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Research Article

A COMPARATIVE STUDY OF EXTRACT OF SUCCULENT LEAVES OF LIVING PLANT WITH METHANOLIC AND AQUEOUS EXTRACT OF *BERLERIA LUPULINA* LINDL. AGAINST PATHOGENIC MICROBES BY DISC DIFFUSION AND SPECTROPHOTOMETRY

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ABSTRACT: *Berleria lupulina* Lindl. was evaluated for its reported antimicrobial activity in a novel way. The extract of succulent leaves collected from living plant was studied along with conventional methanolic and watery extracts made from the dry leaves of the plant. The extracts were tested on three pathogenic bacteria and the antimicrobial activity was tested both by conventional single disc diffusion method and a novel Spectrophotometric method. In disc diffusion study, it was found that the methanolic extract (100 mg/ml. and 200 mg/ ml.) diluted in 70% of methanol and extract of succulent leaves can induce 12 mm, 13 mm and 14 mm diameter zone of inhibition comparable with 24 mm of Ceftriaxone against *Escherichia coli.* The zone of inhibition against *Staphylococcus aureus* were 13 mm, 14 mm, 15 mm and 25 mm and against *Salmonella enteritides* were 12 mm, 14 mm, 15 mm and 28 mm correspondingly. The watery extract made from the dry plant and the methanolic extract diluted in water failed to induce any inhibition in growth of the organisms. In spectrophotometric study, the methanolic extract showed antimicrobial efficacy in the concentration of 10 mg/ml. or above against *Salmonella enteritides* and *Staphylococcus aureus*. But against *Escherichia coli,* effective control was found in 20 mg/ml concentration. The fresh extract of the plant showed antimicrobial efficacy in the concentration of 16.5%. The anti microbial efficacy above that concentration cannot be detected in the available spectrophotometrical method for presence of color material in that fresh extract.

Key words: *Berleria lupulina* Lindl., Extract, Living plant, Methanolic, Watery, Antimicrobial, Disc diffusion, Spectrophotometry.

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INTRODUCTION

Development of resistance among microorganisms against antimicrobial agents and spread of the plasmid based genetic materials related with such resistance to many other new species of microorganisms continuously with accelerated speed is becoming a threat for antimicrobial chemotherapy. The spread is mainly due to indiscriminate, unnecessary use and residual effect of antibacterial substances (Pattanayak 2011). Resistance in bacterial population spread from person to person by bacteria, from bacterium to bacterium by plasmids, from plasmid to plasmid or chromosome by transposons (Rang *et al.,* 2005). So, many of the present antimicrobial substances facing resistance and use of these as the means to kill or control the growth of the microorganisms fail in many cases. The continuous development of new antimicrobial agents may not solve the problem.

The plant derived antimicrobial agents may work through different pathways than commonly used antibiotics and chemotherapeutic agents and thus may be an additional or alternative way to combat the problem.

Berleria lupulina Lindl. (Family: Acanthaceae) commonly known as Vishalyakarani in Bengali and Hophead and Philippine Violet in English, is generally used to stop instant bleeding. It is also used to cure all types of wound and ulcers, both new and old (Pattanayak *et al.,* 2012).The plant is externally used as an anti-inflammatory against insect bites, snake bites, herpes simplex, herpes zoster and varicella zoster virus lesions and it also has a diuretic effect and anti-amoebic activities (Kanchanapoom *et al*., 2001; Lans *et al*., 2001; Sawangjaroen *et al*., 2006). The methanolic extract of this plant is having anti-ulcer activity (Suba *et al.,* 2004).

A study was conducted for validation of the antimicrobial efficacy of this plant, an essential quality for inducing wound healing and all other related activities.

MATERIALS AND METHODS

Contemporary methods of study: In the previous study related with validation of reported medicinal property of plants, generally the plant parts were collected, dried and preserved. Then methanolic, ethanolic, acetone, aqueous etc. extracts of the preserved plant parts were made and stored at different manner. Then these were tested for their reported medicinal use *in vitro* or among *in vivo* animal models, either in that form or in semi-purified or purified form after identification of active principles (Pattanayak *et al.,* 2013).

Validation of crude fresh extract of plant parts collected from living plants:

Generally, the ethnic and other rural people traditionally use the plants in its crude, fresh form in most of the time. It was argued that the concept of contemporary research have the limitation of loss of many aromatic and other phytochemicals present in the living plant, which may have very important role when used together (Pattanayak *et al.,* 2013).

So, the present study was designed for validation of antimicrobial efficacy of methanolic and aqueous extracts of dry plant leaves as well as the extract of succulent leaves of living plant.

