GC- MS analysis and anticancer effect against MCF-7 and HT-29 cell lines and antioxidant, antimicrobial and wound healing activities of plant- derived compounds

Tahereh Valadbeigi[1*], Somayeh Rashki[1]

1. Department of Biology, Faculty of Science, Ilam University, Ilam, Iran

***Corresponding author:** Tel: +98 9126092197 Fax: +98 32227022
Address: School of Biology, College of Sciences, University of Ilam, Ilam, Iran
E-mail: tvaladbeigi@yahoo.com
Received; 2015/04/10 revised; 2015/05/2 accepted; 2015/06/12

Abstract

Introduction: Antioxidants are compounds that eliminate free radicals, which reduce tissue damage and allow the organs and blood vessels to properly heal. In this research, the anticancer effect against MCF-7 and HT-29 cell lines, and antioxidant, antimicrobial and wound healing activities of the lichens extracts have been investigated.
Materials and methods: Anticancer activity was assayed with standard MTT colorimetric procedure against MCF-7 and HT-29 cell lines. Antioxidant activity was studied by measuring DPPH, reducing power, total phenols and flavonoids assays. In vivo antimicrobial property and gas chromatography mass spectrometry analysis were evaluated.
Results: Metanolic extract of *Umbilicaria decussata* showed nearly 50 % HT-29 cell line inhibition at 200 µg/mL tested dose. *Protoparmeliopsis muralis* extract showed stronger antibacterial activity (MIC= 125 mg/mL) than *Fulgensia fulgens*. Also, it had a largest free radical scavenging activity (65.67%). The results revealed that there was a strong positive correlation between flavonoids and total phenolics (r= 0.952). In excision wound model, there was a significant reduction in both wound surface area and bacterial colony count in *F. fulgens* and especially *P. muralis* methanolic extracts. These significant activities are found due to the presence of unique compounds as Usnic acid, 8S,14-Cedrandiol and 3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol in *P. muralis* and 3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid and 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione in *F. fulgens*.
Conclusion: These results show that compounds as 3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid, 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione and usnic acid are responsible for accelerating the wound healing process by antimicrobial and antioxidant activities in these studied species.

Keywords: Antioxidant; DPPH, Excision wounds, HT-29, MTT

Introduction

Free radicals play an important role in many chemical processes in the cells, but they are also associated with unwanted side effects, causing cell damage (1). Synthetic antioxidants as butylated hydroxytoluene (BHT) and propyl gallate (PG) are used widely in the food industry because of their effectiveness and generally being less expensive than natural antioxidants. However, since synthetic antioxidants are often carcinogenic, finding natural substitutes is of great interest (2, 3). In search of new bioactive preparations of natural origin, lichens are the subject of many research teams.
Lichens are symbiotic association between fungi and algae. They synthesize various secondary metabolites (4-6). Those

sometimes make even more than 30% of the dry mass of thallus. They are unique with respect to those of higher plants (5). Theses extracellular secondary metabolites are produced by fungus alone and secreted onto the surface of lichens hyphal cell walls. 350 components are known from lichens and approximately 200 components have been characterized. These substances exhibit a great diversity of biological actions including antimicrobial, anti-inflammatory, analgesic, antipyretic and antiproliferative and cytotoxic activities (6).

In this study, we aimed to identify some biological active metabolites produced by lichens, using gas chromatography combined with mass spectrometry (GC-MS).

Materials and methods

Sample collection and extraction: Four lichen species as *Rhizoplaca chrysoleuca*, *Umbilicaria decussata*, *Prptoparmeliosis muralis* and *Fulgensia fulgens* were collected from Changooleh area in Ilam province (Iran) during the summer of 2014. Determination of the investigated taxa was accomplished using standard key (10). The lichen specimens were shade dried in a well-ventilated place at room temperature. Then fresh lichen material was milled by an electrical mill. Finely dry ground thalli of the studied lichens (50 g) were extracted by Soxhlet apparatus using methanol and distilled water as solvents. The extracts were filtered through Whatman No. 1 filter paper. Then, they were concentrated using a rotary evaporator at 30°C under reduced pressure. The resulting extracts were stored at −18°C until the analysis. Afterward they were dissolved in 5% dimethyl sulfoxide for the experiment (11).

GC-MS analysis: The GC/MS apparatus was Agilent technology 5975 system, capillary column of HP-5MS (30 m × 0.25 mm × 0.25 μm). The oven temperature program was initiated at 70 °C, held for 2 min, and then raised up to 300 °C at a rate of 10 °C /min (held for 10 min). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector temperature was 280 °C.

Identification of components: The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Interpretation of GC-MS was conducted using the database of NIST (National Institute of Standards and Technology) and Wiley 7n.1 mass computer library. The name, molecular weight and structure of the components of the test materials were ascertained.

