Antibacterial Effect of Some Iraqi Lichen Extracts

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ABSTRACT

The research includes study of antibacterial effects of acetone & ethanol extracts of some Iraqi lichens in addition to find out their minimal inhibitory concentration (MIC). For this purpose three lichen species, grown in the mountainous region of north Iraq, were chosen, they are Diploschistes ocellatus, Lecanora muralis & Physconia distorta in addition to 8 bacterial species, half of them were gram (+) & the others were gram (-). The results revealed that the inhibition activity were varied considerably according to extract type in addition to lichen & bacterial species. Generally, acetone extracts of the three lichens have higher antibacterial inhibition activity than ethanol extracts, the gram (+) bacteria were more sensitive than the gram (-), Bacillus sp. & Micrococcus luteus were the most sensitive bacteria while Escherichia coli & Klebsiella pneumoniae were resistant to all extracts of the lichens species. Physconia distorta extracts revealed clear priority in number of inhibited bacterial species followed by Lecanora muralis then Diploschistes ocellatus. The minimum value of (MIC) 0.781 mg/ml was obtained using acetone & ethanol extracts of Lecanora muralis and acetone extract of Physconia distorta against Micrococcus luteus in addition to ethanol extract of Lecanora muralis against Bacillus sp.

Key Words: Iraqi Lichens, Acetone and Ethanol Extracts, Antibacterial Effect, MIC.

1. INTRODUCTION

There are close to 14,000 species of lichens in the world, tremendously diverse in size, form & color. They are found from the poles to the tropics, from the intertidal zones to the peaks of mountains & on every kind of surface from soil, rock & tree bark to the back of living insects (Brodo et al., 2001). Many sorts of lichens are used for human nutrition, animal nutrition, as indicator for air pollution, for getting colors, perfumes, alcohol & in the medicine industry. Lichens have also, for hundreds of years, been used in many European country as a cure for stomach diseases, diabetes, cough, pulmonary tuberculosis, wounds curing, dermatological diseases (Baytop, 1999; Huneck, 1999; State et al., 2011).

The majority of organic compounds found in lichens are secondary metabolites of the fungal component like depsides, depsidones, depsones, anthraquinones, xanthones, chromones, & steroids, which are deposited on the surface of the hyphae rather than within the cells. These products are usually insoluble in water & can only be extracted with organic solvents (Nash III, 2008).

Lichens & their metabolites have manifold biological activity: cytotoxic effect, anti-inflammatory, antitumor, antipyretic, antioxidant, plant growth inhibitory, analgesic & enzyme inhibitory (Gyathri & Swamy, 2012).

A number of investigators have studied antibacterial and antifungal activity of lichens. The first study of the antibiotic properties of lichens was carried out by Burkholder et al. (1944), since then researchers have been reported antibacterial activity for several lichens against gram-positive and gram-negative bacteria, as well as antifungal activity (Silva et al., 1986; Turk et al., 2003; Karagoz et al., 2009; Devi et al., 2011; Baral & Maharjan, 2011).

The present work aimed to investigate inhibition activity of three Iraqi lichen species against growth of (8) different pathogenic bacterial species. The lichens are: Diploschistes ocellatus, Lecanora muralis and Physconia distorta. They were chosen on the basis of their presence, in natural habitat, in quantities enough to conduct this pioneer study.

2. MATERIALS AND METHODS

2.1. Lichen species and collection area:

Specimens of three lichen species were collected, during March 2013, from Amadiyah and Rowanduz physiographic districts within mountain region in the north part of Iraq (Guest, 1966).

The three lichen species are:

1- Diploschistes ocellatus (Vill) Norm: A crustose saxicolous lichen, collected from Gali Zanta in Aqra district with coordination 36° 45’ 3” N and 43° 58’ 32” E.

2- Lecanora muralis (Schreb.) Rabenh.: A crustose saxicolous lichen.

3- Physconia distorta (With.) J. R. Laundon: A foliose corticolous lichen growing on bark of Oak trees.

The last two species were collected from Gali Balkaif in Atrash district, with coordination 36° 52’ 22” N and 43° 20’ 45” E.

2.2. Bacterial species:

For purpose of the present study, a total of (8) bacterial species were chosen, half of them were gram (+) and the other were gram (-). The gram (+) are: Bacillus sp., Micrococcus luteus, Staphylococcus aureus and Staphylococcus intermedius. The gram (-) are: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and salmonella typhimurium.
These species were obtained from the Bacterial Strain Bank of Biology Department, College of Sciences, University of Mosul.

