ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF A CHEMICALLY INDUCED MUTANT OF XANTHORIA PARIETINA

S. Ali* and H. N. Hameed

Institute of Industrial Biotechnology (IIB), GC University Lahore, Pakistan
*Corresponding Author e-mail: dr.sikanderali@gcu.edu.pk

ABSTRACT

Biological active secondary metabolite parietin was extracted from wild-type and mutant strain of lichen Xanthoria parietina. Six different solvents (acetone, ethanol, methanol, hexane, chloroform and distilled water) were used to extract parietin. The parietin activity was found to be maximum when extraction was carried out with 3 ml of methanol at 30°C for 1h. The extracts were proceeded to antibacterial activity against different strains of bacteria. Anion DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging, reducing power and total flavonoid content were used to analyze the antioxidant activity. HPLC analysis represented more than 90% purity of methanol extract with some trace elements. The antioxidant activity increased with increasing concentration of methanol extract. EMS treated strain represented maximum DPPH scavenging activity i.e., 84±3.36% which was 2.08 fold higher than the wild-type. Maximum reduction potential was found to be 0.237±0.0118 which was 1.83 fold higher than wild-type. Mutagen concentration of 0.5 mM exhibited strong parietin activity. The extracts of EMS treated mutant variant revealed strong antimicrobial activity (22 mm for bacteria) as compared to the wild-type and MMS treated variant. It was concluded that by increasing mutagen exposure time, the parietin activity decreased in terms of its antimicrobial activity.

Key words: Xanthoria parietina, parietin, chemical mutagen, antioxidant, antimicrobial, secondary metabolite

INTRODUCTION

Lichens are the inter-reliant organisms in which fungi and algae and/or cyanobacteria form an intimate biological union. About 18,500 lichen species inhabited the world (Nash, 2008). Lichens possess a wide range of primary and secondary metabolites. Almost 800 such metabolites have been recognized (Hunek and Yoshimura, 1996). These metabolites are involved in many biological actions such as antibiotic, antimycobacterial, virucidal, inflammation preventive, pain-relieving, antipyretic, anticancer and cytotoxic effects (Sisodia et al., 2013). Secondary metabolite production is the necessity of lichens which enables them to live in harsh environmental conditions (Manojlovic et al., 2010). Xanthoria parietina is foliose lichen which synthesized various secondary products (Solhaug and Gauslaa, 1996). The thallus of the lichen is 3.1 inch broad and diameter of lobe ranges from 1 to 4 mm (McCune and Geiser, 1997). X. parietina was distributed around the world. It could be present on the stem of trees, shrubs, roofs, walls and rocks (Honegger et al., 2003). X. Parietina contains an anthraquinone physcion (parietin) which gives orange-yellowish color to lichen. Parietin was secreted by mycobiont absorbs blue light and provides protection to photobiont against drastic sun rays (Gauslaa and McEvoy, 2005). It is found in the upper cortex of the lichen (Honegger, 1990). Its molecular weight is 284.26 g/mol and chemical formula is C_{16}H_{12}O_{9}. In its pure form, it forms needle like crystals whose melting point ranges from 209 to 212°C (Edwards et al., 2003). Ultra violet rays produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) which cause irritation, suppression of immune system, alteration in gene sequence and uncontrolled proliferation of cells by disturbing cell cycle (Russo et al., 2008). Antioxidants provide protection from diseases which occurs due to ROS & RNS by combining with free radicals. Antioxidants neutralize the ROS and RNS species before any damage to lipids, proteins, enzymes and DNA (Sannigrahi et al., 2009; Pal et al., 2009). Biomass improvement strategies and improved manufacture conditions play an important role in higher production of secondary metabolites (Burg et al., 1979; Comini et al., 2017). Mutagenesis is a preferable and commonly used method to enhance metabolite production (Volf and Altenbuchner, 1998). Mutagens used different mechanisms to induce mutagenesis. Some chief groups of chemical mutagens include alkylating agent, intercalating agents and base analogs (Sloczynska et al., 2014). The aim of the present study was to determine the antibacterial & antioxidant activity of chemically induced variant of X. parietina by different methods.

