ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Efficacy of Extracts of Some Lichens for Potential Antibacterial Activity.

Lina Abuiraq[1], Ghassan Kanan[1], Mohammed Wedyan[2] and Ahmed El-Oqlah[1]*.

[1] Al Yarmouk University , Department of Biology, Irbid, Jordan.
[2] Al Hashmite University, Department of biology and Biotechnology, al zarqa, Jordan Corresponding author: Ahmed El-Oqlah , Al Yarmouk University , Department of Biology, Irbid, Jordan.

ABSTRACT

Lichens and lichen products have been used in traditional medicines for centuries. The lichens collected from various geographic regions in Jordan have remained unexplored for which this research has been conducted with an aim of testing the phytochemical and antimicrobial properties of lichens present there. Three lichen species were investigated in this study for potential antimicrobial activityand these are; *Xanthoria parietina*, *Physconia* sp., and *Tornabenia atlantica* again steleven bacterial species , namely *Staphylococcus aureus, S. epidermidis, Bacillus cereus, Staphylococcus lentus, Micrococcus luteus, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aeruginosa and, Serratia marcescens* bacterial species.The antibacterial activity of aqueous methanol and ethanol extracts was determined by agar disk diffusion and agar well diffusion method. Themethanol extracts were more active than the aqueous extracts for all 3 lichens studied. The lichen extracts were more active against Gram-positive bacteria than against Gram-negative bacteria. The efficacy of the fractions of *Tornabenia atlantica* crude extracts may be due to the presence of constituents such as usnic acid, diffractaic acid, protocetraric acid, alkaloids, and flavonoids.Generally the lichenextracts tested demonstrated antimicrobial effect which suggests a possibility of their use intreatment of various diseases caused by these and similar microorganisms.

Keywords: Lichens extract, antibacterial, secondary metabolites, Jordan

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Lichens are two or three different organisms living together symbiotically. The main partners in this symbiotic relationship are fungal and algal types, but sometimes cyanobacteria may present within the lichen's thallus (Nash, 1996; Rankovic, *et al*., 2007; Akpinar, 2009). This symbiotic relationship provides slow growing successful alliance between its organisms. In addition, enables the partners to tolerate stress conditions by producing protective secondary metabolites.This secondary metabolites serve as antimicrobial either by killing microbes (cidal agent) or inhibiting their growth (static agent)(Gomes, *et al*., 2002; Piovano, *et a*l., 2002; Suberu, 2004).

Lichens have potentials in medical exploration. Since ancient times, lichens were used for medical purposes. Studies have shown that Lichens secondary metabolites produced by the fungal partner alone,have significant antibacterial and antifungal activities (Elix, 1996; Huneck, 1999, 2001; Piovano, *et al.*, 2002; Tay, *et al*., 2004; Yilmaz, *et al*., 2005). (Huneck, 1999, 2001; Öztürk *et al*., 1999).While other organic compounds such as the primary metabolites are produced by either the lichen's algal or cyanobacterial partners (Lawrey, 1986; Richardson, 1988; Lawrey, 1989; Elix, 1996). Many researchers have investigated the antimicrobial activities of lichens extracts against many Gram positive, Gram negative bacteria and fungi (Türk, *et al*., 2003; Tay *et al*., 24; Yilmaz, *et al*., 2005).These extracts have shown antibiotic properties that may serve as valuable sources of antimicrobial agents for pharmaceutical industry in the near future (Lawrey, 1986, 1989; Richardson, 1988; Elix, 1996; Hunneck and Yoshimura, 1996; Sharnoff, 1997).

The main aim of this studyis to explore the potential antimicrobial (i.e. antibacterial) properties of lichen's natural products, or secondary metabolites, as alternatives to massively used synthetic chemicals This was achieved by address the antimicrobial activity of selected lichen species collected from various geographic regions in Jordan and exploring its mechanism of action.

MATERIALS AND METHODS

Lichen materials

Three lichen materials were collected various geographic regions in Jordan . The specimens were provisionally identified as *Xanthoria parietina, Physconia sp.,* and *Tornabenia atlantica*. Specimens of the samples are stored at the Herbarium of Department of Biology, Yarmouk University, Jordan.

Microorganisms

Bacteria listed below were obtained from the stock culture of Microbiology Research Laboratory,Department of Biology, Yarmouk University. Five Gram-positive bacteria *Bacillus cereus, Micrococcus luteus, Staphylococcus aureus,Staphylococcusepidermidis, and Staphylococcus lentus* and six Gram-negative bacteria *Enterobacteraeruginosa,Escherichia coli, Klebsiellapneumonia, Pseudomonas aeruginosa,Salmonella typhimurium, and Serratiamarcescens*.. (El-Oqlah and Lahham, 1985; Lahham, and EL-Oqlah, 1986).

Preparation of lichen extracts

Lichen's material was dried in the shade, ground to a fine powder in liquid nitrogen. *Tornabenia atlantica*powder was then extracted by soaking either in methanol orabsolute ethanol for 72 hours, while *Xanthoria parietina*and *Physconia* sp. extractionswere by using soxhlet extraction apparatus, using either methanol or absolute ethanol as solvents (Ndukwe, *et al*. 2006). Solvents were then removed using rotary evaporator under reduced pressure at temperatures below 50˚C. The resulting crude extracts were stored at -20°C until assayed. Stock solutions and serial dilutions of extracts were prepared in dimethylsulphoxide (DMSO) (Ambrozin, *et al*. 2004). Antibiotics and DMSO were used as positive and negative controls respectively.

