Antibacterial Properties of Some Lichens in Sri Lanka

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ABSTRACT

Intensive use of antibiotics leads to antibiotic resistance; resulting strains limit the spectrum of therapeutically active antibiotics drastically. Since it is believed that lichens have got the resistant power towards bacteria, this study was carried out to investigate the antibacterial properties of selected lichen species. Secondary metabolites from four lichen species were extracted and tested against ten bacteria species. Then thin layer chromatography was performed to develop the solvent system and to identify the available lichen metabolites. Ethanol: Methanol (three: one) was found be the most effective solvent mixture to extract secondary metabolites and *Roccella montagnii* showed the highest zone of inhibition. In thin layer chromatography solvent system Toluene: Chloroform: Formaldehyde (Twelve: Seven: One) and Toluene: Dioxane: Formaldehyde (Twenty Five: Forteen : One)the highest number of spots with clear, distinct, and round spots. Atronorin was available in *Roccella montagnii*. Therefore *Roccella montagnii* has the capability to develop into antibactic in near future with further studies.

KEYWORDS: Antibiotic, Bacteria, Lichen, Pharmaceutical, *Ralstonia sollanacearum, Roccella montagnii, Salmonella stanley,* Solvent system, Thin layer Chromatography, *Xanthomonas campestris betlicola.*

INTRODUCTION

Nature, in general is a valuable source for novel, pharmaceutically relevant compounds. Historically large proportion of the world's medicine including antibiotics derived from plants and fungi (Crockett and Kageyama, 2005). Intensive use of these antibiotics leads to antibiotic resistance and also facilitates the spread and multiplication of resistant organisms (Lauterwein, *et al.*, 1995). Therefore the resulting strains limit the spectrum of therapeutically active antibiotics drastically (Lauterwein, *et al.*, 1995). Therefore antibiotic resistance has become a major problem for doctors, researchers, and the people that may, one day, need to take an antibiotic (Hovde, 1995). As a result there is a need of continues modification for the prevailing anti-microbial compounds and detection of novel compounds. Hence the most effective way of finding new compounds could be the use of experience of traditional medicine around the world. Throughout the ages, lichens have been used for various purposes, in particular as dyes, perfumes and remedies in folk medicines (Richardson, 1988, Hawksworth, 2003). The topical use of lichen extracts has its origin in ancient Egyptian times (Vartia, 1973). And their biological activities were recognized by Native Americans, Haitian, Indians, Chinese and Europeans to treat a variety of ailments in their traditional medicines (Dayan and Romagni, 2002). By investigating traditional use of these lichens, modern science is given a foundation for exploration of lichen species and their chemical constituents (Crockett and Kageyama, 2005).

One of the fiercest public debates at present concerns the use of antibiotics in agriculture and veterinary practice. The reason for concern is that the same antibiotics (or, at least, antibiotics with the same mode of action on bacteria) are also used for human therapy.

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (photobiont) (Nash, 1996). The photobiont can be green algae, cyano bacteria or both (Nash, 1996) . In all lichens, the fungus forms the thallus or lichenized stroma that may contain characteristic secondary compounds (Ahmadjan and Reynolds, 1993). These are biologically active metabolites with a great variety of effects, including antibiotics, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic activities (Muller, 2001). However, only very limited number of lichen species have been screened for their biological activities and their therapeutic potential in medicine (Bouslin and Martin, 2005). This is certainly due to the difficulties encountered in identification of species, collection of bulk quantities and the isolation of pure substances for structure determination and testing activity (Bouselie and Martin, 2005). Lichen produce protective secondary metabolites serve to deter herbivory and colonization by pathogens (Crockett and Kageyama, 2005). Usnic acid, Stictic acid and Vulpinic acid are a few of the seven hundred plus secondary compounds that are produced by lichen (Lawrey, 1989). A secondary compound that generated a high amount of interest and considerable research was Usnic acid. Infact, sixty four papers are known to have been published on Usnic acid between 1950 and 1959 (Cocchietto, 2002). However most of these experiments were carried out more than forty years ago, so that the data obtained are not comparable to standardized test results (Lauterwein, *et al.*, 1995). Once again there is an interest in the potential uses of antibiotics derived from lichens