MATERIALS AND METHODS

Sample collection and identification: The samples of lichen were collected from Murree (Pakistan). After collection, the samples were brought to laboratory and
dried at room temperature at low light intensity. These were stored at −10°C (Honegger, 2003). A spot test was used to identify the lichen X. parietina. Ten percent potassium hydroxide (KOH) solution was used to identify the lichen type (Lucking, 2014).

**Microcrystal test:** Small fragments (1–2 mm) of lichen biomass were placed on glass slide and 5 to 6 drops of acetone were added. Slides were left open to evaporate the solvent. After evaporation of solvent, biomass fragments were removed and 3–4 drops of mixture of glycerol, ethanol, water (1:1:1) and glycerol, acetic acid (3:1) was placed on to the residue. The slides were kept at a warm place for 30 min and later observed under a compound microscope.

**Preparation of lichen extract:** Lichen extract was prepared by using methanol as an extractant. Three milliliters of solvent was added to 100 mg of biomass and placed in a shaking incubator at 160 rpm for 30 min. After shaking, extraction was done by centrifugation at 3000 rpm for 10 min. The samples were re-centrifuged at 3500 rpm for 15 min. The supernatant was decanted off and stored at −4°C.

**Induction of mutation:** Lichen biomass (100 mg) was dispersed in 2.5 ml of EMS and MMS solution. At 30°C, the mixture was stored in incubator for 5 min. Sodium thiosulphate prepared in phosphate buffer, pH 7.2 (0.5 ml of 0.1 M) was supplemented to cease the reaction. The falcons were centrifuged (centrifuge machine, D-78532, Hettich Zentrifugen EBA20, Tuttligen, Germany) at 5500 rpm for 20 min. The biomass was washed twice with phosphate buffer (pH 7.2) and biomass was removed dried at 70°C for 5 min.

**Antibacterial activity:** Antibacterial activity was determined by using disk diffusion assay, against different strains of bacteria. These strains were collected from Institute of Industrial Biotechnology (IIB) GC University Lahore (Pakistan). Bacterial strains included Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.

**Disk diffusion assay:** Muller Hinton agar medium was used for antibacterial susceptibility testing using disk diffusion method. Bacterial strains were dispensed in saline water and their concentration was compared with McFarland 0.5 standard solution. Each strain was swabbed by sterile cotton buds on (MHA) media poured in autoclaved Petri plates. Simple filter paper discs were prepared by punching with paper puncher and autoclaved. Discs were impregnated with 50 and 100 µl of extracts for bacteria and fungi, respectively. Discs were placed on agar plates containing microbial strains and incubated at 37°C for 17 h. Finally, zones of inhibition were noted in millimeter.

**Antioxidant activity**

**DPPH free radical scavenging activity:** The ability of the extracts to scavenge DPPH free radicals was determined by the method of Rankovic (2011). In a test tube 1ml of lichen extract was added to 2 ml (0.05 mg/ml) of DPPH solution, which was prepared in methanol. The mixture was incubated at room temperature (25°C) for 30 min. A517 was read against the control set at zero using a UV/VIS spectrophotometer. DPPH solution was used as control being devoid of lichen extract. The percentage inhibition values were calculated using the following relationship,

\[
\text{Inhibition} (\%) = \frac{(A_c - A_{sd})}{A_c} \times 100
\]

**Reducing power:** The ability of the extracts to reduce Fe³⁺ was analysed by the method of Dorman (2004). The lichen extract (1 ml) along with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) was taken in a test tube. The mixture was vigorously shaken, and potassium ferricyanide [K3Fe(CN)6] was added. All the contents were kept at 50°C for 20 min and then TCA (2.5 ml, 10% w/v) was added. The upper layer was removed and dissolved in 2.5 ml of distilled water, and 2.5 ml of FeCl₃. A700 was measured against blank sample (without extract).

**HPLC analysis of EMS treated (EMS-1) and MMS treated (MMS-1) variants of X. parietina:** Parietin concentration was determined by a double beam high performance liquid chromatography (HPLC) system (153-MPi Hitachi, Shimadzu, Japan) with a refractive index detector using a CZ-8 metabolite column at 65°C. Deionized water was used as eluent at a flow rate of 0.5 ml/min.

**Statistical analysis:** SPSS (version 24) was used to compare treatment effects as described by Snedecor and Cochran (1980). The significance difference was presented as Duncan’s multiple ranges in the form of probability values.