Fractionation of crude extracts

The crude extract (40 grams)were fractionated with a 1:1 ratio of water /dichloromethane (v/v). The resultant aqueous fraction was further extracted with dichloromethane, concentrated to dryness using rotary

evaporation and stored in sterile containers at 4°C until used. While the dichloromethane fraction was concentrated to dryness using rotary evaporation, and partitioned with a 1:1 ratio of n-hexane/90% methanol (v/v). The hexane and methanol fractions were then concentrated to dryness using rotary evaporation and kept in sterile containers at 4°C until used. Each fraction was dissolved in (v/v) dimethylsulphoxide (Souza-Fagundes, *et al*. 2002).

Screening of antibacterial activity

The bacterial suspension was smeared on nutrient agar media using sterile glass-rod, Wells of 6 mm in diameter were then made in the inoculated nutrient agar.Each well was then loaded with one offour different concentrations (100, 400, 800 and 1500 µg/ml) of each tested extract. Tetracycline at two different concentrations(250 µg/ml, 500 µg/ml) and DMSO were included as positive and negative controls, respectively. Inoculated plates were then incubated at 37º C for 20-22 h. The diameter of each resulting inhibition zone was measured in two directions at right angles to each other and that of the well was subtracted. Experiments were carried out in three replicates per treatment and each treatment was repeated at least twice (Ndukwe, etal. 2006).

Determination of MIC and IC50 values for tested extracts

To test the MIC of each crude extract, 50 µl of each overnight bacterial suspension grown in nutrient broth (NB) were added to 5 ml ofdifferent concentrations of crude extracts (100, 200, 400, 700, 1000, 1400, 1700, 2000, 2400, 2700 and 3000 µg/ml) prepared in NB as a diluent.Tubes were then incubated at 37˚ C for 24 h. Tubes were examined for visible signs of bacterial growth (turbidity) and the least concentration which inhibits the growth was considered as the MIC values.

In order to determine the IC_{50}values, the absorbance for each sample tested was read at 600 nm.The concentration which showed half-value absorbance of the control (minus extract) was considered as the IC_{50} value for that extract.

Phytochemical screening

The presence of several chemical compounds in the various fractions of extracts obtained from each of the ethanolic or methanolic crude extract of *Tornabenia* were screened by the chemical tests. Alkaloids (Singh and Kumar, 2011), flavonoids (Ighodaro*et al*., 2010),tannins (Ighodaro*et al*., 2010)and anthraquinones (Ighodaro*et al.*, 2010) were detected.

Bioautographic method using thin layerchromatography

The procedure described previously by white and James (1985) as modified by Orange and co-workers (2001).

RESULTS

Different concentrations (100, 400, 800 and 1500 µg/mL) of ethanolic and methanolic crude extracts obtained from three lichen species (*Xanthoria parietina; Physconia sp* and*Tornabenia atlantica*) were tested for their antibacterial activity to 10 different bacterial species using agar well diffusion method. In addition, liquid fractions (Aqueous, hexane and methanolic fractions) of ethanolic and methanolic crude extracts of the lichen *Tornabeniaatlantica* were also investigated

Sensitivity of the bacteria species to crude extracts and liquid fractions of three lichen species using agar well diffusion method (*In vitro*).

Results of the regression analysis for the relationship between size of bacteria species growth inhibition zone (mm) and the concentration (µg/mL) of lichens ethanolic and methanolic crude extract and their liquid fractions (Log values) are presented in Table 3.1. For all bacteria species, the obtained results indicated significant correlations (at the 0.05 level of significance) between tested concentrations of *Xanthoria parietina* ethanolic and methanolic crude extracts and the mean inhibition zones of bacteria species. However, there were significant correlations (at the 0.01 level of significance) between tested concentrations

ISSN: 0975-8585

Table 3.1: Sensitivity of the bacteria species to crude extracts and liquid fractions of three lichen species using agar well diffusion method.