Table 1 - Selected lichen species:

Site of Collection	Lichen species
Hambantota.(arid dry zone of Sri Lanka)	*Roccella montagnii*
Hakgala (upper montane rain forest of Sri Lanka/ wet zone)	*Cladonia pityrea*
Hakgala (upper montane rain forest of Sri Lanka/ wet zone)	*Sticta spp*
Hakgala (upper montane rain forest of Sri Lanka/ wet zone)	*Pseudocyphellaria* spp

which may be a valuable source of antibiotics for the pharmaceutical industry as well as in plant pathology in the future (Crockett and Kageyama, 2005).

Therefore this experiment was conducted with the purpose of determination the most effective solvent to extract the secondary metabolites from lichen samples, to examine the secondary compounds present in the selected lichen species, and to determine the potential antibiotics properties of selected lichen species. Apart from that this study refers to the determination of most effective lichen species against selected bacteria species, further the most susceptible bacteria species for the lichen extracts and develop solvent system to run the Coloumn chromatography for the future studies.

MATERIALS AND METHODS

The experiment was carried out at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Karapitiya, Galle and Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila.

Three lichen species from upper montane rain forest at Hakgala and one species from Hambantota were collected (Table 1) and identified from National Herbarium at Royal Botanical Gardens, Peradeniya.

Ten bacteria species (eight human pathogens and two plant pathogens) were used to investigate the antibiotic properties of each selected lichen species (Table 2).

Collected lichen materials were air dried and ground with a mechanical blender. Thimble was prepared using Watman no: 1 filter paper and then it was filled with 20-25g of ground lichen material. Each lichen compound (four species) were extracted using Soxh-let extractor in each solvent such as Acetone, Ethanol: Methanol (3:1) and Water respectively.

Prior to the inoculation of bacteria species in agar plates, ten conical flasks were labeled with species name and date of inoculation. One milliliter of each bacteria broth culture (already prepared) was pipetted out and transferred to the nutrient broth medium (10ml) of each flask and kept overnight in shaker.

Investigation of antibacterial properties

Agar medium of each Petri dish was divided into five sections by marking on the underside of the lower plate. Then four sections were labeled for lichen species while other labeled for standard antibiotics. Using a sterile micropipette, 150 microliters of overnight shacked bacterial broth culture was transferred to a sterile agar plate and spreaded. Disks impregnated with each four lichen

Table 2 - Selected bacteria species

Bacteria Species	Gram reaction	Cell shape	Source/ site of collection
Salmonella stanley	-	rod	Department of Microbiology,
Escherichia coli	-	rod	Faculty of Science, University
Pseudomonas aeruginosa	-	rod	of Kelaniya.
Klebsiella pneumoniae	-	rod	
Bacillus spp	+	rod	
Staphylococcus aureus	+	coccus	
Micrococcus luteus	+	coccus	
Micrococcus ruseus	+	coccus	
Xanthomonas campestris betlicola	-	rod	Department of Horticulture
Ralstonia sollanacearum	-	rod	and Land scape Gardening,
			Wayamba University of Sri
			Lanka.

Table 3 - Constitutes of solvent system and their ratios:

Constitutes of solvent system	Mixing ratio
Toluene : Acetic acid (1)	17:2
Toluene : Dioxane : Acetic acid (2)	17:2:1
Toluene : Dioxane : Acetic acid (3)	17:1:2
Toluene : Dioxane : Methanol (4)	4:2:4
Toluene : Dioxane : Acetic acid : Methanol (5)	16:1:2:1
Toluene : Dioxane : Acetic acid : Methanol (6)	2:1:2
Chloroform : Dioxane : Acetic acid (7)	17:1:2
Toluene : Chloroform : Formaldehyde (8)	12:7:1
Toluene : Dioxane : Formaldehyde (9)	25:14:1

extraction and two of standard antibiotics (Penicillin and Tetracycline) were placed on the surface of the agar (with forceps, the disk were gently tapped to ensure better contact with agar) and it was repeated for all bacteria species. The plates were diametrically sealed with Para films and incubated for 48 hrs at 37 degrees of Celsius. After 48 hrs the zone of inhibition were measured in millimeters.