### 2.3. Identification of the Lichens:

The specimens were identified following the keys mentioned in Brodo et al. (2001), Smith et al. (2009) and Dobson (2011). The identification was confirmed by Dr. Harrie Sipman from Botanical garden- Berlin free University.

### 2.4. Preparation of the lichens extracts:

Two types of solvents, acetone and ethanol, were used for preparation of the extracts as follow:

1. The specimens scraped from the substrate, rocks and tree barks, using sharp razor.
2. Grind with an electrical grinder to get fine powder.
3. (10) gm. of the powder soaked in (100) ml of the solvent.
4. The solution exposed to 2400 frequency/second of ultrasonic waves for 30 minutes discontinuously, 30 seconds per each period.
5. The solution transferred to a conical flask, closed tightly with aluminum foil and stirred for 24 hrs.
6. The coarse suspension particles removed by repeated filtration with gauze.
7. The fine particles removed by filtration with Whatman paper No. 1.
8. To remove most of the solvent, the extract evaporated by rotary evaporator at 40 C°. The evaporation stopped before reaching complete dryness of the solution.
9. The concentrated solution transferred to a weighted glass container and left at room temperature for drying.
10. The dry matter is the dry crude extracts. It kept in a deep freezer (- 80 C°) until test time.

### 2.5. Preparation of 100 mg/ml of extract:

From the dry crude extract, 0.1 g. dissolved in 1.0 ml dimethyl sulphoxide (DMSO) and sterilized by pasteurization in three successive days, using water bath with 60C° for 10 minutes each day.

The culture's media:

1. Nutrient Agar: used to activate the bacterial isolations.

### 2.6. Antibacterial activity assays:

Assays were conducted using agar well diffusion method in nutrient agar media. In each plate 4 peripheral wells were made using 7 mm diameter cork borer.

As a negative control treatment, 10 µl of DMSO were added to the first well and 10 µl of each one of the three lichens extracts were added, separately, to the rest wells. An antibiotic disc of Cefotaxime 10 mcg was placed in plate center as a positive control treatment.

Two sets of plates were prepared, as mentioned above, the first set for ethanol extracts and the other for acetone extracts. From the 8 bacterial species, under study, 24 h. age, 6x10⁸ cell/ml inoculation were prepared. Each plate was inoculated by 0.1 ml inoculation belongs to one bacterial species. The inoculation spread by a sterilized cotton swab before incubating the plates for 24 h. at 37 C°. Inhibition zone diameter around wells and antibiotic disc were measured. The results were statistically analyzed by Duncan's test at 0.05 probability level.

### 2.7. Determination of Minimal Inhibitory Concentration (MIC):

From the main concentration, 100 mg/ml, of the active extracts of the studied lichens, the following series of dilutions were prepared: (50.000, 25.000, 12.500, 6.250, 3.125, 1.562 and 0.781 mg/ml). From each dilution, 10 µl was added to one of the seven peripheral wells that were made in nutrient agar. Plates were inoculated by 0.1 ml of the sensitive bacteria & spread using cotton swab then incubated for 24h. at 37C°. The highest dilution that revealed bacterial growth inhibition was considered as MIC.

### 3. RESULTS AND DISCUSSION

The results revealed that DMSO, the negative control treatment, have no inhibitory effect against all bacterial species under study. This means that bacterial growth was affected only by the active compounds of the lichens.

Antibacterial effect of the lichen extracts varied widely according to the type of lichen, bacteria and solvent of extracts. Generally, acetone extracts of the three lichens were more effective against bacterial growth than ethanol extracts (table1 and plates 1&2). Acetone extracts inhibit 6 out of 8 tested bacterial species while ethanol extracts inhibit 4 out of 8 species only. This result showed similarity with that of Rankovic & Kosanic (2012) Where they found that the acetone extract of lichen Parameilia sulcata achieved highest antibacterial effect.