Determination of anticancer effect: Sensitivities of MCF-7 and HT-29 cells to the lichen extracts were determined individually by the MTT (microculture tetrazolium test) colorimetric assay. The plates including extract (0.2%, and 0.5%) were incubated at 37°C for 48 hours and the MTT assay was performed according to the manufacturer's protocol (12). Dimethyl sulfoxide (DMSO) served as a solvent control. Cells were then treated with MTT reagent (20 μl/well) at 37°C for 4h and then DMSO (200 μl) was added to each well to dissolve the formazan crystals. OD (optical density) was recorded at 492 nm in a microplate reader. Percentage of residual cell viability was determined as [1−(OD of treated cells/OD of control cells)]×100 (12).

Determination of antibacterial activity: The following bacteria were used as test organisms in this study: *Bacillus megaterium* (ATCC 13578), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25213), *S. epidermidis* (PTCC 2405), *Pseudomonas aeruginosa* (PTCC 1047) and *Shigella sonnei* (PTCC 1235). All the used bacteria were obtained from the stock culture of Microbiology Research Laboratory in Shahid Beheshti University (Iran). The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the broth microdilution

method with using 96–well micro–titer plates as described previously (13). A series of dilutions with concentrations ranging from 63 to 1000 mg mL–1 for extracts were used in the experiment against every microorganism tested. Streptomycin, meticilin, vancomycin, gentamicin and clindamycin were used as positive controls. A DMSO solution was used as a negative control for the solvents influence. All assays were done in triplicate.

Determination of in vitro antioxidant activity: Hydrogen donating ability of the extracts was determined by using a method based on the reduction of a methanolic solution of the colored free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl; 0.05 mg mL–1) to the non-radical form (15, 16). Briefly, stock solutions of the extracts (0.2, 0.5, 1 and 1.5 mg mL–1) were prepared in distilled water and methanol, respectively. The synthetic standard antioxidant butylated hydroxytoluene (BHT) and ascorbic acid (10 mg mL–1) were prepared in dehydrated alcohol and water, respectively. Dilutions were made to obtain concentrations ranging from 0.3 to 3.0 mg/mL. The diluted solution was mixed with 0.08 mL of freshly prepared 0.24 mol/L DPPH-methanol solution. The solution was then kept for 30 min in dark at room temperature for its reaction. The absorbance of the solutions was determined by a UV-Vis spectrophotometer at 517 nm. The percentage inhibition of free-radical DPPH was calculated using the following formula (14): $\text{Inhibition } (\%)=[(A_{blank}-A_{sample})/A_{blank}] \times 100$

Where A_{blank} was the absorbance of the control reaction (containing all reagents except the test compound); and A_{sample} was the absorbance of the test compound (15).

Assay of Reducing Power: The procedure was applied by the methods of Meda et al. and Tevfik et al. (16, 17).

Total phenolic content assay: Total phenol content of the extract was determined by the method of Folin–Ciocalteu (18).

Determination of total flavonoid content: The total flavonoid content of the extract was determined according to the method of Meda et al. (16).

Wound Healing activity

Experimental Animals: Male Wistar albino rats (with weigh around 220 ± 5 g) were obtained from the animal house of the Faculty of Veterinary Medicine at Shahid Beheshti University, Iran. All of them were kept at controlled light condition (light: dark, 12:12 hr) and temperature 22± 1º C (17). Authors followed the institutional and national guide for the care and experiments on laboratory animals.

Infliction and infection of wounds: Excision wound (5 X 2 cm) was created as described previously (17). Rats were randomly divided into two groups of 40 rats each. Then wounds were infected with a microorganism [*S. epidermidis* (PTCC2405) and *P. aeruginosa* (PTCC1047) separately]. Each of the two groups by the random divided into four another groups and 10 rats per group. Group I: rats were considered as the control (without any treatment); Group II: rats were treated with tetracycline ointment (Standard); Group III: rats were treated with 5% ointment of methanolic extract; group IV: rats were treated with 10% ointment of methanolic extract. In group III and IV methanolic extract of *P. muralis* was mixed with ointment base (Vaseline 100% pure petroleum jelly) in a concentration of 5% and 10% respectively. The wound contraction was studied by tracing the raw wound area subsequently on day 1, 3, 5, 7, 9 and 11 for all the groups using the graph paper (17). Colony count (CFU mL–1) was done with an automatic Colony Counter (Countermat–Flash IUL–Instruments, Barcelona).

Statistical analysis

All the grouped data were analyzed by SPSS V.21 software. The data were analyzed by one way analysis of variance (ANOVA). P values < 0.05 were considered as statistically significant. Pearson's bivariate correlation test was carried out to calculate correlation coefficients (r) between the content of total phenolic and the DPPH radical scavenging activity. All the results were expressed as mean ±SD for three experiments in each.

Results

GC-MS analysis revealed the presence of 32 and 24 compounds in methanol extracts of *P. muralis* and *F. fulgens*, respectively (Table 1).