Development of solvent system

Pre coated thin layer chromatography sheets (10 x 6 cm) were taken and a line 1cm above was drawn from the bottom of the plate. This lower line represents the start line to spot the extract of lichen substrate. This line in 1 cm distance was ticked and each tick was numbered from 1 to 6. (Number 1, 2 and 3 was standard lichen acid and 4, 5 and 6 were lichen extracts). A second horizontal line at 8cm above the lower line was drawn. (This line was the upper margin of solvent front). A small amount of lichen extract and Methanol were carefully put into the sample tubes. After two to three minutes, samples were spotted in TLC plates by using capillary tube. The plates were allowed to dry for two to five minutes until volatile compounds in spotted sample were evaporated. Ten milliliters of different solvent systems was filled into the solvent glass tank (by changing chemical compounds, and the ratio of the mixture, according to their polarity until clear, distinct and round spot was achieved) (Table 3).

After the addition of solvent system to the TLC tank, four to five minutes, it was allowed to saturate for five minutes. Then the TLC plate was placed in the tank and allowed to run until solvent front meet the upper margin of the TLC plate. The plate was removed from the chamber and the solvent front was marked immediately with a pencil. The plate was let to dry off of. The TLC plates were visualized using UV lamp as a non-destructive technique. The choice of solvent system was selected based on the RF value (Culberson, 1972).

Statistical analysis

Three factor factorial design was used to determine the effect of each factor and the data was analyzed using SAS statistical package.

RESULTS AND DISCUSSION

Investigation of antibacterial properties

Three factor factorial analysis showed that zone of inhibition depends on Extraction methodology (E), Lichen extract (A) and Bacteria species (B) (Table 4).

There was a significant difference in zone of inhibition of bacteria when lichen secondary metabolites extracted by using Ethanol: Methanol (E2 / 8.98mm) with compared to Acetone (E1 / 4.31mm) and water (E3 / 0mm).The highest zone of inhibition was recorded with Ethanol: Methanol extraction.

Solvents and solutes can be broadly classified into polar (hydrophilic) and non-polar (lipophilic). Polar solvents can be further subdivided into polar protic solvents and polar aprotic solvents. Water (H_2O), Methanol (CH_3-OH), Ethanol (CH_3-CH_2-OH) and Acetic acid (CH_3-COOH) are representatives of polar protic solvents. Acetone (CH3-CO-CH_3) is a polar aprotic solvent. The polarity of a solvent determines what type of compound it is able to dissolve and with what other solvents or liquid compounds it is miscible with. Therefore polar solvents dissolve polar compound best and non-polar

Table 4 - Results of 3 factor factorial analysis

Source	DF	F value	Pr>F
E	1	491.99	<0.0001
A	3	308.18	<0.0001
B	9	87.59	<0.0001
E*A	3	61.71	<0.0001
E*B	9	22.60	<0.0001
A*B	27	15.67	<0.0001
E*A*B	27	9.90	<0.0001

Notes: R-square = 89.14 CV= 9.699
Significant at 0.01 probability level.

solvents dissolve non-polar compounds best. Based on that principle, Ethanol: Methanol solvent is more polar than Acetone and less than water. But Ethanol: Methanol solvent extraction gave the highest zone of inhibition of bacteria. Suggested that, polarity of lichen metabolites may be much similar to the polarity of Ethanol: Methanol solvent than Acetone and water.