Lichen species	Extract type	Concentration (µg/ml)	Inhibition Zone (mm) Mean ± SD							
			Bacillus cereus	*Serratia marcescens*	*Micrococcus lentus*	*Staphylococcus epidermis*	*Staphylococcus aureus*	*Staphylococcus lentus*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*
Xanthoria parietina	Ethanolic Crude	100 400 800 1500	5.0±1.0 9.33 ± 0.58 13.0±0.58 14.0±1.53	10.33±1.53 13.7 ± 0.58 15.0±1 17.0±1	5.33±.58 7.33±.58 8±1.0 10.7±1.53	5.3±1.15 9±1 9.7±0.58 11.3±1.15	6±1 8±1 10±1 12±0.58	0 0 6.3±1.53 9.3±1.53	ND ND ND ND	ND ND ND ND
	Methanolic Crude	100 400 800 1500	5.67 ± 0.58 10.33 ±0.58 14.33 ± 0.58 14.33 ± 1.15	7±1 14.3±0.58 14.7 ±1.53 15±1	6.7±1.53 9±1.0 11±1.0 12±1.0	6.3±.58 9.67±.58 13.67±1.15 13.67±1.15	6.3±0.58 7.3±0.58 8.3±0.58 10±1	0 5.33±0.58 7.33±0.58 9.33±0.58	ND ND ND ND	ND ND ND ND
Physconia sp	Ethanolic Crude	100 400 800 1500	6.33 ± 0.58 8.33 ± 0.57 9.33 ± 0.58 10.33 ± 0.58	6.33±0.58 8.33±0.58 14.0±1.0 17.0±1.0	10±1.0 17.7±0.58 18.3±0.58 19.3±0.58	5.3±.58 7.7±.58 8±1 10±1	6.3±.58 9.67±.58 11±1 11±1	5.33±0.58 7.67±0.58 11.33±0.58 11.67±1.15	ND ND ND ND	ND ND ND ND
	Methanolic Crude	100 400 800 1500	0.0 7.67 ± 0.578 8.33 ± 0.57 9.0 ± 1.0	5±1 7±1 8.3±.58 9.3±.58	0 ND ND ND	5.67±.58 7.3±.58 8.3±.58 9.67±.58	0 11.3±.58 12.7±.58 14±1	ND ND ND ND	ND ND ND ND	ND ND ND ND
Tornabenia atlantica	Ethanolic Crude	100 400 800 1500	6.33 ± 0.58 14.7 ±1.15 16.7 ± 0.58 16.7 ± 0.58	6.33±0.47 7.0±0.82 10.7±1.25 13.0±0.82	9.7±2.52 15.33±.58 15.7±1.53 17±1	7±1 9±1 10.3±0.58 11±1	7.77±0.58 12.33±0.58 15.3±0.58 15.3±0.58	0 4.67±0.58 7.33±0.58 7.67±1.155	0 0 6±1 7.3±0.58	6.33±4.62 12.33±0.58 13.7±0.58 14.33±0.58
	Methanolic Crude	100 400 800 1500	0.0 6.33 ± 1.53 7.67 ± 1.15 7.67 ± 1.15	7.3±.58 7.7 ±.58 13.3±.58 16.33±.58	6.7 ±.58 8±1 10.7 ±.58 12.3±.58	6.3±.58 7.3±.58 9.3±.58 10.67±1.15	ND ND ND ND	ND ND ND ND	ND ND ND ND	ND ND ND ND
Tornabenia atlantica	Aqueous fraction/ Ethanolic crude	100 400 800 1280	0.0 0.0 6.0 ± 0.71 7.0 ± 0.71	13.5±0.71 14±0.71 15.5±0.71 17.5±0.71	ND DN ND ND	ND ND ND ND	0 0 8.5±0.71 10.5±0.71	0 0 5±1.414 8.5±0.71	ND ND ND ND	12±1.41 13.5±0.71 13.5±0.71 14.5±0.71
	Aqueous fraction/ Methanolic crude	100 400 800 1280	6.0 ± 1.41 6.0 ± 1.41 7.0 ± 1.41 10.0 ± 1.41	12±1.41 13.5±0.71 16.5±0.71 16.5±0.71	ND DN ND ND	0 0 5.5±0.71 8±1.414	ND ND ND ND	0 0 0 8±1.414	5±1.41 6±1.41 8±1.41 10±1.41	8.5±0.71 10±1.41 12.5±0.71 13.5±0.71
	Hexane Fraction/ Ethanolic crude	100 400 800 1280	4.5 ± 0.7 5.5 ± 0.7 7.5 ±0.7 11.0 ± 1.41	14±1.41 15.5±0.71 16.5±0.71 19±1.41	ND ND ND ND	0 0 8.5±0.71 9.5±0.71	ND ND ND ND	ND ND ND ND	0 0 7.5±0.71 12±1.414	7.5±0.71 10.5±0.71 12.5±0.71 13.5±0.71
	Hexane fraction/ Methanolic crude	100 400 800 1280	6.0 ± 1.41 6.5 ± 0.7 8.0 ± 1.41 9.5 ± 2.1	15.5±0.71 17±1.41 17±1.41 18±1.41	ND ND ND ND	DN ND ND ND	ND ND ND ND	ND ND ND ND	ND ND ND ND	9.5±0.71 10±1.414 10±1.414 16±1.414
	Methanolic fraction/ Ethanolic	100 400 800 1280	7.5± 0.70 8.5 ± 0.70 10.5 ± 0.71 11.5 ± 2.12	11.5±0.71 13.5±0.71 15.5±0.71 17.5±0.71	ND ND ND ND	0 6.5±0.71 6.5±0.71 10±1.414	8±1.41 8.5±0.71 9.5±0.71 10.5±0.71	0 0 5±1.414 7±1.41	4.5±0.71 5.5±0.71 7.5±0.71 9.5±2.12	5.5±0.71 7±1.414 8.5±0.71 11±1.414

ISSN: 0975-8585

	Methanolic fraction/ Methanolic crude	100 400 800 1280	4.5 ± 0.7 5.0 ± 1.4 9.5 ±2.12 15.0 ± 1.4	13.5±0.71 14±1.41 16.5±0.71 16.5±0.71	ND ND ND ND	0 0 5.5±0.71 8±1.414	0 0 8±1.414 9.5±2.121	ND ND ND ND	0 0 8±1.414 9.5±0.71	5±1.414 6.5±2.121 7±2.83 10±1.414

ND: Not Done

Table 3.2: MIC and IC_{50} valuesof the crude extracts and liquid fractions of three lichen speciesagainst bacteria species

Lichen species	Extract type	*Bacillus cereus*		*Serratia marcescens*		*Micrococcus lentus*		*Staphylococcus epidermis*		*Staphylococcus aureus*		*Staphylococcus lentus*		*Klebsiella pneumonia*		*Pseudomonas aeruginosa*	
		MIC [µg/ml]	IC_{50}	MIC[µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}	MIC [µg/ml]	IC_{50}
Xanthoria parietina	Ethanolic Crude	2700	2000	2700	2000	2700	1400	2700	2000	1400	400	2000	200	ND	ND	ND	ND
	Methanolic Crude	400	200	1700	1000	2000	400	3000	2400	1000	200	2700	1400	ND	ND	ND	ND
Physconia sp	Ethanolic Crude	1400	400	700	200	1400	200	2000	1400	1000	200	1000	400	ND	ND	ND	ND
	Methanolic Crude	2000	1400	2400	1000	ND	ND	1700	1000	2000	1400	ND	ND	ND	ND	ND	ND
Tornabenia atlantica	Ethanolic Crude	700	400	2700	1700	2000	1700	1700	700	1700	1400	2000	1700	ND	ND	ND	ND
	Methanolic Crude	2700	1700	700	400	2700	1400	1000	200	ND	ND	ND	ND	2000	700	700	400

of *Physconia sp*ethanolic crude extract and generated inhibition zones , where the obtained correlation value (r-value) was 0.999 for *b. cereus* (P=0.000). In addition, there were significant correlations between tested concentrations of *Tornabenia atlantica* methanolic fraction (obtained from ethanolic crude extract) and inhibition zone. In contrast, there were no correlations between tested concentrations of the remaining extract type or fractions and the generated inhibition zones.