Table 1. Major components identified in the methanol extracts of the studied lichens.

Protoparmeliopsis muralis				
RT*	**Compound name**	**Area %**	**Molecular formula**	**Weight**
17.049	9,12-Octadecadienoic acid (Z,Z)-	2.07	$C_{18}H_{32}O_2$	280.44
17.094	6-Octadecenoic acid	4.35	$C_{18}H_{34}O_2$	282.46
21.598	Phosphonic acid, methyl-, monomethyl ester	2.14	CB41214592	124.07
22.078	Usnic acid	**16.60**	$C_{18}H_{16}O_7$	344.32
22.216	Cedran-diol, 8S,14-	**34.38**	$C_{15}H_{26}O_2$	238.36
23.572	3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol	**6.26**	C12H18O	178.27
25.918	9,10-Dimethylenetricyclo[4.2.1.1(2,5)]decane	4.46	$C_{12}H_{16}$	160.25
32.292	1H-Isoindole-1,3(2H)-dione, 2-buty l-4,5,6,7-tetrahydro-	3.32	$C_{12}H_{17}NO_2$	207.26
Fulgensia fulgens				
RT	**Compound name**	**Area%**	**Molecular formula**	**Weight**
22.261	Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha., 6.beta.,7.alpha.,9.alpha.)-	**36.34**	$C_{15}H_{24}$	204.35
23.348	3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid	**13.85**	$C_{14}H_7ClN_2O_5$	318.66
23.509	3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione	**9.60**	$C_{20}H_{10}Cl_2O_3$	369.19
23.611	Isoquinoline, 1,2,3,4-tetrahydro-7 -methoxy-2-methyl-8-(phenylmethoxy)-	6.86	$C_{18}H_{21}NO_2$	283.36
23.806	7-Chloro-2,3-dihydro-5-phenyl-3-[(pyrrolyl-2)methylene]-1H-1,4-benzo diazepin-2-one	2.75	$C_{20}H_{15}ClN_3O$	348.81
24.567	2,4-Dinitrophenyl hydrazone of 4-trichloromethylbenzaldehyde	6.33	$C_{14}H_9Cl_3N_4O_4$	403.60
32.400	4-Cyclohexene-1,2-dicarboximide, N -butyl-, cis-	6.33	$C_{12}H_{17}NO_2$	207.26
32.441	4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine	3.28	$C_{16}H_{14}N_2O_4$	298.29

* Retention Time

Anticancer activity: Screening of methanolic and aquatic extracts of three studied lichens (*R. chrysoleuca*, *P. muralis* and *F. fulgens*) resulted in no anticancer activities against MCF-7 and HT-29 cell lines. Only *U. decussata* showed nearly 50 % HT-29 cell line inhibition at 200 µg/mL tested dose.

Antimicrobial activity: The methanol extract of the two species *R. chrysoleuca* and *U. decussata* and all aqueous extracts of the investigated lichens were found to be inactive against all tested bacteria ($p > 0.05$). The antibacterial activity of the two other lichens (*P. muralis* and *F. fulgens*) and their main compounds against the tested microorganisms were estimated on the basis of the presence or absences of inhibitory zones, in the results were depicted in Table 2.

Among compounds of *F. fulgens*, 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione had strong antimicrobial activity than others and maximum activity was demonstrated against *S. aureus* (23mm) and *E. coli* (28mm). Among major compounds of *P. muralis*, usnic acid had strong antimicrobial activity. Maximum activity was demonstrated against *S. aureus* (34mm) and *E. coli* (34mm) (Table 2).

Table 2. Antibacterial activity of the lichen extracts and their main compounds.

	Mean (average) inhibition zone [mm][a]					
	P. aeruginosa	E. coli	S. sonnei	S. epidermidis	B. megaterium	S. aureus
Extracts[b] & main compounds[c]						
F	16.01± 0.31	20.11± 0.61	19.32± 0.4	13.61± 0.5	(–)	23.64± 0.1
F- compounds						
T	(–)	(–)	(–)	(–)	(–)	(–)
C	8±0.21	10±0.18	8±0.49	11±0.19	(–)	12±0.51
D	20.8±0.12	28±017	18±0.9	14±0.43	(–)	23±0.18
P	18.98± 0.21	31.71±1	23.88±1	33.1±0.57	18.91±89	31.01±0.57
P- compounds						
U	22±0.84	34±0.91	18±0.49	32±0.75	28±0.70	34±0.81
Ced	14±0.38	18±0.50	16±0.99	12±00.15	12±0.76	10±0.65
Dime	10±0.5	(–)	12±0.87	11±0.81	8±0.19	(–)
Standard[d]						
Meticillin	12.1± 0	8 ± 0	8± 0	16± 01	7± 06	7± 02
Clindamycin	7.2± 0	7.1± 0.1	7.78± 0	9.33± 0.57	8.33± 0.75	8.66± 0.57
Streptomycin	7.1± 0	7± 0.2	13.1± 0	12.33± 0.57	17.33± 0.75	8± 0
Vancomycin	9.4± 0	7.1± 0	7.56± 0	20± 0	14± 1	8± 0
Gentamicin	6.8± 0	18± 0	7± 0	20± 1	22± 1	19.33± 0.57

