Journal of Applied Pharmaceutical Science Vol. 3 (02), pp. 143-147, February, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.30225
ISSN 2231-3354

The Investigation of Antibacterial Activities of Ethanol and Methanol Extracts of *Flavoparmelia caperata* (L.) Hale (*Parmeliaceae*) and *Roccella phycopsis* Ach. (*Roccellaceae*) Lichens Collected from Eastern Blacksea Region, Turkey

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ARTICLE INFO

Article history:
Received on: 18/01/2013
Revised on: 09/02/2013
Accepted on: 21/02/2013
Available online: 28/02/2013

Key words:
Antibacterial, Lichen, *Roccella phycopsis*, *Flavoparmelia caperata.*

ABSTRACT

This study evaluates antibacterial activities of extracts of *Flavoparmelia caperata* and *Roccella phycopsis* by disc-diffusion and broth dilution methods against seven gram positive and nine gram negative bacteria. The solvents used as extractants in this study were ethanol and methanol. The antibacterial activities of lichen extracts were comparable with penicillin, tetracycline and gentamicin, commonly used antibiotics for the treatment of infections. Antibacterial activities of lichen extracts ranged from 14-26 mm. It was observed from the studies that the most resistant bacteria was *Bacillus megaterium* and the most sensitive bacteria was *Proteus vulgaris.* Studied lichen extracts have antibacterial activity on both gram negative and gram positive bacteria. The minimum inhibitory concentration values of the lichen extracts were ranged from 58-7500 µg/mL. Our studies suggest that methanol and ethanol extracts of *Flavoparmelia caperata* and *Roccella phycopsis* could be an alternative of the antibiotic to cure the diseases.

INTRODUCTION

Since 1990s there has been a growing shift in interest towards plants as significant sources for new pharmaceuticals. (Pavithra *et al.,* 2010). Due to having phytochemicals, plants become an important research source. Drugs which are extracted from plants are very effective, easily available and less expensive and they rarely have side effects associated with them (Nazir and Latif, 2012). Lichens are symbiotic associations of a fungus and green algae or cyanobacteria. As a result of this union, lichens have a new anatomical, morphological and physiological properties which unlike organisms that they constitute. Lichens are food resources for many people and animals. They are used for production of dye, perfume as well as pharmaceutical industries. In addition to this, lichens have been used in folk medicine for centuries (Romagni & Dayan 2002). *Roccella phycopsis* mostly grows on rocks near to the coast..

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In the past centuries, *Roccella* spp. was used as a dye source in Europe Due to revealing synthetic dyes, it was ceased to use this lichen (Huneck, 1999).

R. phycopsis dye and alcohol are used in thermometer (Uphof, 1959). Moreover, litmus is obtained from *R. phycopsis* lichens (Mitrović *et al.,* 2011).

Flavoparmelia caperata is a foliose lichen which is grows on trunks and branches of trees, shrubs and fences in open areas, rarely on rocks. The intestinal worms are treated by *F. caperata* and dried powder of the thallus can be applied on burns (Haq *et al.,* 2012). This species was used to dye wools in Man Island (Uphof, 1959).

It also is used as bioindicator for determining atmospheric pollution (Freitas, 2011).

In this preliminary antibacterial assay, we aimed to investigate antibacterial activities of ethanol and methanol extracts of *R. phycopsis* Ach. (*Roccellaceae*) and *F. caperata* (L.) Hale (*Parmeliaceae*) lichens which could serve as a good candidate for the development of new antimicrobial agents.

MATERIAL AND METHODS

Collection and identification of lichen samples

F. caperata and *R. phycopsis* species were collected from the two different localities of Giresun province between 14 October and 25 September 2011. Localities were as shown in Table 1. Lichen species were identified by Kadir Kınalıoğlu. Vouchers are preserved in the herbarium of the Faculty of Science and Arts, Giresun University, Giresun.

Table. 1: The collecting localities.