**RESULTS AND DISCUSSION**

**Identification of lichen X. parietina and secondary metabolite parietin:** Potassium hydroxide (10%) was added drop by drop on the thallus of orange lichen attached with tree bark. The orange color of lichen was diminished and changed into violet color which confirmed the presence of parietin in the upper cortex of lichen X. parietina (Fig. 1). Micro-crystallization test was used for identification of parietin in extract. Crystal formation (Fig. 2a) was observed at a low resolution power. Needle shaped crystals were observed in low quantity. In Fig. 2b needle shaped crystals were observed in high quantity with clear resolution. Manojlovic et al. (2000) isolated physcion (parietin) from the species of
genus *Xanthoria* and observed the needle shape crystals with melting point 208-210°C. Later on Edwards et al. (2003) observed the needle shape crystals of acetone extract of parietin.

**Antibacterial activity of different extracts of wild-type (ISL-3) *X. parietina***: Table 1 shows the antibacterial activity of untreated sample of *X. parietina*. Antibacterial activity of extracts was tested against four strains of bacteria (*B. subtilis*, *E. coli*, *S. aureus*, and *P. aeruginosa*). Significant antibacterial activity was shown by methanol extract followed by hexane and ethanol extract. Aqueous extract didn’t show antibacterial activity against any strain. Maximum zone of inhibition i.e., 8 mm was observed against *P. aeruginosa* and *S. aureus* by ethanol, methanol and hexane extract, respectively. Methanol extract exhibited 18.75% better antibacterial activity against *S. aureus* as compared to *P. aeruginosa*. Antiseptic activity of acetone extract was maximum against *P. aeruginosa* (8 mm). However, chloroform extract didn’t show antiseptic action against *S. aureus*, and *P. aeruginosa*, and 6.5 mm inhibition zone was observed against *B. subtilis* and *E. coli*. Dias and Urban (2009) reported varying degrees of antibacterial activity of *X. parietina* against *B. subtilis*, *T. Mentagrophytes* and *C. resinae*.

**Antibacterial activity of EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina***: Treatment with EMS and MMS at different time intervals represented different zones of inhibition. Maximum inhibition zone (22 mm) was observed in the methanol extract of EMS mutated strain which was treated for 5 min and there was no inhibition zone around methanol extract treated for 30 min (Table 2&3). Similarly, hexane extract showed 3 fold higher antibacterial activity treated for 5 min as compared to strain treated for 30 min. In contrast, MMS mutated strain was not as effective as EMS mutated strain. EMS treated strain showed 11.3, 10 and 22.2% superior antibacterial activity as compared to methanol, ethanol and hexane extracts of MMS mutated strain, respectively. Manojlovic et al. (2000) reported antibacterial activity of lichen anthraquinones against different *Pseudomonas* spp. Physcion showed 15% antibacterial activity against *Pseudomonas* spp. Similarly, Basile et al. (2015) reported the antibacterial activity of parietin. Parietin exhibited strong antibacterial activity against *Pseudomonas aeruginosa* whose MIC value was found to be 31.3±0.3 μg/ml. Strong antibacterial activity against *E. coli* was shown by methanol extract of EMS treated strain extract followed by ethanol extract (18 mm) (Table 4). The methanol extract of EMS mutated strain showed 1.16 fold higher antibacterial activity than methanol extract of MMS mutated strain. Ethanol extract of EMS treated strain revealed 1.18 fold higher activity than methanol extract of MMS treated strain. On the other hand, MMS treated methanol extract showed inhibition zone of 16 mm subsequently ethanol extract 14.5 mm. Inhibition zone of hexane extract was 16.5 and 13 mm of EMS and MMS treated strain, respectively. Ethanol extract of EMS treated strain exhibited 15% higher antiseptic action with respect to ethanol extract of MMS treated strain.

**DPPH scavenging ability of EMS & MMS treated variant of *X. parietina***: DPPH scavenging ability generally increased with increasing concentration of extract. DPPH scavenging ability ranges from 17 to 43% for wild-type, 49 to 84% for EMS mutated strain and from 42 to 79% for MMS mutated strain. EMS mutated strain exhibited 10% higher scavenging ability as compared to MMS treated strain. Fig. 3&4 showing that absorbance and % DPPH scavenging activity are inversely related to each other i.e., with decreasing absorbance % DPPH scavenging activity increased. Oktay et al. (2003) stated the basic phenomenon of DPPH assay. DPPH is a stable free radical with odd number of electron and exhibit deep purple color. The purple color of DDPH diminished and yellow color formed when antioxidant donate electron to DPPH. In a similar kind of study Bag et al. (2015) reported that with increasing concentration of methanolic extract DPPH scavenging activity also increased. Rankovic et al. (2011) studied the DPPH scavenging activity of lichens Cladoniafurcata, Lecanoraatra and Lecanoramuralis. DPPH inhibition was of Lecanoraatra found to be 94.7% greater than the commercial antioxidant.