Note: The values are mean ±SD of three determinations. [a]Includes diameter of disc (6 mm). [b]tested at a concentration of 35 mL/disk. [c]tested at a concentration of 15 mL/disk. [d]tested at a concentration of 10 mL/disk. (–) No inhibition detected. *F*: *Fulgensia fulgens*. *P Protoparmeliopsis muralis*. T: Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha., 6.beta.,7.alpha.,9.alpha.)-. C: 3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid. D: 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione. U: Usnic acid. Ced: Cedran-diol, 8S,14-. Dime: 3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol.

The methanol extract of *P. muralis* had strong antimicrobial activity. Among all the tested gram positive bacteria minimum activity was observed against *B. megaterium* (18.91 mm, MIC=500, MBC=500) and maximum activity was demonstrated against *S. epidermidis* (33.1 mm, MIC=125, MBC=250). While among the examined gram negative bacteria the minimum and maximum activity was showed against *P. aeruginosa* (18.98 mm, MIC=500, MBC=500) and *E. coli* (31.71 mm, MIC=500, MBC=1000), respectively .

The methanol extract of *F. fulgens* showed a good antimicrobial activity. Among all the tested gram positive bacteria no activity was observed against *B. megaterium* and maximum activity was demonstrated against *S. aureus* (23.64 mm, MIC=500, MBC=500). While among the examined gram negative bacteria, the minimum and maximum activity was showed against *P. aeruginosa* (16.01 mm, MIC=500, MBC=500) and *E. coli* (20.11 mm, MIC=500, MBC=500), respectively.

Antioxidant activity: In this study, no significant antioxidant activity for methanol extract of the two species *R. chrysoleuca* and *U. decussata* and all aqueous extracts of the studied lichens were observed ($p > 0.05$). The scavenging DPPH radical of the lichen extracts *P. muralis* and *F. fulgens* are shown in figure 1-B. Among compounds of *F. fulgens*, 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione showed stronger DPPH radicals and reducing power activity than other compounds. Also, usnic acid showed strong DPPH radicals scavenging activity in *P. muralis*.

Increasing the concentrations of the extract from 0.2 to 1.5 mg/ mL caused increased antioxidative capacity of the reaction mixtures. Also there was a statistically significant difference between extracts and control ($P<0.05$). Extract from *P. muralis* showed larger DPPH radical- scavenging activities (65.57%) than other samples. In other word, the lichen *F. fulgens* showed a weaker DPPH radical scavenging activity (28.93%) (Table 3). The results of the reducing power assay of lichen extracts are summarized in Figure 1A.

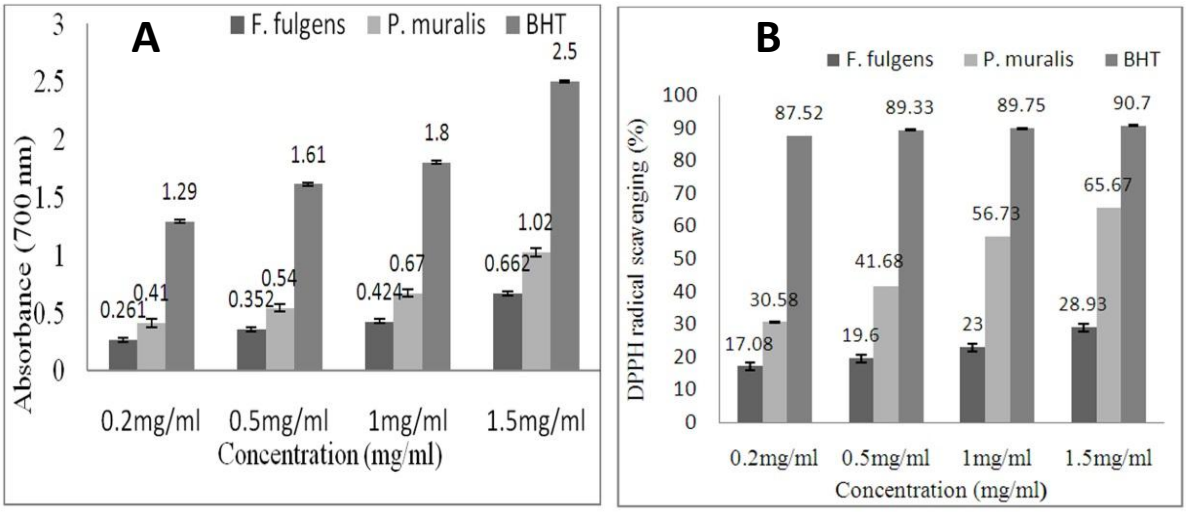

Figure 1. (A): Reducing power and (B): DPPH radical scavenging of the extract of lichens *Protoparmeliopsis muralis* and *Fulgensia fulgens* and BHT.

