Effects of extracts from lichen *Ramalina pacifica* against clinically infectious bacteria

H. Joy Hoskeri [1], V. Krishna[1] and C. Amruthavalli[2]
1. P.G. Department of Studies and Research in Biotechnology and Bioinformatics,
Kuvempu University, Shankaraghatta – 577 451, Karnataka, India.
2. Bioinformatics Division, Centre for Information Science and Technology, University of Mysore,
Mysore, Karnataka, India.
E.mail: joybioinfo@gmail.com

Abstract Bactericidal activity of crude extracts from lichen *Ramalina pacifica* were screened against 20 clinical pathogenic strains isolated from different infectious sources which belong to *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi, Salmonella paratyphi, Echerichia coli,* and *Staphylococcus aureus*. The minimal inhibitory concentration of petroleum ether extract and ethanol extract was determined against AmericanTypeCellCulture and MicrobialTypeCellCulture strains. Both the extracts exhibited predominant antibacterial activity against all the multi-resistant strains isolated from infected patient's sample with significant zone of inhibition at MIC=100µg/100µL. The bactericidal activity was assessed comparatively with the reference ATCC strains (*Pseudomonas aeruginosa*- ATCC-20852; *Staphylococcus aureus*- ATCC 29737), (*Salmonella typhi* – ATCC-19430), (*Salmonella paratyphi* – ATCC-9150), (*E. coli* – ATCC-25922) and MTCC strains (*Klebsiella pneumoniae* – MTCC-618) respectively. Ciproflaxin at the concentration 50µg/100µL was used as standard. Ethanolic extract exhibited significant zone of inhibition against the clinical strains of *S. aureus* (16.67±1.05mm) and *E. coli* (17.00±1.24mm) isolated form the abscess and hospital effluent respectively. The results were promising and supported the traditional use of lichens for the treatment of respiratory infections, urinary tract infections and pneumonia. [Researcher. 2010;2(3):81-85]. (ISSN: 1553-9865).

Keywords: Bactericidal activity, clinical isolates, AmericanTypeCellCulture, MicrobialTypeCellCulture, Minimal inhibitory concentrations, and Sensitive radial diffusion technique.

1. Introduction

Natural products are proposed as a therapeutic alternative to conventional antimicrobial treatment, whose effectiveness is often limited by the resistance that the infectious agents have developed against antibiotics (Ali *et al*., 1999; Nimri *et al*., 1999). Pathogenic microbes pose serious threats to human health and are increasing in prevalence in institutional health care settings (James *et al.,* 1997). New alternatives for combating the spread of infection by antibiotic resistant microbes in future are necessary tools for keeping pace with the evolution of 'super' pathogens. The most successful antibiotics that have been applied to combat disease are small molecule, secondary metabolites, including penicillin derivatives that were originally isolated from fungi (Babita *et al.,* 2008).

New antibiotics that are active against resistant bacteria are required. Bacteria have lived on Earth for several billion years. During this time, they encountered in nature a wide range of naturally occurring antibiotics. To survive, bacteria developed antibiotic resistance mechanism (Raja *et al.,* 2010). Raw meat remains an important and probably the major source of human food borne infection with pathogenic bacteria. In spite of decades of effort it has been difficult to obtain food animals free of pathogenic bacteria (Purabi and Joshi, 2010).

Lichens represent a symbiotic association of a fungus with an algal partner, and are important constituents of many ecosystems. Lichens have been used for medical purposes since ancient times and are known to produce unique secondary metabolites, a number of which have considerable biological activities such as antimicrobial, antiherbivore, and antibiotic (Vartia, 1973; Richardson, 1988; Lawrey, 1989; Elix, 1996). These secondary metabolites fall into various chemical classes, which are, as a group, distinct from those produced by higher plants. These include: diterpene, triterpene, dibenbenzofuran, dibenzopyranone, depside, depsidones, anthraquinone, xanthones, usnic acids and pulvinic acids (Dayan and Romagni, 2001). Lichen secondary metabolites exhibit numerous biological activities including: antimycobacterial (Ingolfsdottir *et al.*, 1998), antiviral (Neamati *et al.*, 1997), antioxidant (Hidalgo *et al.*, 1994), analgesic (Okuyama *et al.*, 1995), cytotoxic, antimicrobial, fungicidal,

herbicidal, antifeedant, photosystem inhibitory (Dayan and Romagni, 2001). Some *Ramalina* species are usually used as food in some Central and South Eastern Asian countries. Since the beginning of the 20th century hair powder of *Ramalina* species have been used in cosmetics in Europe (Richardson, 1974), and India (Smith, 1921) respectively.