From the results it was declare that gram (+) bacterial species, under study, were the most sensitive against lichen's extracts as growth of all gram (+) species were inhibited by one or more extracts comparing with only 2 of gram (-) species which inhibited only by extracts of Physosonia distorta. This variation in the sensitivity between gram (+) and gram (-) bacteria may belong to the differences in cell wall structure. The gram positive cell wall consists of a single, thick homogeneous layer of peptidoglycan (murein) lying outside the plasma membrane. In addition, gram positive cell walls usually contain large amounts of teichoic acids.
Table (1) Effect of lichens extracts on growth of bacteria under study

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Gram stain reaction</th>
<th>Control treatments</th>
<th>Lichen extracts</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+) Cefotaxime 10 mcg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diploschistes ocellatus</td>
<td>Lecanora muralis</td>
<td>Physciona distorta</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>+</td>
<td>A</td>
<td>E</td>
<td>A</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>+</td>
<td>10 h</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>+</td>
<td>in j</td>
<td>14 g</td>
<td>3</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>in b</td>
<td>18 d</td>
<td>3</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>-</td>
<td>in b</td>
<td>22 c</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
<td>in f</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Salmoneella typhimurium</td>
<td>-</td>
<td>in d</td>
<td>20 b</td>
<td>1</td>
</tr>
</tbody>
</table>

Lichen’s extract concentration=100 mg/ml

Inhibition zone diameter including 7mm of the diameter’s well

Diameter of each treatment is a Mean of two replic. At row level, same letters indicate no significant differences between the numbers

A= Acetone extract E= Ethanol extract in= inactive

Gram negative cell walls are much more complex than gram positive walls.

The thin peptidoglycan layer next to the plasma membrane and bounded on either side by the periplasmic space usually constitutes only 5 to 10% of the wall weight. The outer membrane lies outside the thin peptidoglycan layer, the most unusual constituents of the outer membrane are its lipopolysaccharides (LPSs). A major function of LPS is that it helps create a permeability barrier. The geometry of LPS and interactions between neighboring LPS molecules are thought to restrict the entry of bile salts, antibiotics, and other toxic substances that might kill or injure the bacterium. LPS also plays a role in protecting pathogenic gram-negative bacteria from host defenses (Willey et al., 2009).

Almost same result gain by Angelique et al. (2010), they found that gram (-) species were less affected by extracts of Ramalina sp. and Usnea sp. than gram (+) one.

Logically, the three lichen species were varied in their inhibition range, the widest one was Physciona distorta then Lecanora muralis and the narrowest one was Diploschistes ocellatus, whereas the inhibition effect of P. distorta include 6 species, L. muralis 3 species and only acetone extract of D. ocellatus was able to inhibit growth of 2 bacterial species. With regard to Molina et al. (2003) results, the active compounds that present in P. distorta were the reason behind this wide inhibition range. After analyzing same lichen with HPLC technique, they found that it contains phenolic acid as malonprotocetraric and usnic acids besides two bis-anthraquinones (i.e) eumetrin T and secalonic acid in addition to two depsides compounds, atranorin and chloroatranorin.

Among the three lichen species under study, only Physciona distorta extracts was able to inhibit growth of gram (-) bacteria, whereas acetone and ethanol extracts of this species cause 28 and 17 mm of inhibition zone respectively in culture of Pseudomonas aeruginosa and acetone extract cause 15 mm zone in Salmonella typhimurium.

Among bacterial species, Bacillus sp. was the most sensitive one against lichen extracts. The inhibition effect for most treatments surpasses the positive control treatment by Cefotaxime. The inhibition zone diameter of acetone extracts that belong to the three lichens (i.e) Physciona distorta, Lecanora muralis and Diploschistes ocellatus reached 30, 23, and 14 mm and for ethanol extracts reached 26, 18 and 0 mm while Cefotaxime inhibition zone diameter was 10 mm. In 2006 Cansaran et al. get similar result , they found that acetone extracts of Usnea subflorida revealed higher inhibition for Bacillus subtilis & B. megaterium.

Concerning Micrococcus luteus, the acetone extracts of the three lichens showed stronger inhibition power than ethanol extracts. The inhibition zone diameter formed as a consequence of using acetone extracts of Physciona distorta, Lecanora muralis and Diploschistes ocellatus, were 30, 31 and 18 mm respectively, while those of ethanol extracts were 21, 18 and 0 mm respectively, in addition the inhibition zone diameter of the antibiotic Cefotaxime was 30 mm which is closer to the acetone extracts of Physciona distorta & Lecanora muralis.

According to the results shown in table1and plates 1&2, Staphylococcus aureus can be considered as a resistant species since its growth was affected by acetone extracts of Physciona distorta only which form 16 mm diameter of inhibition zone.