The amount of total phenolic compounds was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph (y=0.0021x – 0.0092, R2=0.9934). So that high absorbance indicates high reducing power. Measured values of absorbance varied from 0.2 (concentration 0.2 mg mL–1 extract of *F. fulgens*) to 1.02 (concentration 1.5 mg mL–1 extract of *P. muralis*).

Results of the study showed that the phenolic compound of the extracts F. fulgens and P. muralis varied from 29.90 to 91.80 µg of pyrocatechol equivalent. Highest phenolic compound was identified in methanol extract of P. muralis (91.80 µg of pyrocatechol equivalent in 1.5 mg mL–1 concentration). Although, the high phenolic content was identified in methanol extract of F. fulgens (43.71 µg of pyrocatechol equivalent in mg mL–1 concentration). Correlation coefficient between the phenolic compound content of the tested extracts and free radical scavenging activity was r= 0.952.

Table 3. Total Phenol (mg GAEs/g dry wt.) and flavonoid (mg QEs/g dry wt.) content of different lichens extracts and their main compounds.

Lichen species	***Protoparmeliopsis muralis***				***Fulgensia fulgens***			
Conc. (mg/mL)	0.2	0.5	1	1.5	0.2	0.5	1	1.5
Flavonoid	33.54± 0.01	43.86± 0.01	74.78± 0.07	99.27± 0.06	39.61± 0.01	93.152± 0.08	97.12± 0.06	104.19± 0.07
Phenol	61.80± 0.04	69.42± 0.03	74.66± 0.09	91.80± 0.01	29.90± 0.09	33.71± 0.01	39.42± 0.08	43.71± 0.01

	Protoparmeliopsis muralis			***Fulgensia fulgens***		
Compound	U	Ced	Dime	T	C	D
Conc. (mg/mL)	0.5	0.5	0.5	0.5	0.5	0.5
Flavonoid	196.1± 0.01	98.21± 0.01	(-)	45.71± 0.03	19.321± 0.05	218.61± 0.05
Phenol	185.0± 0.09	90.54± 0.05	(-)	64.14± 0.06	26.189± 0.09	61.71± 0.08

Note: The values are mean ±SD of three determinations. T: Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha., 6.beta.,7.alpha.,9.alpha.)-. C: 3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid. D: 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione. U: Usnic acid. Ced: Cedran-diol, 8S,14-. Dime: 3, 9-Dimethyltricyclo [4.2.1.1(2,5)] dec-3-en-9-ol.

Table 4. Wound healing activity (%) of lichens extracts and their main components on rats.

Extracts and compounds	Wound healing (%)			
	wound infected with S. epidermidis			
	5th	7th	9th	11th
P(5%)[a]	16	37	97	100
P (10%)[b]	36	51	98	100
P- compounds				
U	35	56	68	82
Ced	30	48	72	80
Dime	(-)	(-)	26	35
F (5%)	61	76	96	100
F (10%)	66	87	97	100
F- compounds				
T	(-)	(-)	(-)	(-)
C	50	68	72	80
D	(-)	9	23	29
	wound infected with P. aerogens			
P(5%)	48	68	71	89.46
P(10%)	62	75	94	100
P- compounds				
U	45	70	83	91
Ced	43	68	76	80
Dime	(-)	11	22	28
F (5%)	36	74	75	80.37
F (10%)	48	81	86	89.46
F- compounds				
T	(-)	(-)	(-)	(-)
C	31	50	57	68
D	(-)	(-)	15	21

Note: [a]Ointment with 5% of lichen extract. [b]Ointment with 10% of lichen extract. *F*: *Fulgensia fulgens*. *P Protoparmeliopsis muralis*. T: Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha., 6.beta.,7.alpha.,9.alpha.)-. C: 3-[5-(2-Chloro-5-nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid. D: 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione. U: Usnic acid. Ced: Cedran-diol, 8S,14-. Dime: 3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol.

As shown in Table 3, excellent flavonoid content was found in extracts of F. fulgens (104.19 μg of rutin equivalent in 1.5 mg mL–1 concentration). Also good flavonoid content was found in the methanol extract of P. muralis (99.27 μg of rutin equivalent in 1.5 mg mL–1 concentration). These two tested extracts exhibited the highest radical scavenging activity and ferric reducing power with the greatest amount of phenolic content.

Wound healing activity of compounds: Among compounds of F. fulgens, 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione had strong wound healing activity than others. Also usnic acid, 8S,14-Cedrandiol and 3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol showed wound healing property in P. muralis (Table 4).