Depending on the generated MIC and IC_{50} values against bacteria species (Table 3.2), the tested lichens extracts and fractions were ranked in the following order; *Xanthoria parietina* methanolic extract <*Tornabenia atlantica*ethanolic extract <*Physconia sp*ethanolic extract <*Physconia* sp methanolic extract <*Tornabenia atlantica* methanolic extract <*Xanthoria parietina* ethanolic extract.

The sensitivity of tested bacteria species to the control antibiotic are shown in table 3.3.

Table3.3: Sensitivity of bacteria species to control antibiotic (Tetracycline).

Bacterial Species	Tetracycline concentration µg/mL	
	250*	500*
	inhibition zone (mm)	
Serratia marcescens	8	9
Staphylococcus aureus	11	14
Staphylococcus epidermis	8	9
Staphylococcus lentus	1	4
Bacillus cerrus	6	4
Micrococcus luteus	8	8
Escherichiacoli	4	9
Salmonella typhimurium	6	8
Pseudomonas aeruginosa	8	9
Enterobacter aeruginosa	5	7
Klebsiella pneumonia	5	9

Phytochemical screening

Results presented in table 3.4 , indicate that methanolic, and hexane fraction of ethanolic crude extracts and methanolic fractions of ethanolic crude extracts of *Tornabenia atlantica* showed a positive response in preliminary detection test of flavenoid. Moreover, Methanolic, and hexane fraction of ethanolic crude extracts and ethanolic fractions of methanolic crude extracts and hexane fraction of methanolic crude extract of *Tornabenia atlantica* showed a positive response in preliminary detection test of alkaloid. While no tanins and anthraquinones were presented in all fractions.

Table 3.4Phytochemical analysis for secondary metabolites in tested fractions of ethanolic and methanolic extracts of *Tornabenia atlantica.*

Ethanol Extract			Methanol Extract			
Hexane Fraction	Methanol Fraction	H_2O Fraction	Hexane Fraction	Methanol Fraction	H_2O Fraction	
-	+	+	+	+	-	Alkaloids
+	+	+	-	+	+	Flavonoids
-	-	-	-	-	-	Tannins
-	-	-	-	-	-	Anthraquinones

TLC-bioautography was usedto identify bioactive compounds of *Tornabenia atlantica* fractions (obtained from ethanolic and methanolic*Tornabenia atlantica* crude extract).Four solvents system (A, B, C, and D) were used to detect lichen spots. Interestingly, specific lichenic acids were detected as follow: solvent A detected usnic acid (Rf= 0.71) whilesolvent B detected diffractaic acid, and protocetraric acid (Rf= 0.25, and Rf= 0.10) and several unidentified spots were also detected (Santiago *et al*., 2010). Results are shown in tables (3.5, 3.6, 3.7 and 3.8)

ISSN: 0975-8585

Table**3.5**:**TLC profile of lichen species as detected using Solvent Systems A**

Sulfuric acid			U-V			After H_2O			Before H_2O			Extraction type A
Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	
–	–	–	–	–	–	–	–	–	–	–	–	*Tornabenia atlantica* ethanol H_2O fraction
–	–	–	–	–	–	–	0.48 0.73 0.73	3	Pale green	0.74	1	*Tornabenia atlantica* methanol H_2O fraction
Violet	0.49	5	Brown	0.83	4	–	0.12	5	Pale yellow	0.47	4	*Tornabenia atlantica* ethanol Hexane fraction
Black	0.72		Brown	0.91			0.47		Pale yellow	0.69		
Brown	0.77		Brown	0.98			0.50		Dark green	0.71		
Green	0.78		Pink	0.86			0.59		Yellow	0.95		
Dark green	0.95						0.67					
Violet	0.5	4	Yellow	0.54		–	0.48	1	Yellow	0.5	7	*Tornabenia atlantica* methanol Hexane fraction
Black	0.72		Pale brown	0.59					Pale green	0.67		
Dark red	0.75		Pale brown	0.60					Pale green	0.67		
Dark red	0.95		Pale brown	0.65					Yellow	0.71		
			Brown	0.86					Pale green	0.73		
			Brown	1					Dark green	0.74		
									Dark yelloo	0.95		
Violet	0.50	1	Pale brown	0.46	4	–	0.05	4	Pale green	0.73	2	*Tornabenia atlantica* ethanol Methanol fraction
			Pale brown	0.56			0.35		Yellow	0.75		
			Pale brown	0.68			0.47					
			Pale brown	0.83			0.71					
Violet	0.78	2	Pale brown	0.56	2	–	0.05	3	Yellow	0.47	4	*Tornabenia atlantica* methanol Methanol fraction
							0.34		Yellow	0.71		
Pale green	0.79		Pale brown	0.83			0.62		Pale green	0.72		
									Pale green	0.73		

Solvent A: Toluene / Dioxane / Acetic acid (180: 45: 5)

ISSN: 0975-8585

Table 3.6: TLC profile of lichen species as detected using Solvent Systems B

Solvent B: Hexane / Diethylether / Formic acid (130: 80: 20)

