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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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 Kınaşoğlu. Vouchers are preserved in the herbarium of the Faculty of Science and Arts, Giresun University, Giresun.

Table 1: The collecting localities.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality Name</th>
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<tbody>
<tr>
<td><em>R. phycopsis</em></td>
<td>Giresun Center, Gedikkaya hill, 225 m</td>
</tr>
<tr>
<td><em>F. caperata</em></td>
<td>Giresun, Bulancak, Ahmetli village, 350 m</td>
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</table>

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 were used in the study as follows: _Enterococcus faecium_ (laboratory isolate), _Staphylococcus calnii_ (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 typhimurium_ 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. calnii_ (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 typhimurium_ 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 Molecular 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 separately by using a Soxhlet apparatus for 7 h at a temperature not exceeding the boiling point of the solvent, separately. 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 using the following equation: % Extract yield = (W₁ x 100) / W₂

W₁ shows the remaining solid lichen extract weight after evaporation of the solvent used in extraction, W₂ 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 separately. 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 10⁸ 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), separately. 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 _R. phycopsis_ and _F. caperata_ 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 separately. 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>
<thead>
<tr>
<th>Lichen</th>
<th>Extract</th>
<th>Extract Yield (%)</th>
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<tbody>
<tr>
<td>R. phycopsis</td>
<td>Ethanol</td>
<td>16.28</td>
</tr>
<tr>
<td>R. phycopsis</td>
<td>Methanol</td>
<td>19.38</td>
</tr>
<tr>
<td>F. caperata</td>
<td>Ethanol</td>
<td>12.34</td>
</tr>
<tr>
<td>F. caperata</td>
<td>Methanol</td>
<td>14.65</td>
</tr>
</tbody>
</table>

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 typhimurium 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 typhimurium, 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 typhimurium and P. vulgaris didn’t show any inhibition on exposure to methanol extract of R. phycopsis; S. cohnii, S. enterica serovar typhimurium, 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 typhimurium, 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 typhimurium 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 values of 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 H37Rv and M. tuberculosi H37Ra with an MIC of 250 µg/mL (Gupta et al., 2007). According to a study was conducted by Mitrović 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 typhimurium (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 typhimurium ATCC 14028. Using different lichen concentration against test bacteria and using different bacteria strains are the reasons of differences between Mitrović 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.
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 and C. albicans. Logesh et al. (2012) examined antimicrobial activity of chitosan which isolated from R. phycopsis and it was suggested that methanolic extracts of lichen had antimicrobial activity against methicillin-resistant Staphylococci. 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.

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REFERENCES