Wound healing activity of methanolic extract *P. muralis* on wound infected with *S. epidermidis*: A very rapid closure of the wound (100%) in both treated groups (groups with the different concentrations as 5% and 10% of the lichen extract) observed (figure 2-D). There was a statistically significant difference between the treatment and control groups during this study ($p<0.05$). In all of the extract treatment groups and standard group, wounds completely were healed on 11th day. The result of colony count showed that antibacterial activity significantly was increased with increasing concentration of extract. So that found no traces of the bacteria on the wound in the groups treated with ointment 5% and 10% on the tenth and eighth days respectively ($p < 0.05$). Also on 10th day there were not any *S. epidermidis* colonies in standard group.

Wound healing activity of methanolic extract *P. muralis* on wound infected with *P. aeruginosa*: A better healing pattern with complete wound closure was observed in treated group with ointment 10% comparing to the control and ointment 5% groups (Figure 2A). On 11th day wound completely healed in standard

and extract 10% groups. In extract 5%, on 11th day, percentage of wound healing was 89.46. The result of colony count showed that there was a significant antibacterial activity in groups treated with lichen extracts, standard and control ($p < 0.05$). So that found no traces of the bacteria on the wound treated with extracts 5% and 10% and also standard group on 10th day.

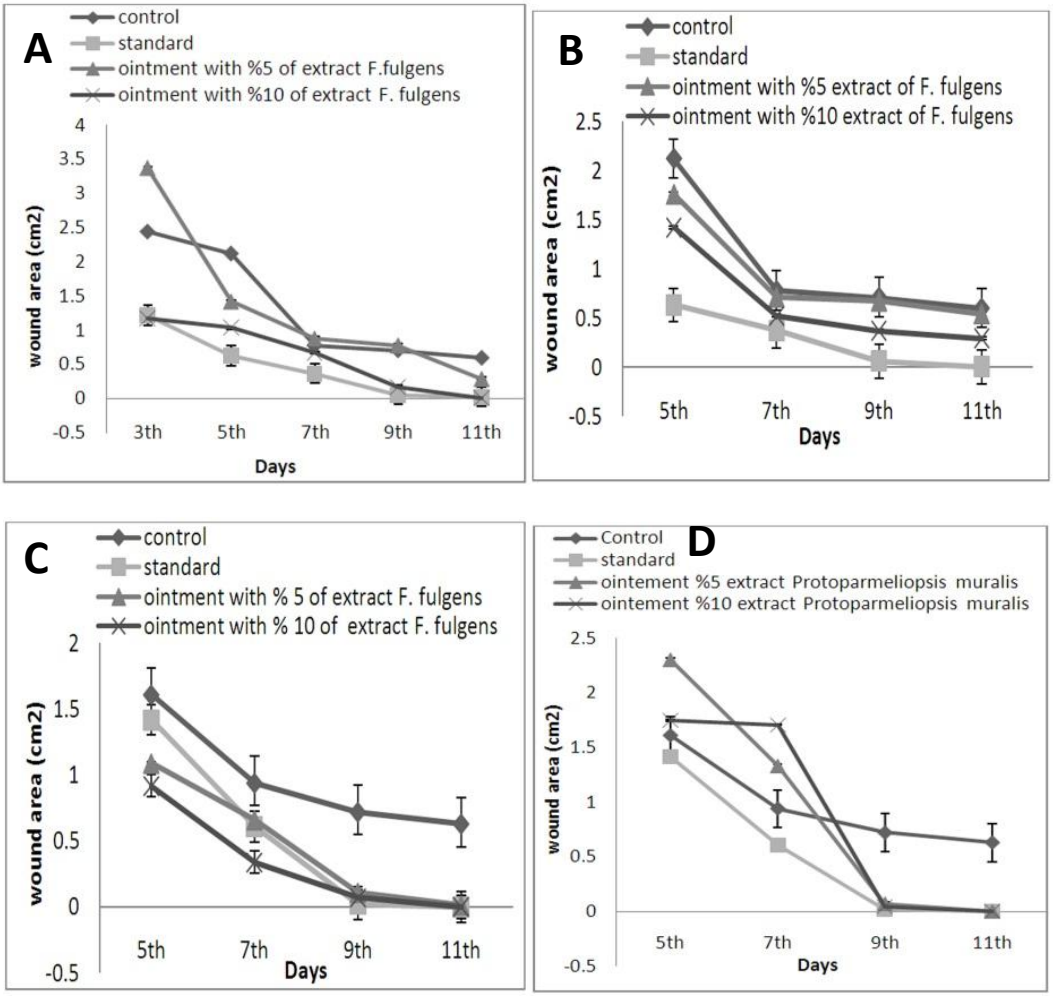

Figure 2. Comparative mean wound area of different groups in wound infected with (A, B): *Staphylococcus epidermidis* and (C, D): *Pseudomonas aeruginosa.*

Wound healing activity of methanolic extract *F. fulgens* on wound infected with *S. epidermidis*: The significant increase in the wound–healing activity was observed in the groups treated with the extract compared to control group. So that, wound in groups treated with ointment 5% and 10% was completely healed on 11th day (figure 2-C). The result of colony count showed that complete loss of the bacteria was found in both concentrations of the lichen extracts and also standard group on 11th day.