The isolation Staphylococcus intermedius was somewhat resistant, it showed sensitivity only against acetone extracts of Physciona distorta and Lecanora muralis in addition to ethanol extracts of Lecanora muralis.

Regarding gram(-) bacteria, the results showed that E. coli and Klebsiella pneumoniae were resistant to both types of extracts, acetone & ethanol, in addition to the antibiotic Cefotaxime. These two bacterial species were also resistant to acetone and ethanol extracts of Xanthoparmelia pokornyi (Candan et al., 2007).

Growth of the isolation Pseudomonas aeruginosa was sensitive only to extracts of one lichen species (i.e) Physciona...
distorta. The inhibition zone of acetone extract of this lichen surpasses those of the antibiotic Cefotaxime and Ethanol extract, the inhibition zone diameters were 28, 22 and 17 mm respectively.

The results also showed that Salmonella typhimurium exhibited resistance for all extracts except for acetone extract of Physconia distorta, diameter of inhibition reached 15mm in front of 20mm for Cefotaxime.

The Minimal Inhibitory concentration (MIC)

Concerning acetone extracts, The minimum MIC value achieved was 0.781 mg/ml when extracts of Physconia distorta and Lecanora muralis used against Micrococcus luteus, While the maximum value (50 mg/ml) achieved after using Physconia distorta extracts against Salmonella typhimurium and also Diploschistes ocellatus with Bacillus sp. (table 2 and plate 3).

Regarding ethanol extracts, the minimum MIC value (0.781mg/ml) noticed when Lecanora muralis extracts were tested with Bacillus sp. and Micrococcus luteus while the maximum value (25mg/ml) exhibited by Physconia distorta extracts with Pseudomonas aeruginosa (table 2 and plate 4).

Table (2) Values of the minimal inhibitory concentration (MIC) mg/ml of the lichen's active extracts

<table>
<thead>
<tr>
<th>Sensitive bacterial species</th>
<th>Lichens extracts</th>
<th>Diploschistes ocellatus</th>
<th>Lecanora muralis</th>
<th>Physconia distorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>E</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>50</td>
<td>-</td>
<td>1.652</td>
<td>0.781</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>12.5</td>
<td>-</td>
<td>0.781</td>
<td>0.781</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>-</td>
<td>-</td>
<td>3.125</td>
<td>1.562</td>
</tr>
</tbody>
</table>

A= Acetone extract  E= Ethanol extract
Plate (1) Effect of acetone extracts of lichens on bacterial species under study

D= Diploschistes ocellatus extract         L= Lecanora muralis extract
P= Physconia distorta extract

(-)= negative control treatment (DMSO)  
(+) = positive control treatment (cefotaxime 10mcg)
Plate (2) Effect of ethanol extracts of lichens on bacterial species under study

D = Diploschistes ocellatus extract
L = Lecanora muralis extract
P = Physconia distorta extract
(-) = negative control treatment (DMSO)
(+) = positive control treatment (cefotaxime 10mcg)
Plate (3) The minimal inhibitory concentration (MIC) of the acetone's active extracts for the lichens under study

(B) Bacillus sp.  (M) Micrococcus luteus  (Si) Staphylococcus intermedius
(Pa) Pseudomonas aeruginosa  (Sa) Staphylococcus aureus  (St) Salmonella typhimurium  (Pd) Physconia distorta  (Lm) Lecanora muralis
(Do) Diploschistes ocellatus

(Ac) Acetone extract

Numbers 1 to 7 represent the following dilutions: (50.000, 25.000, 12.500, 6.250, 3.125, 1.562, 0.781) mg/ml respectively.
Plate (4) The minimal inhibitory concentration (MIC) of the ethanol's active extracts for the lichens under study

(B) Bacillus sp. (Mi) Micrococcus luteus  (Si) Staphylococcus intermedius
(Pa) Pseudomonas aeruginosa  (Pd) Physconia distorta  (Lm) Lecanora muralis
(Eth) Ethanol extract

Numbers 1 to 7 represent the following dilutions: (50,000, 25,000, 12,500, 6,250, 3,125, 1,562, 0.781) mg/ml respectively.

In conclusion, the results indicate that the native Iraqi lichens could be a good source of antibiotics for treatment of diseases caused by pathogenic bacteria as the challenges today for pharmaceutical industry lies in the discovery & development of new pharmacological active molecules.

Execution of more elaborate and deeper consideration about isolation and purification of the active compounds from Iraqi lichens is recommended.
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REFERENCES


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