**Reducing potential of methanol extract of wild-type (ISL-3), EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina***: Data in Fig. 5 showed that EMS mutated variant had 18% and 86% strong reduction potential as compared to MMS treated, and wild-type for 0.5 ml, respectively. Reducing power progressively increased with concentration of methanolic extract. Absorbance increased with increasing concentration of methanol extract. The methanol extract (3 ml) of EMS treated variant revealed 14.3 and 45% strong reduction potential than MMS mutated variant and wild-type strain, respectively. In previous literature it was reported that with increasing concentration of methanol extract reducing potential also increased (Bag et al., 2015). Chanda and Dave (2009) reported that compounds with reducing power have antioxidant activity as these compounds have ability to donate electron to oxidize species and reduced the intermediates of lipid peroxidation.

**HPLC analysis of EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina***: HPLC analysis showed the number of peaks informed about the compounds present in mixture. Chromatogram of both EMS & MMS mutated strain showed one major peak with retention time of 19.5-20 min (Fig 6 a&b). However,
from chromatogram it was noted that some trace compounds were also present with retention time of 40 min. Peak area represented the concentration of parietin. Yoshimura et al. (1994) reported the HPLC analysis of various lichen secondary metabolite with photodiode array detector. The authors reported the relative retention time of parietin 21.83 min. Cornejo et al. (2016) reported presence of parietin in lichen Ramalina terebrata with the help of HPLC (27.21 min retention time). Backorova et al. (2011) in their finding reported the 98.5% of final purity of parietin by HPLC.

**Conclusion:** The present study deals with the extraction of parietin from treated and untreated strain of lichen *X. parietina*. EMS treated strain of lichen *X. parietina* revealed better antimicrobial and antioxidant activity than MMS treated strain. The chemical mutation of lichen *X. parietina* was proved to be a better mutagenic technique that not only increased the parietin activity but also improved the antimicrobial and antioxidant activity for its potential application in health biotechnology. The overall parietin activity was also significantly increased (p≤0.05).

**Figure 1:** Identification of lichen *X. parietina* attached with tree bark by spot test. (a) Lichen sample attached with tree bark, (b) *X. parietina* with 10% KOH solution.

**Figure 2 (a & b):** Micro-crystallization test for identification of parietin at 40X & 100X.
Figure 3: DPPH scavenging activity with different extracts concentration (absorbance / concentration). The error bars indicate standard deviation (±sd set at 5 %) among the values of three parallel replicates. The sum means values differ significantly at p≤0.05 from each other in one set, under one way ANOVA.

Figure 4: % DPPH scavenging activity with different extracts concentration. The error bars indicate standard deviation (±sd set at 5 %) among the values of three parallel replicates. The sum means values differ significantly at p≤0.05 from each other in one set, under one way ANOVA.
Figure 5: Reducing potential of methanol extract of EMS, MMS and wild-type strain of *X. parietina*. The error bars indicate standard deviation (±sd set at 5%) among the values of three parallel replicates. The sum means values differ significantly at *p* ≤ 0.05 from each other in one set, under one way ANOVA.

Figure 6: Chromatogram of high performance liquid chromatography of extract of EMS (a) and MMS (b) treated variant of *X. parietina*. (CZ-8 metabolite column, 65°C, eluent; deionized water, flow rate; 0.5 ml/min).
Table 1. Antibacterial activity of different extracts of wild strain (ISL-3) of *X. parietina*.