Wound healing activity of methanolic extract *F. fulgens* on wound infected with *P. aeruginosa*: On day 11, the wound size was significantly decreased in treated group with extract 10% (11.12% more than control group) (figure 2-B). So that the activity wound healing of ointment 10% was much faster than control and ointment 5% groups. In other word, percentage of wound healing was calculated at 80.37% and 89.46% respectively in the treated groups with ointment 5% and 10%. The results of

cultured microbial colony count obtained from the wound, showed significant antibacterial activity between treated and control groups ($p < 0.05$). On day 11, no colony in standard group and 80 and 23 colonies in treatment groups with the extract 5% and 10% were demonstrated, respectively.

Discussion

Infection is a factor that can raise the level of free radicals and ROS. During the last years, there has been growing evidence for the persistency of chronic wounds which is caused by bacterial infections in many cases (18). Among microorganisms, *S. epidermidis* and *P. aeruginosa* are the major human pathogens which are present in high amounts that cause significantly impaired wound healing in their infected wounds (19-21). So, in this study healing the wounds infected by these two bacteria were investigated. Our result of wound healing activity indicating that both extracts concentrations of *P. muralis* and *F. fulgens* were showing 100 % *S. epidermidis* infected– wound healing property on 11th day. Also on 11th day or before it (on 10th day in extract 5% and on 8th day in extract 10% *P. muralis*) there were not showed colonies of *S. epidermidis* bacteria. On 11th and 13th days (13th day not shown in Figures) *P. aeruginosa* infected–wound size completely reduced by the extract 10 % and 5% of the lichen *P. muralis* respectively. Also on 10th day complete loss of the bacteria was found in these both concentrations of the lichen extracts. Just in wounds infected by *P. aeruginosa* did not show complete reduction of the wound size and bacteria. In other word there was a directly related between the number of colonies and wound area. So that the size of the wound area was reduced by reducing the number of colonies and wound fully recovered by complete loss the bacteria.

According to Ranković et al. (13) and Celenza et al. (8), the lichens inhibit mostly gram-positive bacteria, but from these results it could be concluded that gram–positive bacteria significantly were inhibited as gram–negative. Our result showed that standard antibiotics significantly had less activity than the lichen extracts of *P. muralis* and F. *fulgens* and their main compounds (Table 2). Differences in biological activity of different species of lichens are probably a consequence of the presence of different components with biological activity (13-15). Anticancer activity of various lichens components are known, such as: usnic acid, lecanoric acid, gyrophoric acid, salazinic acid, lobaric acid, evernic acid, vulpinic acid and protolichesterinic acid (2, 7, 12, 13).

In our research, the investigated lichen extracts show a relatively strong antioxidant activity but the antioxidant activity of their component (e.g. usnic acid and 3-[2, 4-Dichlorophenyl]-1-hydroxy-9, 10-anthracenedione in this study) was much stronger. This means that lichen components are responsible for the antioxidant activity of lichens. Several researches found a high correlation between antioxidant activities and phenolic content (13, 14, 17). Interestingly, Kosanic et al. (2) reported that the antioxidant activity of different lichens may also depend on other, non-phenol components.

In general, lichens are rich in chemical compounds; their properties clearly indicate their potential for pharmaceutical purposes, although some properties of lichens still need consideration. Further studies are still required to clarify the molecular processes and signaling pathways involved in their activity.

Conclusions

Unique compounds as Usnic acid, 8S,14-Cedrandiol and 3,9-Dimethyltricyclo[4.2.1.1(2,5)] dec-3-en-9-ol in *P. muralis* and 3-[5-(2-Chloro-5-

nitro-phenyl)-furan-2-yl]-2-cyano-acrylic acid and 3-[2,4-Dichlorophenyl]-1-hydroxy-9,10-anthracenedione in *F. fulgens* decrease the necessary duration of complete wound healing by antimicrobial and antioxidant activities and can be replaced the chemical antibiotics. Also, the lichen *U. decussatacan* be a potential candidate for the development of anticancer agent. Further, toxicity studies and isolation of active principles from the lichen extract is to be carried out.