Sulfuric acid			U-V			After H_2O			Before H_2O			Extraction type B
Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	
–	–	–	–	–	–	–	–	–				*Tornabenia atlantic*a ethanol H_2O fraction
–	–	–	Dark blue	0.04	2	–	–	–	Yellow	0.21	31	*Tornabenia atlantica* methanol H_2O fraction
			Orange	0.52					Green	0.24		
									Green	0.27		
Dark green	0.25	4	Pale blue	0.39	2	–	0.33	6	Pale green	0.20	6	*Tornabenia atlantica* ethanol Hexane fraction
Pale green	0.26		Yellow	0.59			0.37		Green	0.22		
Brown	0.31						0.43		Green	0.24		
Brown to pink	0.37						0.47		Green	0.27		
							0.51		Green	0.28		
							0.63		Yellow	0.58		
Pale green	0.22	5	Pale blue	0.71	3	–	0.33	6	Pale green	0.21	5	*Tornabenia atlantica* methanol Hexane fraction
							0.41					
Pale green	0.23		Orange	0.43			0.47		Pale green	0.24		
Pale green	0.25		Violet	0.51			0.62		Dark green	0.25		
Green to Brown	0.27						0.86		Dark green	0.28		
brown	0.31						0.68		Yellow	0.94		
			Pale blue	0.38	2	–	0.03	3	Pale green	0.10	3	*Tornabenia atlantica* ethanol Methanol fraction
			Yellow	0.59			0.24		Green	0.17		
							0.28		Pale green	0.20		
			Pale orange	0.08	3	–	0.18	2	Pale green	0.24	3	*Tornabenia atlantica* methanol Methanol fraction
			Violet	0.17			0.40		Green	0.27		
			Yellow	0.56					Green	0.28		

ISSN: 0975-8585

Table 3.7: .TLC profile of lichen species as detected using Solvent Systems C

Sulfuric acid			U-V			After H_2O			Before H_2O			Extraction type C
Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of band	
–	–	–	–	–	–	–	–	–	–	–	–	*Tornabenia atlantica* ethanol H_2O fraction
–	–	–	–	–	–	–						*Tornabenia atlantica* methanol H_2O fraction
Black	0.24	4	Pale brown	0.19	5	–	0.64	2	Pale green	0.31	6	*Tornabenia atlantica* ethanol Hexane fraction
Violet	0.71		Brown	0.31			0.78		Pale green	0.39		
Brown	0.91		Brown	0.40					Green	0.45		
Violet	0.54		Yellow to pink	0.07					Green	0.55		
			Yellow	0.76					Yellow	0.82		
									Yellow	0.91		
Pale green	0.11	9	Brown	0.08	5	–	0.80	1	Pale green	0.07	5	*Tornabenia atlantica* methanol Hexane fraction
Pale green	0.20		Brown	0.17					Pale green	0.14		
Pale green	0.29		Brown	0.24					Pale green	0.21		
Green	0.36		Pink	0.62					Dark green	0.36		
Dark green	0.44		Pink	0.68					Dark green	0.41		
Violet	0.72											
Brown to pink	0.89											
Pale green	0.25											
green	0.52											
Violet	0.20	1	Brown	0.27	3	–			yellow	0.20	3	*Tornabenia atlantica* methanol Methanol fraction
									Yellow to green	0.31		
			Brown	0.18					Pale yellow	0.82		
			Brown	0.07								

Solvent C: Hexane / Methyl tetr-butyl ether / Formic acid (140: 72: 18)

ISSN: 0975-8585

Table 3.8:TLC profile of lichen species as detected using Solvent Systems D

Sulfuric acid			U-V			After H_2O			Before H_2O			Extraction type D
Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of bands	Color	RF	# of band	
–	–	–	–	–	–	–	–	–	–	–	–	*Tornabenia atlantica* ethanol H_2O fraction
–	–	–	–	–	–	–			Pale green	0.26	2	*Tornabenia atlantica* methanol H_2O fraction
									Pale green	0.28		
Pink	0.18	5	Black	0.46	2	–	0.36	2	Pale green	0.30	3	*Tornabenia atlantica* ethanol Hexane fraction
Dark green	0.31		Black	0.46			0.57		Dark green	0.31		
Pink	0.32								Yellow	0.54		
Pale orange	0.54											
Pale brown	0.90											
Brown	0.22	3	Brown	0.54		–	0.57	1	Yellow	0.21	5	*Tornabenia atlantica* methanol Hexane fraction
Dark green	0.31		Black	0.57					Pale green	0.29		
Pink to brown	0.36		Black	0.74					Dark green	0.30		
			Black	0.71					Dark green	0.31		
									Pale yellow	0.53		
Black	0.24	4				–	0.22	1	Pale green	0.25	4	*Tornabenia atlantica* ethanol Methanol fraction
Pale green	0.25								Pale green	0.28		
Pale green	0.28								Pale green	0.30		
Pale green	0.30								Pale yellow	0.61		
Black	0.24	4	Pale brown	0.40	7	–	0.05	3	yellow	0.23	4	*Tornabenia atlantica* methanol Methanol fraction
							0.34		Pale green	0.29		
Pale green	0.30		Black	0.46			0.62		Pale green	0.30		
Pale green	0.31		Black	0.46					Pale yellow	0.57		
Dark red	0.95		Pink	0.50								
			Pale black	0.53								
			Orange	0.59								
			Yellow	0.65								

Solvent D: Toluene / Acetic acid (170: 30)

DISCUSSION

Despite tremendous progress in human medicine, infectious disease caused by bacteria remain a major threat to public health. The impact of disease is great in developing countries because of the relative unavailability of medicines and the emergence of widespread drug resistance (Swathi *et al*., 2010) .