A. Identification, extraction and preservation of plant materials:

The taxonomic identification of plant was performed by plant taxonomist before proceeding for the study.

The leaves of the plant, *Berleria lupulina* Lindl., were collected from Belda, Paschim Medinipur,West Bengal, India during the month of September – October, 2013. After collection, the green, succulent leaves were washed with tap water, rubbed slightly individually with cotton soaked with distilled water to remove adhered particle, if any, and then were rinsed with distilled water and air dried.

Fresh extract preparation: A part of the washed, cleaned plant leaves was taken and paste of the plant material (leaves) was made by pestle and mortar. Afterwards the material was filtered by three layers of clean, white cotton cloth, centrifuged at 1000 rpm for 10 minutes and the supernatant was stored in airtight bottles at 4^0C for two hours and then at - 20^0 C for further use.

Dry leaf preparation: Another part of the plant material were air dried in shade for seven days with changing of position twice daily and the dry leaves were stored in airtight nylon bottles at 4^0 C for further use (Dey *et al.*, 2010).

Aqueous and methanolic extract preparation: The methanolic and aqueous extracts from the dry leaves following conventional methods (Dey *et al.*, 2010).

Preparation of semisolid methanolic extract: The materials (methanolic extracts of plant parts) was attached with Vacuum evaporator, where temperature was adjusted at 37^0C to get concentrated material. After evaporation of five hours, a portion of water was still present in the material. The material was then placed in sterile petridishes inside the U.V ray sterilized Laminer flow for further evaporation of water. The remains were some pasty materials, which was collected in sterile vials and kept at – 20^0C.

B. *In vitro* antibacterial study (Bauer *et al.*, 1966).

Microorganisms used:

1. *Escherichia coli.*
2. *Salmonella enteritides.*
3. *Staphylococcus aureus*.

Source of bacteria: Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Kolkata,West Bengal, India.

Dilution of plant extracts for experiment:

i) Methanolic extract in water:

Semisolid methanolic plant leaf extract (200 mg) was mixed with 1 millilitre of distilled water to obtain 200 mg/ml concentration.

ii) Methanolic extract in methanol:

a) Semisolid methanolic plant leaf extract (100 mg) was mixed with 1 millilitre of 70 % methanol to obtain 100 mg/ml concentration.

b) Semisolid methanolic plant leaf extract (200 mg) was mixed with 1 millilitre of 70 % methanol to obtain 200 mg/ml concentration.

ii) Fresh extract: The fresh extract of the plants were used as it was without any dilution or modification.

Procedure

The bacterial broth culture was adjusted to the bacterial concentration of 10^6/ml following the procedures described by Willey *et al.* (2011). Then 100 µl of this was added in each plate and spreaded on the surface by a spreader. Then

one sterile empty disc (Diameter of the disc is 7 millilitre, HiMedia) was taken each time to dip it for 30 seconds in the fresh plant extract/ watery extract of dry plant leaves/ methanolic extract of plant diluted in water (200 mg/ml)/ methanolic extract of plant diluted in methanol (100 or 200 mg/ml). Then that was allowed to dry for a few seconds and placed individually on the agar and pressed by the forceps slightly. The control discs were also placed on the agar. The plates were incubated for 37°C for 24 hours (Ayyappa das *et al*., 2009). The process was repeated several times according to the requirement. The zone of inhibition of growth of bacteria was measured at the next day after bringing the plates from the incubator.

C. Study of antimicrobial efficacy and determination of Minimum Inhibitory Concentration by spectrophotomety:

The Minimum inhibitory concentration (MIC) was evaluated by a novel type Spectrophotometrical study. The study was designed following partially the dilution method of Willey *et al.*, 2011, Beth *et al.*, 2002, recommendation of European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID 2000), and Doss *et al.* (2012).

Doss *et al.*, 2012 compared only reduction of turbidity of the culture media than control to standardize the concentration of microorganisms in broth, but Spectrophotometric study was also performed by some others (Carmen Dominguez *et al*, 2001, Willey *et al.*, 2011). The wave lengths used were 405 nm by Beth *et al*. (2002) and 450 nm by Carmen Dominguez *et al.* (2001).

During our study, we have selected both the wavelengths to study our samples, at 405 nm and 450 nm wavelengths setting the plain bacterial broth media as control.

Procedure:

Dilution of extracts.

The sterile bacterial tubes were added with 3 milliliter of Broth media (HiMedia). Then the previously made methanolic plant extracts (200 mg/ml in 70% methanol) was added to get desired concentration in the broth media (Table1). Likewise, the fresh extract of the plant leaves was diluted (Table 2).

