphylococcus aureus	8	4
Streptococcus pyogenes	16	8
Gram – negative		
Escherichia coli	8	4
Shigelladysenteriae	16	8
Pseudomonas aeruginosa	16	8
Salmonella typhi	64	16

Negative control containing water had no MIC value. Thus, it has not been shown

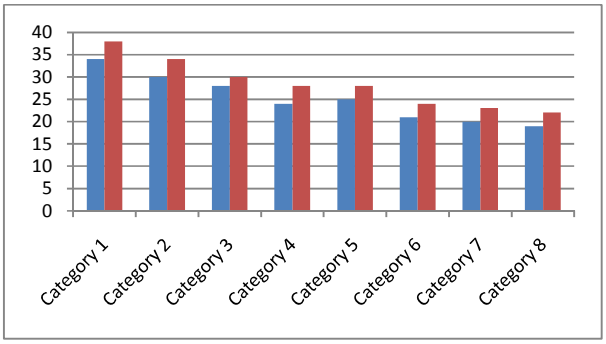

Fig 1: *In vitro* antibacterial activity of the isolated compound from *A. rivularis* leaves and kanamycin [Zone of inhibition (diameter in mm)] against Gram positive and Gram negative bacteria.

Category 1: *Bacillus subtilis*, Category 2: *Bacillus megaterium*, Category 3: *Staphylococcus aureus*, Category 4: *Streptococcus pyogenes* Category 5: *Escherichia coli* Category 6: *Shigella dysenteriae* Category 7: *Pseudomonas aeruginosa* Category 8: *Salmonella typhi*

■Isolated compound ■ Kanamycin

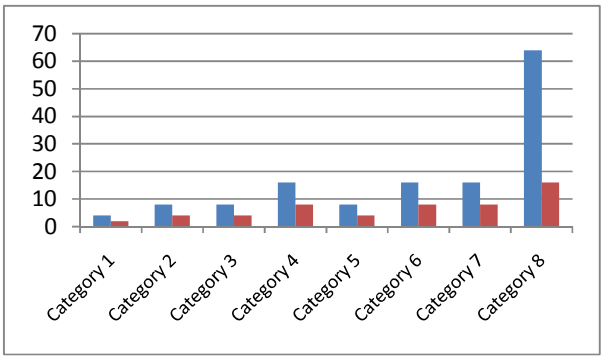

Fig 2: Minimum inhibitory concentration of the isolated compound from *A. Rivularis* leaves and kanamycin against Gram positive and Gram negative bacteria

Category 1: *Bacillus subtilis* Category 2: *Bacillus megaterium* Category 3: *Staphylococcus aureus* Category 4: *Streptococcus pyogenes* Category 5: *Escherichia coli* Category 6: *Shigella dysenteriae* Category 7: *Pseudomonas aeruginosa* Category 8: *Salmonella typhi*

■Isolated compound ■Kanamycin

Hyperenone A, hypercalin B andhyperphorin were isolated from different species of the plant genus *Hypericum (Hypericum acmosepalum; Hypericumolympicum*). Isolated compoundswere foundresponsible for anti bacterial activity on resistant *Staphylococcus aureus*and on*Mycobacterium tuberculosis* [32 ,33]. Araujo et al. isolated two compounds viz. xanthone 8-carboxymethyl-1,5,6 trihydroxy3 methoxyxanthone and 6-hydroxy-7-methoxyluteolin from the leaves of *Leiothrix spiralis*, a South American plant belonging to the Eriocaulaceae family. The compounds showed anti bacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*[34].Catechins and theoflavins of tea extracts have anti bacterial effect against *Bacillus cereus*[35].2´,5-di-*O*-galloyl d-hamamelose, a natural nonpeptide compound isolated from the bark of *Hamamelis virginiana*, was found to inhibit*S. aureus* and *S. epidermidis*[36]. 3,5,4´-trihydroxystilbe isolated from grapes and other plants showed direct antibacterial activity against *Neisseria gonorrhoeae*

and*Neisseria meningitides*[37] .Kadota et al. isolated trichorabdal from *Rabdosia trichocarpa* and noted its anti bacterial effect against *Helicobacterpylori*[38]. Alkaloid berberine, isolated from *Mahonia aquifolia,*could in activate *Plasmodium* Trypansomes[39] .

In the present study by solvent extraction, acid hydrolysis, solvent treatment and chromatographic experiments we have isolated a compound from *A. rivularis* leaves. The compound showedin vitro anti bacterial activity against four Gram positive bacteria viz. *Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus* and *Streptococcus pyogenes* and four Gram-negative bacteria *viz*. *Pseudomonas aeruginosa, Shigella dysenteriae, Escherichia coli* and *Salmonella typhi*as evidenced by disc diffusion technique. Results were comparable to that of standard antibiotic kanamycin (Figure – 1). The MIC (minimum inhibitory concentration) value of the compound was also found comparable to that of standard antibiotic kanamycin (Figure – 2). Isolated compound now requires characterization. In isolation process methanol was used as extraction solvent. Compound may, therefore, be anthocyanin, saponin, tannin, terpanoid or polyphenol[40]. Work on characterization of the compound is now going on in our laboratory.

5. CONCLUSION

In this study we have isolated a compound from the leaves of *A. rivularis*. The compound showed anti bacterial activity against four Gram positive and four Gram negative bacteria. Anti bacterial activity of the compound was comparable to that of kanamycin, a standard antibiotic. The isolated compound, therefore, provides a scientific rationale for its use as anti bacterial agent in future.

6. ACKNOWLEDGEMENT

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