According to the results (Table 5), *Roccella montagnii* extraction (A3) gave the highest zone of

Table 5 - Zone of inhibition of each bacteria for each antibiotic extraction:

	Penicillin (A1)	Tetracycline (A2)	*Roccella Montagnii* (A3)	*Cladonia Pityrea* (A4)	*Sticta Spp* (A5)	*Pseudo Cyphellaria* (A6)
Xanthomonas campestris betlicola(B1)	11.17b	14.50b	**21.50a**	15.33b	11.00b	7.17b
Escherichia coli(B2)	9.00b	**18.17a**	**19.00a**	**15.83a**	9.67b	8.00b
Bacillus spp (B3)	9.33c	**15.33ab**	**17.83a**	14.00b	8.50c	6.33c
Salmonella stanley (B4)	10.50b	15.33b	**20.33a**	14.67b	8.50c	6.67c
Ralstonia solanacearum (B5)	3.33c	0.00c	**24.83a**	8.33b	0.00c	0.00c
Klebsiella pneumoniae (B6)	5.66b	4.50b	**15.00a**	6.50b	0.00c	0.00c
Staphylococcus aureus (B7)	3.67b	7.00b	**11.33a**	7.00b	6.83b	6.33b
Micrococcus ruseus (B8)	10.83b	3.83b	**16.50a**	7.83b	6.83b	6.33b
Micrococcus luteus (B9)	3.16bc	0.00c	**10.17a**	6.33b	1.16c	0.00c
Pseudomonas aeruginosa (B10)	0.00b	3.50ab	**7.33a**	**6.33a**	0.00b	0.00b

inhibition for all bacteria species. But in *Escherichia coli* (B2), there was no significant difference between *Roccella montagnii* (A3) *Cladonia pityrea* (A4) extraction and Tetracycline (A2). In *Bacillus spp* (B3), there was no significant different among *Roccella montagnii* (A3) and Tetracycline (A2) and also with *Pseudomonas aeruginosa* (B10), *Roccella montagnii* (A3) and *Cladonia pityrea*(A4) extraction did not show any significant difference in zone of inhibition.

One of the methods that determines the effectiveness of an antibiotic is measuring its zone of inhibition. In the agar diffusion method one species of bacteria is uniformly swabbed on to the nutrient agar plate. Chemicals/antibiotics are added to paper disks and those discs are placed on the surface of the agar. An effective agent will inhibit bacterial growth, and measurements can be made to quantify the size of the zones of inhibition around the disks. The relative effectiveness of a compound is determined by comparing the diameter of the zone of inhibition with values of standard table (Table 6).

Table 6 - Interpretation of inhibition zones of test cultures:

	Diameter of Zones of inhibition (mm)
Resistant	10 or less
Intermediate	>10 or < 15
Susceptible	>15

Source: Johnson and Case, Laboratory experiment in Microbiology (1995).

Based on this principle, each and every lichen extract was tested with ten bacteria species and two standard antibiotics. According to the results, *Roccella montagnii* extraction (A3) gave the highest inhibitory activity with compared to two standard antibiotics. However Penicillin can't inhibit gram negative bacteria. But Tetracycline consider as a broad spectrum antibiotic which have capability to inhibit the growth of most of the bacteria. According to the present study, Tetracycline showed an intermediate activity. Hence *Roccella montagnii* extraction showed higher activity against both negative and positive bacteria than two standard antibiotics. It revealed the *Roccella montagnii* extraction may have broad spectrum activity compared to all other selected lichen species.

When *Roccella montagnii* extraction (A3) is considered, *Xanthomonas campestris betlicola* (B1/ 21.50mm), *Bacillus spp* (B3/ 17.83mm), *Salmonella stanley* (B4/ 20.33mm) and *Ralstonia solanacearum* (B5/ 24.83mm) recorded more than 15mm diameter zone of inhibition. Suggested that, they are susceptible to the *Roccella montagnii* extraction (A3). *Escherichia coli* (B2) and *Micrococcus ruseus* (B8) were not significantly different from 15mm diameter, but significantly differe from 10mm diameter. Therefore they show intermediate resistant for *Roccella montagnii* extraction. *Klebsiella pneumoniae* (B6), *Staphylococcus aureus* (B7), *Micrococcus luteus* (B9) and *Pseudomonas aeruginosa* (B10) were not significantly different from both 15mm and 10mm. Therefore their zone of inhibition was less than 10mm and they are resistant to the *Roccella montagnii* extraction (Table 7).