In this study, we evaluated the bactericidal activity of petroleum ether and ethanolic extracts of *Ramalina pacifica* against 6 clinically pathogenic bacteria *viz., Staphylococci aureus*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi* and *Escherchia coli* isolated from different infectious sources.

2. Materials and Methods

2.1 Lichen material

Ramalina pacifica growing on barks of ziziphus trees were collected from the forest area of Bhadra wildlife sanctuary - a South west region of India, which is located between 28°58′ and 43.49″ North latitude and 77°16′ and 29.50″ East longitude, 2,308ft. Bhadra wildlife sanctuary is a deciduous forest located almost at the central part of Karnataka state in the malnad region bounded by Western Ghats on the east direction.

The voucher specimens of the selected lichens *Ramalina pacifica* Asahina (Voucher no. KU00934) was deposited in the Department of Applied Botany, Kuvempu University, India for future reference.

During 01 June, 2009, the fresh whole lichen material was shade dried, powdered mechanically and was subjected for soxhlet extraction using petroleum ether and ethanol as solvent system for about 48 h successively. The extract was filtered and concentrated in vacuum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and allowed it for complete evaporation of the solvent.

2.2 Evaluation of minimal inhibitory concentrations (MIC)

The minimal inhibitory concentrations (MIC) of the crude petroleum ether and ethanolic extract were determined by micro dilution techniques (Islam *et al.,* 2008) in Luria-Bertini broth, according to National Committee for Clinical Laboratory Standard, USA guidelines. The inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard colony forming units and diluted 1:10 for the broth micro dilution procedure. The microtiter plates were incubated at 37^0C and MIC was determined after 24 h of incubation.

2.3 Antibacterial screening

The antibacterial activity of the petroleum ether and ethanolic extracts was screened by agar well diffusion method (Carron *et al*., 1987) against twenty clinical isolates of bacterial strains belonging to *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi, Salmonella paratyphi* and *Escherchia coli* respectively. The bacterial strains were collected from different infectious status of the patients with the help of authorized physicians, in district health center of Gulberga, Karnataka state, India. The clinical isolates were identified in Microbiology Laboratory, Gulberga University following the standard method (Cowan and Steel, 1993).

The different infectious sources of pathogen are mentioned in the Tables 1. The petroleum ether and ethanolic extract were dissolved in 10% aqueous dimethyl sulfoxide (DMSO; that enhances compound solubility) to get stock solutions. Commercial bactericide ciprofloxacin was used as standard (100µg/100µL of sterilized distilled water) concomitantly with the test samples. The activity was screened comparatively with the reference ATCC strains (*Pseudomonas aeruginosa*- ATCC-20852; *Staphylococcus aureus*- ATCC 29737), (*Salmonella typhi* – ATCC-19430), (*Salmonella paratyphi* – ATCC-9150), (*E. coli* – ATCC-25922) and MTCC strains (*Klebsiella pneumoniae* – MTCC-618).

A sensitive radial diffusion technique (Lehrer *et al.,* 1991) was used for the assessment of antibacterial activity of the test samples. Sterilized Luria- Bertini agar medium was poured into sterilized petridishes. Luria- Bertini broth containing 100µL of 24 h incubated cultures of clinical isolates and the AmericanTypeCellCulture (ATCC) and MicrobialTypeCellCulture (MTCC) strains were spread on the agar medium. Wells were created using a sterilized cork borer in an aseptic condition. 100µL of crude petroleum ether extract, 100µL of ethanolic and 100µL of standard drug ciprofloxacin were loaded on the corresponding wells. The plates were incubated at 37^0 C for 24 h. The diameter of the zone of complete inhibition of the bacteria was measured to the nearest around each well and readings were recorded in mm. The results of these experiments are expressed as mean ± SE of three replicates in each test.

Table 1. *In vitro* antibacterial activity of petroleum ether and ethanolic extract of *Ramalina pacifica* against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi* and *Escherchia coli*.