Plant product drugs have been employed since prehistoric times to treat human and animal diseases. Moreover, plants are known to produce bioactive molecules that inhibiting the growth of bacterial and fungal

species. Lichen which is a symbiotic association of fungi and algae, produces several metabolites (the lichen substances) such as: amino acid derivatives, sugar alcohols and aliphatic acids, quinones (Karagos*et al*., 2009). Antibiotic substances found in lichen were reportedfor the first time in 1944by Burkholder *et al*.. An increasing interest in the lichen secondary metabolite is emerging because of the wide spread of microbial resistance to antibiotics and the ineffectiveness of some previous drug. Secondary metabolites are compounds produced by the fungus part of lich (Sati and Joshi, 2011; Karthikaidevi *et al*., 2009). In this study, the antimicrobial activity of ethanolic and methanolic crude and fraction extract of *Tornabenia atlantica, Physconia sp., and Xanthoria parietina* were evaluated for their antibacterial activity using agar well diffusion and amended methods. According to antimicrobial activity of lichen extracts, it seems that inhibition zone depends on lichen extract, solvent used for extraction, method of extraction and bacteria in use (Mitrovic *et al*., 2011).

In this study,soxhlet extraction for*Xanthoria parietina* and *Physconia sp* and soaking extraction for*Tornabenia atlantica*were used.Soxhelt extraction improves the efficiency of extraction because samples are continually exposed to fresh solvent. Many researchers used soxhlet extraction for dried plant using organic solvents and the method worked well, taking in consideration that compounds being extracted can withstand the temperature of boiling solvent.. However, Soaking method with shaking give a high yield and bioactivity and important with thermolabile compounds that prolonged heating lead to degradation of compounds and this maybe that *Tornabenia atlantica* ethanolic crude extract is potent against bacteria.

Our results showed that themost potent crude extract affecting the growth of bacteria is *Tornabenia atlantica* ethanolic crude extract and the most potent fraction extract affecting the growth of bacteria was aqueous fraction of ethanolic *Tornabenia atlantica* crude extract. We also showed that the Gram negative bacteria *S.marcescens., K.pneumonia, E.coli, E.aeruginosa, S.typhimorium*, and *P.aeruginosa* were more resistant to the extract when compared to the gram positive bacteria used in this study (*B. cerrus, M.luteus, S.lentus, S.aureus and S.epidermis*). The reason of the difference in sensitivity between the two groups(Gram positive and negative)could be of morphological differences, difference in porousness of cell wall and the transparency of cell wall. The cell wall of gram positive bacteria ismade mainly of peptidoglycan (mureine) and teichoic acids while in gram negative bacteria the cell wall ismade mainly of peptidoglycan, lipopolysaccharide, and lipoprotein (Rankovic and Misic. 2008; Marijana *et al*., 2010). Biologically active components are believed to disturb cytoplasmic membrane and thereby facilitate influx of antibiotics (Stefanovic *et al*., 2009). Moreover, Lichen metabolites play a significant role in their bioactivity of various modern pharmaceutics and medicine (Mitrovic *et al*., 2011). Depending on the results, it was found that the antimicrobial activity of the extracts rest on the specie of bacteria being treated. Phytochemical constituents such as tannins, flavonids, alkaloids and several other compounds of plant was found to play a broad spectrum in antimicrobial activity (Singh and Kumar, 2011). Singh and Kumarin 2011found that,the bioactivity of the *Tornabenia atlantica* extracts is denoted to the presence of secondary metabolites such as alkaloids, and flavonoids. According to ourresults, it was found that inhibition zones of lichen extract increased drastically when the concentration of lichen extractincrease. Karagoz et al. in 2009 reported that Xanthoria parietina ethanolic extracts had no bioactivity effect on the growth of *B. subtillis*, *S. aureus*, *P. aeruginosa, S. epidermis*, *E. coli*, and *K. pneumonia*. Our results also showed that Xanthoria parietina ethanolic extracts had no bioactivity effect on *P. aeruginosa*, *E. coli* and *K. pneumonia* which is compatible with the results of Karagoz et al.. In the other hand our results showed that Xanthoria parietina ethanolic extracts inhibited the growth of *S. aureus* and *S. epidermis*(6±1, and 5.3± 1.15, respectively) at 100µg/mL concentration.

Determiningthebiologically active compounds from plants depends onthe type of solvent used in extraction. In this study, we found thatmethanolic extract of *Xanthoria parietina*has a betterbioactivity effect against bacteria. This refers to polarity of methanol that is more polar than ethanol andsolubilize polyphenolic compounds such as flavones and other bioactive compounds. Methanolisconsidered anorganic solvent that solve compounds and showed the ability to extract more chemical compounds and sapiens that play a significant role in antimicrobial activity (Ncube *et al*., 2008).. However, ethanolic extracts of *Tornabenia atlantica and Physconia* sp.were better than methanolic extracts against bacteria while ethanolic extracts of *Xanthoria parietina* and *Tornabenia atlantica* were better against fungus.

An antimicrobial agent with high activities against an organism yields a low MIC while a low activity against an organism has a high MIC value **(**Aboaba and Efuwape, 2001**;** Ncube *et al*., 2008**)**. In general,Our results indicate that the MIC for Gram positive bacteria was lower than the MIC forGram negative bacteria this could be related to the porousness of the cell wall, another explination could be thatGram negative bacteria like *E.coli* and

P.aeruginosa have efflux pump thatremove the harmfull substances out of the cell (Rankovic and Misic. 2008; Marijana *et al*., 2010**;** Poole *et al*., 1993**)**. However, we found that all bacteria responded to the extract and reached to the complete inhibition.