Species	Locality Name
R. phycopsis	Giresun Center, Gedikkaya hill, 225 m
F. caperata	Giresun, Bulancak, Ahmetli village, 350 m

Test microorganisms

Nine gram negative and seven gram positive bacteria strains were used to determine the antibacterial activities of ethanol and methanol extracts of *R. phycopsis* and *F. caperata* lichens. Bacteria are used in the study as follows: *Enterococcus faecium* (laboratory isolate), *Staphylococcus calmii* (laboratory isolate), *Proteus mirabilis* (laboratory isolate), *Bacillus megaterium* (laboratory isolate), *Acinetobacter baumannii* (laboratory isolate), *Erwinia amylovora* (laboratory isolate), *Gordonia rubripertincta* (laboratory isolate), *Proteus vulgaris* ATCC 7829, *Yersinia enterocolitica* ATCC 27729, *Klebsiella pneumoniae* ATCC 13385, *Listeria monocytogenes* ATCC 7644, *Salmonella enterica* serovar *typhimirium* ATCC 14028, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Escherichia coli* ATCC 35218, *Yersinia pseudotuberculosis* ATCC 911, *Enterococcus faecalis* ATCC 29212 and *Bacillus cereus* 702 ROMA. *E. faecium* (laboratory isolate), *S. calmii* (laboratory isolate), *P. mirabilis* (laboratory isolate), *B. megaterium* (laboratory isolate), *A. baumannii* (laboratory isolate), *E. amylovora* (laboratory isolate), *G. rubripertincta* (laboratory isolate), *P. vulgaris* ATCC 7829, *Y. enterocolitica* ATCC 27729, *K. pneumoniae* ATCC 13385 were obtained from Genetics and Bioengineering Department, Yeditepe University; *L. monocytogenes* ATCC 7644, *S. enterica* serovar *typhimirium* ATCC 14028, *S. aureus* subsp. *aureus* ATCC 25923 were obtained from Control Laboratory in Giresun province, *E. coli* ATCC 35218 was obtained from Biology Department, Giresun University and *Y. pseudotuberculosis* ATCC 911, *E. faecalis* ATCC 29212, *B. cereus* 702 ROMA were obtained from Moleculer Biology Department, Rize University.

Preparation of lichen extracts

Lichen samples were dried at room temperature for 48 h and powdered with a blender. Powdered lichens (48 g) were extracted with 480 mL of ethanol and methanol separetely by using a Soxhlet apparatus for 7 h at a temperature not exceeding the boiling point of the solvent, separetely. The extracts were filtered using Whatman filter paper (No.1) and then concentrated in vacuo at 40°C using a rotary evaporator. Extracts were stored at-80°C for further assays (Kumar *et al.*, 2012).

Extract yield (%) of extracts

Extract yields of dried extracts were calculated the following equation: % Extract yield = $(W_1 x100) / W_2$

W_1 shows the remaining solid lichen extract weight after evaporation of the solvent used in extraction, W_2 shows the weight of lichen powder form to be used in extraction.

Antibacterial susceptibility testing of the lichen extracts

The dried ethanolic and methanolic lichen extracts were dissolved to obtain 30 mg/mL final concentration in ethanol separetely.

Then, ethanol and methanol extracts were sterilized by filtration through 0.45 µm Millipore filters (Aslan et al. 2006). Antibacterial tests were carried out by the disc diffusion method. Inocula (contains 10^8 CFU bacteria), corresponding to a value of 0.5 on the McFarland optical density scale, was prepared in Muller Hinton Broth and cultivated onto Mueller Hinton agar plates. Steril discs, 5 mm diameter, were put on each agar surface.

Discs were impregnated with 20 µL of ethanol extract of *R. phycopsis*, methanol extracts of *R. phycopsis*, ethanol extracts of *F. caperata*, methanol extracts of *F. caperata* and ethanol (negative control), separetely. Besides standart antibiotic discs (positive control) were put the agar surface. Plates waited in refrigerator for 2 h and then all the plates were incubated at 37°C for 24 h. After incubation the antibacterial activity was evaluated by measuring the inhibition zone diameter observed. Each test was performed twice (Šarić *et al.*, 2009).