<table>
<thead>
<tr>
<th>Extracts (ml)</th>
<th><em>B. subtilis</em></th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>N.O</td>
<td>7.5±0.375</td>
<td>6.5±0.400</td>
<td>8±0.400</td>
</tr>
<tr>
<td>Chloroform</td>
<td>6.5±0.325</td>
<td>6.5±0.325</td>
<td>N.O</td>
<td>N.O</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.5±0.375</td>
<td>6.5±0.325</td>
<td>7.5±0.375</td>
<td>8±0.400</td>
</tr>
<tr>
<td>Methanol</td>
<td>7.5±0.375</td>
<td>7±0.350</td>
<td>8±0.400</td>
<td>6.5±0.325</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>7.5±0.375</td>
<td>6.5±0.325</td>
<td>8±0.400</td>
<td>8±0.400</td>
</tr>
<tr>
<td>Distilled water</td>
<td>N.O</td>
<td>N.O</td>
<td>N.O</td>
<td>N.O</td>
</tr>
</tbody>
</table>

*100 mg biomass, 5 ml solvent, temperature 30ºC, N.O= Not observed, ± Indicate standard deviation (±sd set at 5 %) among the values of three parallel replicates. The sum-mean values differ significantly at p≤0.05 from each other in one set, under one way ANOVA.

Table 2. Antibacterial activity of ethanol, hexane and methanol extract of EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina* against *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>EMS treated variant (EMS-1)</th>
<th>MMS treated variant (MMS-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.E</td>
<td>E.E</td>
</tr>
<tr>
<td>5</td>
<td>22±1.10</td>
<td>20±1.00</td>
</tr>
<tr>
<td>10</td>
<td>17±0.85</td>
<td>16.5±0.82</td>
</tr>
<tr>
<td>15</td>
<td>15±0.75</td>
<td>15±0.75</td>
</tr>
<tr>
<td>20</td>
<td>11±0.55</td>
<td>9±0.45</td>
</tr>
<tr>
<td>25</td>
<td>8±0.40</td>
<td>7±0.35</td>
</tr>
<tr>
<td>30</td>
<td>6±0.30</td>
<td>6.5±0.32</td>
</tr>
</tbody>
</table>

*EMS= ethyl methane sulphonate, MMS= methyl methane sulphonate, E.E= ethanol extract, M.E= methanol extract, H.E= hexane extract, mutant concentration optimized 0.5 mM, mutant exposure time vary. ± Indicate standard deviation (±sd set at 5 %) among the values of three parallel replicates. The sum-mean values differ significantly at p≤0.05 from each other in one set, under one way ANOVA.

Table 3. Antibacterial activity of ethanol, hexane and methanol extract of EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina* against *E. coli*.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>EMS treated variant (EMS-1)</th>
<th>MMS treated variant (MMS-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.E</td>
<td>E.E</td>
</tr>
<tr>
<td>5</td>
<td>18±0.90</td>
<td>18±0.9</td>
</tr>
<tr>
<td>10</td>
<td>16.5±0.82</td>
<td>13±0.65</td>
</tr>
<tr>
<td>15</td>
<td>14.5±0.72</td>
<td>11.5±0.57</td>
</tr>
<tr>
<td>20</td>
<td>9±0.45</td>
<td>8±0.85</td>
</tr>
<tr>
<td>25</td>
<td>7.5±0.37</td>
<td>7±0.35</td>
</tr>
<tr>
<td>30</td>
<td>6±0.30</td>
<td>6±0.30</td>
</tr>
</tbody>
</table>

*EMS= ethyl methane sulphonate, MMS= methyl methane sulphonate, E.E= ethanol extract, M.E= methanol extract, H.E= hexane extract, mutant concentration optimized 0.5 mM. ± Indicate standard deviation (±sd set at 5 %) among the values of three parallel replicates. The sum-mean values differ significantly at p≤0.05 from each other in one set, under one way ANOVA.

Table 4. Antibacterial activity of ethanol, hexane and methanol extract of EMS treated (EMS-1) & MMS treated (MMS-1) variant of *X. parietina* against *S. aureus*.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>EMS treated variant (EMS-1)</th>
<th>MMS treated variant (MMS-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.E</td>
<td>E.E</td>
</tr>
<tr>
<td>5</td>
<td>19±0.95</td>
<td>17±0.85</td>
</tr>
<tr>
<td>10</td>
<td>17.5±0.87</td>
<td>13.5±0.67</td>
</tr>
</tbody>
</table>
Acknowledgements: We are grateful to the Director IIB and Vice Chancellor of the University for providing research facilities and moral assistance.

REFERENCES