Phytochemical screening revealedthe presence of alkaloids and flavonoid (table 3.4). These are believed to be responsiblefor the observed antibacterial effects of Lichens extracts. Thishighlights the continuous interest in laboratoryscreening of medicinal lichens, not only to determinethe scientific rationale for their usage, but also todiscover new active ingredients.

The data obtained from this study demonstrated that flavonoids and alkaloids are among the chemical classes responsible for the antibacterial activity. Several authors have documented the antibacterial potency of flavonoids (Cowan, 1999; Koysomboon et at., 2006; Kuete et al., 2007a; Kuete et al., 2007b; Kuete et al., 2008). This activity may be due to its ability to complex with bacterial cell wall (Cowan, 1999) and thus inhibiting microbial growth. Though the lichens extract is used traditionally, the results this study showed that the fractions from *Tornabenia atlantica* could be used alone with good efficiency. TLC has been used to detect specific group of lichen products. (table3.5, 3.63.7, and 3.8). Moreover, usnic acid which is a very active lichen substance used in pharmaceutical preparation. Usnic acid, diffractaic acid, and protocetraric acid play a significant role in antibacterial activity (Mitrovic *et al*., 2011; Molnar and Farkas, 2010; Hanus *et al*., 2008). The activity of hexane fraction and methanolic fraction (obtained from methanolic crude extracts of *Tornabenia atlantica*) maybe refers to presence of usnic acid and diffractaic acid in. However, methanolic fraction (obtained from ethanolic crude extracts of *Tornabenia atlantica*) has protocetraric acid and this maybe responsible of bioactivity of the extract.

CONCLUSION

The results obtained in the present study can be concluded that Lichenshave broadspectrum of antibacterial potentiality. All the organic solvent extract of this lichenethanol and methanol possess significant inhibitory activity against the plant andanimal pathogenic bacteria. Hence, these lichens can be a potential source forevolving newer antibacterial compounds.

ACKNOWLEDGMENT

The authors wish to thank

REFERENCES

[1] Aboaba OO and Efuwape BM. Antibacterial properties of some Nigerian spices. *Biolo.Res Commun*, 2001;13:183 – 188.

[2] Akpinar A, Ozturk S and Sinirtas M. Effects of some terricolous lichens *Cladonia rangiformis*Hoffm.,*Peltigeraneckerii* Hepp ex Müll. Arg., *Peltigera rufescens* (Weiss) Humb.on soil bacteria in natural conditions. *Plant Soil Environ* 2009;55(4): 154:158.

[3] Ambrozin ARP, et al. Trypanocidal activity of *Meliaceae* and *Rutaceae* plant extracts. *Mem.Inst. Oswaldo. Cruz*. 2004;99: 227-231.

[4] Burkholder PR, Evans AW, McVeigh I, Thornton HK. Antibiotic activity of lichens.Proc. Natl. Acad. Sci., USA., 1944;30:250-55

[5] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12: 564-582.

[6] Elix JA. Biochemistry and secondary metabolites. In: *LichenBiology*, (Nash III T.H., ed.) *Cambridge University Press*, Cambridge, 1996;pp.154-181.

[7] El-Oqlah AA and Lahham JN. Lichens from the northern parts of Jordan.*Nova Hedwgia*. 1985;42 (1): 200-205.

[8] Gomes A, Honda N, Rose F, Muzzi R and Marques M. Bioactive derivatives obtained from lecanoric acid, a constituent of the lichen *Parmotrema tinctorum (Nyl.)*Hale (Parmeliaceae*).RevistaBrasileira de Farmacognosia*. 2002;12 :74-75.

[9] Hanus L, Temina M, and Dembitsky V. Biodiversity of the chemical constituents in the epiphytic lichenized ascomycete *Ramalina lacera* grown on difference substrates *Crataegus sinaicus*, *Pinus halepensis*, and *Quercus calliprions*. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2008;152 (2):203-208.

[10] Huneck S. The significance of lichens and their metabolites.*Naturwissenschaften*. 1999;86: 559-570.