Determination of minimal inhibition concentration

The minimal inhibition concentration (MIC) were also studied for the microorganisms which were determined as sensitive to studied lichen extracts. The inocula of microorganisms were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The dry ethanol and methanol extracts of *F. caperata* and *R. phycopsis* were evaluated against the test microorganisms using broth dilution method described by Yiğit et al. (2009) with some modifications. The dried lichen extracts were dissolved to obtain 30 mg/mL final concentration in ethanol separetely. Then, ethanol and methanol extracts were sterilized by filtration through 0.45 µm Millipore filters. 950 µL volume of Muller Hinton Broth was added to each tube. Then, 1000 µL volume of the extract sample was transferred to the first tube and serial 2-fold dilutions were performed, the remaining 1000 µL was discarded. Finally, 50 µL bacteria suspension added to tubes. The tubes for antibacterial tests were incubated at 37 °C overnight. The growth of the bacteria was determined by turbidity. Clear tubes indicated absence of bacteria growth. The MIC values were defined as the lowest concentration of test samples that completely inhibited the visible growth. The tests were carried out in duplicate.

RESULTS AND DISCUSSION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents.

The first step towards this goal is the *in vitro* antibacterial activity assay (Mahesh and Satish, 2008). Table 2 shows extraction yields of compounds that can be extracted from plants. Methanol extractions have the highest extraction yield.

Table. 2: Extract yields (%) of lichen extracts.

Lichen	Extract	Extract Yield (%)
R. phycopsis	Ethanol	16.28
R. phycopsis	Methanol	19.38
F. caperata	Ethanol	12.34
F. caperata	Methanol	14.65

Antibacterial activity of ethanol and methanol extracts of *F. caperata* and *R. phycopsis* were determined by disc diffusion method against *E. faecium, B. megaterium, S. aureus* subsp. *aureus* ATCC 25923, *G. rubripertincta, S. cohnii, B. cereus* 702 ROMA, *E. faecalis* ATCC 29212, *A. baumannii, P. mirabilis, E. amylovora, E. coli* ATCC 35218, *S. enterica* serovar *typhimirium* ATCC 14028, *Y. pseudotuberculosis* ATCC 911, *P. vulgaris* ATCC 7829, *Y. enterocolitica* ATCC 27729 and *K. pneumoniae* ATCC 13385. Ethanol and methanol extracts of lichens were employed to observe the inhibition of test bacteria. The inhibitory activities of the extracts of *F. caperata* and *R. phycopsis* are given in table 3. If inhibition zones are small than 14 mm, it means microorganism is resistant, if inhibition zones are between 14-17 mm, it means microorganism is less sensitive, if inhibition zones are bigger than 17 mm, it means microorganism is sensitive (Albayrak, 2006). In the present study, bacteria responded differently against lichen extracts and antibiotics. Antibacterial activities of lichen extracts ranged from 14-26 mm. Some extracts created bigger zones than antibiotics which used for control group. This situation shows studied some extracts are more efficient than antibiotics. Growth inhibition potency of tested lichen extracts shown in table 3 clearly demonstrates *B. megaterium* was the most resistant bacteria against studied lichen extracts and *P. vulgaris* was the most sensitive bacteria against studied lichen extracts.

B. megaterium, *S. cohnii*, *B. cereus*, *E. faecalis*, *S. enterica* serovar *typhimirium*, *Y. pseudotuberculosis* and *K. pneumoniae* didn't show any inhibition on exposure to ethanol extract of *R. phycopsis*; *S. cohnii*, *E. amylovora*, *S. enterica* serovar *typhimirium* and *P. vulgaris* didn't show any inhibition on exposure to methanol extract of *R. phycopsis*; *S. cohnii*, *S. enterica* serovar *typhimirium*, *Y. pseudotuberculosis* and *P. vulgaris* didn't show any inhibition on exposure to ethanol extract of *F. caperata*; *S. cohnii, E. coli, S. enterica* serovar *typhimirium, Y. pseudotuberculosis* and *P. vulgaris* didn't show any inhibition on exposure to methanol extract of *F. caperata*. In addition to, any of the extracts didn't show inhibitory effect against *S. cohnii* and *S. enterica serovar typhimirium* bacteria. While, ethanol and methanol extracts of *F. caperata* lichen show higher activity than ethanol and methanol extracts of *R. phycopsis* against *E. faecium*, *B. megaterium*, *B. cereus*, *E. faecalis*, *A. baumannii* and *E. amylovora*; ethanol and methanol extracts of *R. phycopsis* lichen show higher activity than ethanol and methanol extracts of *F. caperata* against *P. mirabilis*, *E. coli*, *P. vulgaris* and *Y. enterocolitica* bacteria.