Development of solvent system

Solvent system 3 (Toluene: Dioxane: Acetic acid /17:1:2),8(Toluene: Chloroform: Formaldehyde/ 12:7:1) and 9 (Toluene: Dioxane: Formaldehyde /25:14:1) gave the highest separation of compounds or spots and those spots are more clear distinct and round in shape. Further solvent system 8 (Toluene: Chloroform: Formaldehyde/12:7:1) and 9 (Toluene: Dioxane: Formaldehyde /25:14:1) showed a compatible spot (Rf value for Atronorin = 0.78) among *Roccella montagnii* extraction (A3) and Atronorin (reference sample 3). But there were no compatible spots with Usnic acid (reference sample 1) and Norstictic acid (reference sample 2).

Thin Layer Chromatography (TLC) is an extremely useful technique for monitoring reactions. It is also used to determine the proper solvent system

Table 7 - Effect of *Roccella montagnii:*

	Bacteria Mean (mm)	H0=15,H1>15		H0=10,H1>10	
		T	P	T	P
Xanthomonas campestris betlicola(B1)	21.50	**3.64**	**0.0074**	-	-
Escherichia coli(B2)	19.00	2.26	0.0370	**5.08**	**0.0019**
Bacillus spp (B3)	17.83	**4.33**	**0.0037**	-	-
Salmonella stanley (B4)	20.33	**8.68**	**0.0002**	-	-
Ralstonia solanacearum (B5)	24.83	**8.23**	**0.0002**	-	-
Klebsiella pneumoniae (B6)	15.00	0.00	0.5000	2.50	0.0270
Staphylococcus aureus (B7)	11.00	-2.38	0.9700	0.86	0.2100
Micrococcus ruseus (B8)	16.50	1.10	0.1600	**4.78**	**0.0025**
Micrococcus luteus (B9)	10.17	-	-	-	-
Pseudomonas aeruginosa (B10)	7.33	-	-	-	-

Notes: Significant at 0.01 probability level.

for performing separations using column chromatography. TLC uses a stationary phase, usually alumina or silica that is highly polar (standard) or non-polar (reverse phase). The mobile phase is a solvent whose polarity can be changed. Reaction mixture in solution will be applied to the chamber and then "run" the plate by allowing a solvent (or combination of solvents) to move up the plate by capillary action. Depending on the polarity of the components of the mixture, different compounds will travel different distances up the plate. More polar compounds will "stick" to the polar silica gel and travel short distances on the plate. Non-polar substances will spend more time in the mobilesolvent phase and travel larger distances on the plate. A major objective of TLC is to obtain clear, distinct, and round spot with more separation of compounds. It could be achieved in solvent system, 8 (Toluene: Chloroform: Formaldehyde/12:7:1) and 9 (Toluene: Dioxane: Formaldehyde /25:14:1).

CONCLUSIONS

Roccella montagnii extraction shows more antibacterial properties than two standard antibiotics and other three lichen extracts. Therefore it is clear that, antibiotics can be developed with further studies using active compounds of *Roccella montagnii* extraction. *Roccella montagnii* extraction can be used to control *Xanthomonas campestris betlicola*, *Bacillus spp*, *Salmonella stanley* and *Ralstonia solanacearum* (bacteria wilt) successfully.

Solvent system 8 (Toluene: Chloroform: Formaldehyde/12:7:1) and 9 (Toluene: Dioxane: Formaldehyde /25:14:1) can be used to perform the Coloumn chromatography. Atronorin is available in *Roccella montagnii* extraction.

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