Three sterile test tubes were added only with 3 ml of broth media for media control. Another three tubes with only 3 ml of broth media and all other test tubes having plant extracts were added with 100 µl of previously made bacterial broth culture having bacterial concentration of 10^6/ml, made following the procedure of Willey *et al.* (2011).

Then the media controls were kept at 4^0C and the other tubes containing the materials were incubated for 24 hours at 37^0C. At the next day, the Optical Density (O.D.) of the materials were measured in UV VIS spectrophotometer of Systronics (India) model no.119 and the values were noted.

RESULTS AND DISCUSSION

The result of disc diffusion assay shows that the methanolic and the fresh extract of *Barleria lupulina* Lindl. showed antimicrobial effect on all the tested microorganisms. The methanolic extract of 200 mg/ ml concentration shows a better effect than 100 mg/ ml concentration. Mubarack *et al.* (2011) and Doss *et al.* (2012) took both 100 mg/ml and 200 mg/ml and got better result in the higher concentration in some other plants.The further higher concentrations were not taken as most of the plant extracts

remain at almost thick and semisolid state in higher concentration (300 mg/ml or more) and remain adhered with disc. The methanolic extract cannot able to show its effect when diluted in water, instead of methanol. This may be due to change of diluents, *ie*, ineffective dilution in separate media, in water in place of methanol. This is definitely a limitation of the methanolic extract.

In the study, the aqueous extract made from the dry leaves of the plant fail to show any effect on any of the tested micro organisms.

The previous study reports did not show same findings. In one report, the methanolic and aqueous extracts were not found effective against *E.coli*, but found effective against *S.aureus* (Dey *et al.,* 2014). In another report, the methanolic extract showed 12 mm zone against *E.coli* and 16 mm zone against *S.aureus* and the aqueous extract failed to show any effect (Doss *et al.,* 2011). In our study, the zone of inhibition against *E.coli* is 12 mm and 13 mm for two concentrations of methanolic extracts (Table 3).

As the people residing at the rural areas are accustomed with the use of readily available herbal preparations made from the locally available plants mainly at its raw, pure, fresh and crude form for cure of the ailments of themselves as well as of their domestic animals for same or related type of ailments, validation of the extracts closer to their use is important. In our study, the fresh extract of the plant showed better antimicrobial efficacy than methanolic extracts of two concentrations. The zones of inhibition were found higher in fresh extract treated disc all the three microorganisms (Table 3).

A novel type of study was done to measure the effect of plant extracts on growth of micro - organisms by spectrophotometry. The concept of measurement of antimicrobial efficacy was taken from the procedures described for determination of Minimum Inhibitory Concentration (MIC) by Willey *et al.*, 2011. It was expected that the O.D value of the mixture of plant leaf extract with added bacteria should

Table 1 : Dilution of Methonotic Extract.

Parameter	**Dilution procedure**			
Methanolic extract of the plant (200 mg/ml)	75 µl.	150 µl.	300 µl.	750 µl.
Broth media	3 ml.	3 ml.	3 ml.	3 ml.
Concentration	5 mg/ml	10 mg/ml	20 mg/ml	50 mg/ml

Table 2 : Dilution of Fresh Extract.

Parameter	**Quantity of materials added**			
Fresh extract	1.5 ml	1.0 ml	0.5 ml	0.25 ml
Broth media	1.5 ml	2.0 ml	2.5 ml	2.75 ml
Concentration of Fresh Extract	50%	33%	16.50%	8.25%

Table 3: Average diameter of zone of inhibition of bacterial growth (mm) against extracts of *Barleria lupulina* Lindl.

Parameters/Bacteria	*Escherichia coli*	*Salmonella enteritides*	*Staphylococcus aureus*
Watery extract	0	0	0
Methanolic extract in water (200 mg/ml)	0	0	0
Methanolic extract in 70% methanol (100 mg/ml)	12	12	13
Methanolic extract in 70% methanol (200 mg/ml)	13	14	14
Fresh plant extract	14	15	15
Ceftriaxone (control)	24	28	25
Distilled water (control)	0	0	0
Methanol (70%) (control)	0	0	0

Table 4: Spactro photometrical study on effect of extracts of *Barleria lupulina* Lindl. on the growth of *Escherichia coli*.

Plant: *Barleria lupulina* Lindl.
Bacteria: *Escherichia coli*.
Broth media used as blank.
Bacterial culture reading: At 405 nm: Pre inoculation 0.055; Post inoculation 1.058.
At 450 nm: Pre inoculation 0.048; Post inoculation 1.008.