The solvent residue diluted with ethanol (the control) showed no inhibitory zone.

Antimicrobial spectrum of lichen species used in studies make up a potential activity against both Gram-positive and Gram-negative bacteria. According to the cell envelope property there is no differences in lichen efficiency.

Quantitative evaluation of antibacterial activity of all extracts was carried out against test bacteria by broth dilution techniques. MIC values of ethanol and methanol extracts of *F. caperata* and *R. phycopsis* lichen are presented in table 4. The minimum inhibitory concentration valuesof the lichen extracts ranged from 58-7500 µg/mL.

Lichen extracts have been effectively proven for their utilization as source for antimicrobial compounds. Previous researches showed significant bioactive characteristics of *F. caperata*. Acetone extract of *F. caperata* didn't show antimicrobial activity against *E. coli*, *E. faecalis*, *P. mirabilis*, *P. vulgaris* (Duman, 2009). Seaman et al. (2007) found that acetone extracts of *F. caperata* exhibited good activity against *K. pneumoniae*, with an MIC of 1 mg/mL. In our study ethanol and methanol extracts of *F. caperata* exhibited 7.5 mg/mL MIC value against *K. pneumoniae*. This means acetone extracts of *F. caperata* is more efficient than methanol and ethanol extracts of *F. caperata* against *K. pneumoniae*. Ethanol extract of *F. caperata* inhibited *Mycobacterium tuberculosis* H_{37}Rv. and *M. tuberculosis* H_{37}Ra with an MIC of 250 µg/mL (Gupta *et al.*, 2007). According to a study was conducted by Mitroviç et al. (2011) methanol extracts of *F. caperata* lichen had 78.1 µg/mL, 39.1 µg/mL, 156 µg/mL, 10000 µg/mL, 2500 µg/mL and *S.* 10000 µg/mL MIC values against *E. faecalis* ATCC 29212, *B. cereus* (clinical strain), *S. aureus* subsp. *aureus* ATCC 25923, *E. coli* ATCC 25922, *P. mirabilis* (clinical strain) and *S. enterica* serovar *typhimirium* (clinical strain). In our study; methanol extracts of *F. caperata* lichen had 234 µg/mL, 58 µg/mL, 937 µg/mL and 1875 µg/mL MIC values against *E. faecalis* ATCC 29212, *B. cereus* (laboratory isolate), *S. aureus* subsp. *aureus* ATCC 25923 and *P. mirabilis* (laboratory isolate). No activity observed against *E. coli* ATCC 35218, *S. enterica* serovar *typhimirium* ATCC 14028. Using different lichen concentration against test bacteria and using different bacteria strains are the reasons of differences between Mitroviç et al. 2011 and our study. So far, antibacterial activity of *R. phycopsis* hasn't been tried against bacteria, but antibacterial activity of *Roccella belangeriana* and *Roccella montagnei* of *Roccella* species was tried against bacteria. Antimicrobial activity of water, acetone, methanol, ethyl acetate, chloroform, ethanol, diethyl ether and petroleum ether extracts of *R. belangeriana* species investigated by Karthikaidevi et al. (2009).

It was reported that ethanol extracts of lichen exhibited activity against *E. coli*, *Staphylococcus* sp., *Proteus* sp. and methanol extracts of lichen exhibited activity against *K. pneumoniae*, *Staphylococcus* sp., *Proteus* sp. and *Salmonella* sp. Findings by Dahake et al. (2010) have demonstrated that acetone extracts of *R. belangeriana* had antimicrobial activities against *Bacillus subtilis*, *S. aureus*, *Pseudomonas aeruginosa* and *E. coli*.