Pathogens	Bacterial strains tested	Source of collection	Petroleum ether extract	Ethanolic extract	Reference drug Ciprofloxacin
	Zone of inhibition (in mm)				
Staphylococcus aureus					
	Sa-1	ATCC-29737	17.33 ± 1.28	16 ±1.75	21±0.86
	Sa-2	Abscess	15.33 ± 2.79	16.67 ± 1.05	21±1.63
	Sa-3	Urine	16.67 ± 1.05	13.67±2.28	19.5±0.96
	Sa-4	Wound	17.17 ± 1.54	12±0.86	18.67±1.41
	Sa-5	Hospital effluents	13 ± 1.84	16.5±1.34	21±1.81
Pseudomonas aureginosa					
	Pa-1	ATCC-20852	9.83 ± 0.54	11.33 ± 0.49	22.67±0.8
	Pa-2	Urine	11.67 ± 0.88	11 ± 0.89	21±1.13
	Pa-3	Pus	12.17 ± 0.79	10.83±0.7	18.83±1.01
	Pa-4	Stool	9.67 ± 0.33	9±1.06	19.17±0.95
Klebsiella pneumoniae					
	Kp-1	MTCC-618	10.33 ± 0.67	11.33 ± 0.56	16±0.58
	Kp-2	Urine	11.67 ± 0.8	8.5 ± 0.67	16.83±1.4
	Kp-3	Feaces	8.33 ± 0.84	11.33±0.67	14.33±1.31
	Kp-4	Sputum	9.67 ± 0.88	8.67±1.09	16±1.32
Salmonella typhi					
	St-1	ATCC-19430	12.33 ± 0.42	13.33 ± 0.88	21±0.58
	St-2	Blood clot	12 ± 0.86	12 ± 1.34	20.83±0.98
Salmonella paratyphi					
	Spt-1	ATCC-9150	13.83 ± 0.75	10.83 ± 0.6	16.83±1.3
	Spt-2	Blood clot	10.67 ± 0.67	ND	20±1.06
Escherchia coli					
	Ec-1	ATCC-25922	18.67 ± 1.15	13.83 ± 0.75	17.33±0.49
	Ec-2	Hospital effluents	16.83±1	17 ± 1.24	16.33±1.76
	Ec-3	Urine	13.83 ± 0.75	13.67±0.28	20±1.06

The value of each constituents consisted of ± S.E. of 03 replicates, ND – Not Defined.

3. Results and Discussion

100µL of petroleum ether extract and 100µL of ethanolic extract in 100µL (10% DMSO in distilled water v/v) was found to be the minimum concentration at which they showed inhibition of bacteria under study. Evaluation of anti-bacterial activity revealed that the petroleum ether and ethanolic extracts showed effective activity against all the six bacterial pathogens. Specifically, petroleum ether extract was more efficient than ethanolic extract, but less potent than standard against *S. aureus*. The effects of the extracts on the clinical isolates can be depicted from table 1.

Petroleum ether extract showed significant results in inhibiting S. aureus with 17.33 ± 1.28 mm, when compared to ethanol extract which showed 16 ±1.75 mm zone of inhibition. Whereas, ethanol extract proved to be significantly active against *P. aureginosa* with 11.33 ± 0.49 mm zone of inhibition than petroleum ether extract with 9.83 ± 0.54 mm.

Ethanolic extract exhibited significant zone of inhibition against *K. pneumoniae* (11.33 ± 0.56mm) and S. *typhi* (13.33 ± 0.88 mm) isolated form the abscess and hospital effluent respectively than petroleum ether extract. But petroleum ether extract show significant inhibitory activity against *S. paratyphi* (13.83 ± 0.75mm) and *E. coli* (18.67 ± 1.15mm) respectively, than ethanol extract. This study revealed that the extracts of

Ramalina pacifica possesses potent activity against both Gram-negative and Gram-positive bacteria.

A great number of lichen species have proved to be a source of important secondary metabolites for the food and pharmaceutical industries (Crittenden & Porter, 1991). Although many natural lichens and cultured lichens have been screened for their biological activities and several novel compounds have been isolated and identified, lichens have been essentially ignored by the modern pharmaceutical industry because of their slow growth in nature (Yamamoto et al., 1998; Lauterwerwein et al., 1995). Industrial-scale harvests are neither ecologically sensible nor sustainable and, for many species, are not feasible (Miao et al., 2001).

Results of *in-vitro* bactericidal activity are shown in Table 1. Both petroleum ether and ethanolic extract were significantly effective in controlling the growth of all the bacterial strains under study. In particular, Ramalina species were most commonly used for medicinal, perfumery, and cosmetics. Usnic acid as a pure substance has been formulated in creams, toothpaste, mouthwash, deodorants, and sunscreen products, in some cases as an active principle, in others as a preservative. Up to now about 350 secondary metabolites are known from lichens and approximately 200 have been characterized. The antibiotic mechanism of many lichens results from usnic acid or lichenic acids. The antimicrobial agent's usnic acid or lichenic acids has activity against Gram-negative, Gram-positive bacteria and mycobacteria. In addition to antimicrobial activity against human and plant pathogens, usnic acid has been reported to exhibit antiprotozoal, antiproliferative, anti-inflammatory, analgesic, and antiviral activity (Ingolfsdottir, 2002).

Extracts of *Ramalina pacifica* proved to be effective bactericidal agents against clinically potent bacterial pathogens. This investigation is in support to the traditional use of lichens for the treatment of respiratory infections, urinary tract infections and pneumonia.

4. References

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