Type of Extract	Concentration	Pre 405	Post 405	Pre 450	Post 450
Methanolic extract	5 mg/ml	1.481	≥2.00	1.374	≥2.00
	10 mg/ml*	1.689	1.762	1.503	1.587
	20 mg/ml*	1.846	1.760	1.703	1.649
	50 mg/ml	=2.00	≥2.00	≥2.00	≥2.00
Fresh Extract	100%	=2.00	≥2.00	≥2.00	≥2.00
	50%	=2.00	≥2.00	≥2.00	≥2.00
	33%	=2.00	≥2.00	≥2.00	≥2.00
	16.5%*	1.898	1.823	1.654	1.581
	8.25%	1.485	≥2.00	1.312	≥2.00

Table 5: Spactro photometrical study on effect of extracts of *Barleria lupulina* Lindl. on the growth of *Salmonella enteritides.*

Plant: *Barleria lupulina* Lindl.
Bacteria: *Salmonella enteritides*
Broth media used as blank.
Bacterial culture reading: At 405 nm: Pre inoculation 0.148; Post inoculation 1.150.
At 450 nm: Pre inoculation 0.136; Post inoculation 1.141.

Type of Extract	Concentration	Pre 405	Post 405	Pre 450	Post 450
Methanolic extract	5 mg/ml	1.571	≥2.00	1.485	≥2.00
	10 mg/ml*	1.787	1.679	1.625	1.537
	20 mg/ml*	1.903	1.827	1.786	1.712
	50 mg/ml	≥2.00	≥2.00	≥2.00	≥2.00
Fresh Extract	100%	≥2.00	≥2.00	≥2.00	≥2.00
	50%	≥2.00	≥2.00	≥2.00	≥2.00
	33%	≥2.00	≥2.00	≥2.00	≥2.00
	16.5%*	1.903	1.816	1.784	1.695
	8.25%	1.610	≥2.00	1.531	≥2.00

Table 6: Spactro photometrical study on effect of extracts of *Barleria lupulina* Lindl. on the growth of *Staphylococcus aureus.*

Plant: *Barleria lupulina* Lindl.
Bacteria: *Staphylococcus aureus*
Broth media used as blank.
Bacterial culture reading: At 405 nm: Pre inoculation 0.098; Post inoculation 1.171.
At 450 nm: Pre inoculation 0.0666; Post inoculation 1.157.

Type of Extract	Concentration	Pre 405	Post 405	Pre 450	Post 450
Methanolic extract	5 mg/ml	1.488	1.597	1.392	1.445
	10 mg/ml*	1.715	1.691	1.581	1.472
	20 mg/ml*	1.881	1.722	1.746	1.582
	50 mg/ml	≥2.00	≥2.00	≥2.00	≥2.00
Fresh Extract	100%	≥2.00	≥2.00	≥2.00	≥2.00
	50%	≥2.00	≥2.00	≥2.00	≥2.00
	33%	≥2.00	≥2.00	≥2.00	≥2.00
	16.5%*	1.872	1.767	1.695	1.559
	8.25%	1.575	≥2.00	1.432	≥2.00

increase if the bacteria could grow and multiply. The O.D value should decrease if the plant extract was able to kill or inhibit the growth of bacteria. As the present extracts were having color material, so the O.D values of extracts were always higher than the value of the broth with bacteria at post incubation.

The effective control of growth of bacteria was considered only at negative absorbance value in spectrophotometry of incubated samples. According to that idea, the methanolic extract of *Barleria lupulina* Lindl. showed anti microbial efficacy in the concentration of 10 mg/ml. or above against *Salmonella enteritides* and *Staphylococcus aureus* in spectrophotometrical study (Table 5 and Table 6). But against *Escherichia coli*, the 10 mg/ml concentration of the plant extract was able to show some level of control of growth of bacteria, but effective control was found in 20 mg/ml concentration (Table 4). The fresh extract of the plant showed antimicrobial efficacy in the concentration of 16.5% against allthe three bacteria. The antimicrobial efficacy above that concentration cannot be detected in the present in spectrophotometrical method for presence of color material in that fresh extract (Table 4, 5 and 6).

The methods used by Willey *et al.* (2011), Recommendation of European Committee for Antimicrobial Susceptibility Testing (EUCAST) etc. were developed to test antibacterial substances, mainly of synthetic origin. There was no interference of color of those synthetic antibacterial chemicals. But both the methanolic as well as fresh extracts of the plants are having some color, sometimes very deep. That caused limitation in the spectrophotometric study of the antibacterial effect of different plant extracts.

CONCLUSION

After thorough analysis of the study results, it can be concluded that the fresh extract made from the succulent leaves collected from the living *Barleria lupulina* Lindl. plant showed better antimicrobial efficacy than methanolic and aqueous extracts. The spectrophotometric evaluation of antimicrobial efficacy of fresh extract as well as methanolic extract face the limitation after a certain concentration due to the presence of color material in them.

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