[11] Huneck S. New results on the chemistry of lichensubstances. In: *Progress in the Chemistry of Organic Natural Products*, Herz, New York, 2001; 81: 224-230.
[12] Huneck S and Yoshimura I.Identification ofLichenSubstance*s.Springer*, Brelin. 1996;pp 113-123.
[13] Karagoz A, Dogruoz N, Zeybek Z, Aslan A. Antibacterial activity of some lichen extracts. *J.Med.plant.Res*. 2009;3 (12):1034: 1039.
[14] Koysomboon S, Altena IV, Kato S, Chantrapromma K. Antimycobacterial flavonoids from *Derris indica*. Phytochemistry 2006;67: 1034-1040.
[15] Kuete V, et al. Antimicrobial activity of the methanolic extract and of chemical constituents isolated from *Newbouldia laevis*. Pharmazie 2007;62: 552-556.
[16] Kuete V, Konga Simo I, Ngameni B, Bigoga DJ, Watchueng J, Nzesse Kapguep R, Etoa FX, Ngadjui TB, Penlap BV 2007a. Antimicrobial activity of the methanolic extract, fractions and four flavonoids from the twigs *of Dorstenia angusticornis* Engl. (Moraceae). J Ethnopharmacol 112: 271-277.
[17] Kuete V, et al. Antimicrobial activity of the crude extracts and compounds from *Ficus chlamydocarpa* and *Ficus cordata* (Moraceae). J Ethnopharmacol 2008;120: 17-24.
[18] Lahham JN and EL-Oqlah AA.Common lichens in Jordan.*J. Biol. Sci. Res*. 1986.
[19] Lawrey JD. Biological role of lichen substances.*Byrologist.* 1986;89: 111-122.
[20] Lawrey JD. Lichen secondary compounds: evidence for a correspondence between antiherbivore and antimicrobial function. *Bryologist*. 1989;92: 326-328.
[21] Marijana, K., Branislav, R., and Slobodan, S. 2010. Antimicrobial activity of the lichen *Lecanora frustulosa* and *Parmeliopsis hyperopta* and their divaricatic acid and zeorin constituents.*African J.Microbiol. Res*. 4 (9): 885:890.
[22] Mitrovic T, et al. Lichen as source of versatile bioactive compounds. *Biol.Nyssana*. 2011;2(1): 1-6.
[23] Molnar K and Farkas E.Current results on biological activities of lichen secondary metabolites.*Z. Naturforsch*. 2011;65:157: 173.
[24] Nash TH. *Lichen Biology*. *Cambridge University Press*, U.K. 1996.
[25] Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends .Afr J Biotech, 2008;7:1797-1806.
[26] Ndukwe IG, Habila JD, Bello IA and Adeleye EO. Phytochemical analysis and antimicrobial screening of crude extracts from the leaves, stem bark and root bark of *Ekebergia senegalensis A. Juss*. *Afri. J. Biotech*. 2006;5(19): 1792-1794.
[27] Nwachukwu EO and Umechuruba CI. Antifungal activities of some leaf extracts on seedborne fungi of Africa Yam bean seeds, seed germination and seedling emergence. *J. Appl. Sci. Environ. Mgt*. 2001; 5(1): 29-32.
[28] Orange A, James PW and White FJ. Microchemical methods for the identification of lichens. *British Lichen Society*. 2001; pp.101.
[29] Öztürk, S., Güvenc, S., Arıkan, N. and Yılmaz, Ö.. E ffect of usnic acid on mitotic index in root tips of *Allium cepa* L. *Lagascalia* 1999;21: 47-52.
[30] Piovano M, et al. Evaluation of antifungal and antibacterial activities of aromatic metabolites from lichens.*Bol. Soc. Chil. Quim*. 2002;47: 235-240.
[31] Poole K, et al. Multiple antibiotic resistances in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J. Bacteriol.* 1993;175: 7363 – 7372.
[32] Rankovic, B., Misic, M. 2008. The antimicrobial activity of the lichen substances of the lichens *Cladonia furcata, Ochrolechia androgyna, Parmelia caperata* and *Parmelia conspresa. Pharm.biotech.* 1013-1016.
[33] Rankovic B, Misic M and Sukdolak S..Evaluation of antimicrobial activity of the lichens *Lasallia pustulata*, *Parmalia sulcata*, *Umbilicaria crustulosa* and *Umbilicaria cylindrical*.*Mikrobiologya* 2007;76 (6): 817-821.
[34] Richardson DHS. Medicinal and other economic aspects of lichens. In: *CRC* Handbook of Lichenology, (Galun,M., Ed.) CRC Press, Boca Raton, FL, 1988;3: 93-108.
[35] Santiago K, et al. Antibacterial activities of fruticose lichens collected from selected sites in Luzon Island, Philippines. Philipp Sci lett. 2010;3: 18-29.
[36] Sati S, and Joshi S. Antibacterial activity of the Himalayan lichen *Parmotrema nilgherrense* extracts. *J. British. Microbiol. Res.* 2011;1 (2): 26:32.
[37] Sharnoff SD. Lichens and people. Online: http://www.lichen.com/people.html. 1997.
[38] Singh G and Kumar P. Antimicrobial efficacy of alkaloids of Withania somnifera (Ashwagandha) an indigenous medicinal plant against some pathogen.*J. Pharm. Res.* 2011;4 (3): 807:809.

[39] Souza-Fagundes EM, et al. Screening and fractionation of plant extracts with antiproliferative activity on human peripheral blood mononuclear cells.Mem.Inst. Oswaldo. Cruz. 2002;97 (8): 1207–1212.

[40] Stefanovic O, Comic L, Stanojevic D and Solujic- Sukdolak S. Antibacterial activity of Aegopodium podagraria L. extracts and interaction between extracts and antibiotics. *Turk. J.Biol*. 2009;33:145:150.

[41] Stevanovic O, Stenojevic D, Comic L. Inhibitory effect of Torilis anthriscus on growth of microorganisms. Central European Journal of Biology. 2009;4:493-498.

[42] Suberu H. Preliminary studies of inhibitions in *Aspergillus flavus* with extracts of two lichens and Bentex-T fungicide.*Afri. J. Biotech*. 2004;3(9):468-472.

[43] Swathi D, Suchitha Y, Prashith Kekuda TR, Venugopal TM, Mallikarjun N, Raghavendra HL. *Int. J. Drug Dev*. and *Res* . 2010;2(4): 780:789.

[44] Tay T, Turk AO, Yilmaz M, Turk H and kivanc M. Evaluation of the antimicrobial activity of the acetone extract of the lichen *Ramalina farinacea* and its (+)- usnic acid, and protocetraric acid constituents.*Z.* Naturforsch. 2004;59:384-388.

[45] Türk AO, Yilmaz M, Kivanc M and Türk H. Antimicrobial activity extracts of the lichen *Cetraria aculeate* and its protolichesterinic acid constituent, Verlag diseases in human and plant diseases. *Z.* Naturforsch. 2003;58: (11-12): 850-854.

[46] White FJ and James PW. New Guide to microchemical techniques for the identification of lichen substances.*Bull. Brit. Lich. Soc.* 1985;57: 41.

[47] Yilmaz M, Tay T, Kivanc M and Turk AO. The Antimicrobial activity of extracts of the Lichen *Hypogimnia tubulosa* and It's 3- Hydroxyphysodic Acid Constituent.*Z.* Naturforsch. 2005;60(1-2): 35-38.