References

1. Otzurk S, Guvenc S, Arikan N, Yylmaz O. Effect of usnic acid on mitotic index in root tips of *Allium cepa* L. Lagascalia. 1999; 21: 47-52.
2. Kosanic M, Manojlovic N, Jankovic S, Stanojkovic T, Rankovic B. *Evernia*. *prunastri* and *Pseudoevernia*. *furfuraceae* lichens and their major metabolites as antioxidant, antimicrobial and anticancer agents. Food Chem Toxicol. 2013; 53: 112-8.
3. Pompilio A, Pomponio S, Di Vincenzo V, Crocetta V, Nicoletti M, Piovano M, et al. Antimicrobial and antibiofilm activity of secondary metabolites of lichens against methicillin-resistant *Staphylococcus aureus* strains from cystic fibrosis patients. Future Microbiol. 2013; 8(2): 281-92 .
4. Boustie J, Grube M. Lichens: a promising source of bioactive secondary metabolites. Plant Genet Resour. 2005; 3: 273-87.
5. Kosanić M, Ranković B. Antibacterial and antifungal activity of different lichens extracts and lichen acid. Res J Biotechnol. 2011; 6: 23-6.
6. Sati SC, Joshi S. Antibacterial activity of the Himalayan lichen *Parmotrema nilgherrense* extracts. Br Microbiol Res J. 2011; 1(2): 26-32.
7. Backorova M, Jendzelovsky R, Kello M, Backor M, Mikes J, Fedorocko P. Lichen secondary metabolites are responsible for induction of apoptosis in HT-29 and A2780 human cancer cell lines. Toxicol In Vitro. 2012; 26: 462-8.
8. Celenza G, Segatore B, Setacci D, Bellio P, Brisdelli F, Piovano M, et al. In vitro antimicrobial activity of pannarin alone and in combination with antibiotics against methicillin-resistant *Staphylococcus aureus* clinical isolates. Phytomedicine. 2012: 19: 596-602.
9. Goel M, Dureja P, Rani A, Uniyal PL, Laatsch H. Isolation, characterization and antifungal activity of major constituents of the Himalayan lichen Parmelia reticulata Tayl. J Agric Food Chem. 2011: 59(6): 2299-307.
10. Purvis OW, James PW. *Acarospora* Massal. (1852). In: Purvis OW, Coppins BJ, Hawksworth DL, James PW, Moore DM, editors. The lichen flora of Great Britain and Ireland. 3rd ed. London/ Natural History Museum: British Lichen Society; 1992. P. 58-63.
11. Manjunatha BK, Patil HSR, Vidya SM, Kekuda TRP, Mukunda S, Divakar R. Studies on the antibacterial activity of *Mucuna monosperma* DC. Indian Drugs. 2006; 43: 150-2.
12. Ramachandran C, Nair PK, Alamo A, Cochrane CB, Escalon E, Melnick SJ. Anticancer effects of amooranin in human colon carcinoma cell line in vitro and in nude mice xenografts. Int J Cancer. 2006; 119(10): 2443-54.
13. Ranković BR, Kosanić MM, Stanojković TP. Antioxidant, antimicrobial and anticancer activity of the lichens *Cladonia furcata*, *Lecanora atra* and *Lecanora muralis*. BMC Complement Altern Med. 2011; 11: 97-8.
14. Talaz O, Gulcin I, Goksu S, Saracoglu N. Antioxidant activity of 5, 10–dihydroindeno [1, 2–b]indoles containing substituents on

dihydroindeno part. Bioorg Med Chem. 2009; 17(18): 6583 – 9.
15. Dorman HJ, Bachmayer O, Kosar M, Hiltunen R. Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. J Agric Food Chem. 2004; 52(4): 762-70.
16. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan Honey, as well as their radical scavenging activity. Food Chem. 2005; 91(3): 571-7.
17. Tevfik O, Ibrahim D, Huseyin A. Determination of antioxidant activities of various extracts and essential oil compositions of *Thymus praecox* subsp. skorpiliivar. Skorpilii Food Chem. 2011; 124(1): 58-64.
18. Allen–Gipson DS, Zimmerman MC, Zhang H, Castellanos G, O'Malley JK, Alvarex–Ramirez H, et al. Smoke extract impairs adenosine wound healing: Implications of smoke–generated reactive oxygen species. Am J Respir Cell Mol Biol. 2013; 48(5): 665-73.
19. Stojadinovic A, Carlson JW, Schultz GS, Davis TA, Elster EA. Topical advances in wound care. Gynecol Oncol. 2008; 111(2 Suppl): S70-80.
20. Morton JJ, Malone MH. Evaluation of vulnerary activity by an open wound procedure in rats. Arch Int Pharmacodyn Ther. 1972; 196(1):117-26.
21. Sudharshan SJ, Chinmaya A, Valleesha NC, Prashith Kekuda TR, Rajeshwara AN, Murthuza S. Central Nervous System (CNS) depressant and analgesic activity of methanolic extract of Drypetes roxburghii wall in experimental animal model. Res J Pharm Tech. 2009; 2(4): 854-7.