Table. 3: Results of antibacterial activities of lichen extracts (mm).

	Inhibition Zone (mm)							
Microorganism	**RP et**	**RP met**	**FC et**	**FC met**	**Ethanol**	**CN10**	**P10**	**TE30**
E. faecium	17.5	15	21	18	-	NT	23	NT
B. megaterium	6	14.5	17	17.5	-	NT	NT	16
S. aureus subsp. *aureus*	17	17	17	18	-	NT	NT	20
G. rubripertincta	14	18	14	15	-	16	NT	NT
S. cohnii	9.5	8	9	9	-	14	NT	NT
B. cereus	13.5	17.5	22.5	24.5	-	16	NT	NT
E. faecalis	10.5	17	14	17.5	-	NT	NT	20
A.baumannii	15	14.5	21.5	21.5	-	25	NT	NT
P. mirabilis	14.5	17	14	11.5	-	21	NT	NT
E. amylovora	17	13	18	17	-	18	NT	NT
E. coli	17.5	18	13.5	12	-	19	NT	NT
S. enterica serovar *typhimirium*	11.5	10	8	8.5	-	17	NT	NT
Y. pseudotuberculosis	7.5	15	12	9.5	-	NT	NT	10
P. vulgaris	26	18	11	13.5	-	22	NT	NT
Y. enterocolitica	24	18	15	15	-	NT	NT	22
K. pneumoniae	13	16	14	15	-	22	NT	NT

RP et: *R. phycopsis* ethanol extract; RP met: *R. phycopsis* methanol extract; FC et: *F. caperata* ethanol extract; FC met: *F. caperata* methanol extract; (-): No zone; NT: Not tested; P10: Penicillin 10 µg/mL; TE30: Tetracycline 30 µg/mL; CN10: Gentamicin 10 µg/mL.

Table. 4: MIC values of lichen extracts.

	MIC Value (µg/mL)			
Microorganism	**RP et**	**RP met**	**FC et**	**FC met**
E. faecium	937	1875	117	234
B. megaterium	7500	1875	234	234
S. aureus subsp. *aureus*	1875	3750	937	937
G. rubripertincta	3750	1875	468	937
S. cohnii	-	-	-	-
B. cereus	-	937	117	58
E. faecalis	-	3750	937	234
A.baumannii	1875	937	234	1875
P. mirabilis	7500	3750	7500	1875
E. amylovora	3750	-	117	234
E. coli	3750	7500	7500	-
S. enterica serovar *typhimirium*	-	-	-	-
Y. pseudotuberculosis	-	117	-	-
P. vulgaris	3750	-	-	-
Y. enterocolitica	1875	7500	3750	3750
K. pneumoniae	-	1875	7500	7500

Balaji et al.(2006) investigated antimicrobial activity of hexane, ethyl acetate, acetone, methanol extracts of *R. montagnei* lichen and it was suggested that methanolic extracts of lichen had antimicrobial activity against *K. pneumoniae*, *P. vulgaris*, *Salmonella typhi* ve *C. albicans*. Logesh et al. (2012) examined antimicrobial activity of chitosan which isolated from *R. montagnei* thallus. Chitosan had antimicrobial activity against *Vibrio cholerae* and *E. coli* at 100 µL concentration.

CONCLUSION

The results of present investigation clearly indicate that the antibacterial activity vary with the species of the plants and plant material used. Lichen extract tested possess compounds with antibacterial properties which require further studies to determine antibacterial agents for therapy of infectious diseases in human and plant diseases. Therefore, studies about substances which responsible for antimicrobial activity in lichens should be expanded.

ACKNOWLEDGEMENT

This study was supported by Giresun University FEN-BAP-140411-16.

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How to cite this article:

Sinem AYDIN, Kadir KINALIOĞLU., The Investigation of Antibacterial Activities of Ethanol and Methanol Extracts of *Flavoparmelia caperata* (L.) Hale (*Parmeliaceae*) and *Roccella phycopsis* Ach. (*Roccellaceae*) Lichens Collected from Eastern Blacksea Region, Turkey. J App Pharm Sci. 2013; 3 (02): 143-147.