Production and characterization of exopolysaccharide from marine Bacillus sp. MSHN2016 with studying its effect on isoniazid/ rifampicin-induced hepatic and renal toxicities in rats

Manal S. Selim*, Sahar S. Mohamed, Mohsen M. Asker, Abeer A.A. Salama, Heba M.I Abdallah, Noha N. Yassen

1Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Division, National Research Centre, Dokki, Cairo, Egypt.
2Pharmacology Department, Medical Division, National Research Centre, Dokki, Cairo, Egypt.
3Pathology Department, Medical Division, National Research Centre, Dokki, Cairo, Egypt.

ABSTRACT

Exopolysaccharides (EPSs) are bioactive natural products used in different applications. A preliminary chemical analysis of EPSMSHN indicated that the monosaccharides were arabinose, xylose, glucose and glucuronic acid with a relative ratio of 1: 1: 2: 1, respectively, with a weight average molecular weight ($M_w$) of $5.50 \times 10^5$ g/mol and a number average molecular weight ($M_n$) of $3.45 \times 10^5$ g/mol. INH/RIF deteriorated hepatic and renal functions through decreased hepatic and renal GSH and increasing MDA contents that associated with hepatocellular apoptosis as well as degeneration and fibrosis in many tubules. The treatment with EPSMSHN at different doses (50 and 100 mg/kg) corrected all previously mentioned INH/RFP-induced changes. In conclusion, EPSMSHN at both doses protected from the hepatic and renal toxicity induced by INH/RFP through its antioxidant and antifibrotic influence.

KEY words: Exopolysaccharide, Bacillus sp., isoniazid/rifampicin, hepatic and renal toxicity.

INTRODUCTION

One of the reasons of 50% of the cases of acute liver failure is drug-induced hepatic toxicity which mimics all forms of liver disease (Kaplowitz, 2001). Distinctive sorts of pills, for example, acetaminophen, chloroquine also isoniazid are inducers for that hepatotoxicity over the globe. Isoniazid moreover rifampicin, the initial drugs utilized for tuberculosis help, are connected with hepatotoxicity (Kaplowitz, 2004). Those rate of hepatotoxicity needs to be appeared to being substantially higher for creating nations such as India (8–30%) contrasted with that for propelled nations (Sharma, 2004). Toxic liver injury happens in greatest amount comparing with the other organs, because the liver is a presentable organ for absorbed substances and their metabolism and disposal (Kaplowitz, 2001). A major mechanism evolved in INH and RFP-induced hepatic toxicity is endogenous lipids peroxidation. This mechanism is may be because of the creation of the exceedingly reactive oxygen species (ROS) provokes the destruction of the cell membrane (Sharma, 2004). Many different mechanisms cause hepatorenal toxicity, including cellular membrane disruption and cell death and creation of new adducts from binding between cell proteins and the drug resulting in an immunologic reaction (Robin et al., 1997). Other reasons included as drug metabolism interruption (Yun et al., 1993), actin filaments disruption leading to abnormal bile flow and cholestasis and jaundice (Trauner et al., 1998), induction of pathways of Fas and tumor necrosis factor (TNF) (Reed, 2001), also ROS accumulation due to inhibition of mitochondrial dysfunction (Pessayre et al., 2001). INH and RFP caused instinctive sorts of hepatic metabolic aberrations because the liver is the major site for detoxifying these anti-tubercular drugs (Sharma, 2004). Expanding confirmation recommends that harmful metabolites potentiate oxidative stress reaction in the rats’ liver (Saad et
(al., 2010; Chen et al., 2012). It was recorded that INH oxidized by itself to a reactive metabolite that binds to hepatic proteins mediating immune liver injury (Yamamoto et al., 2005). EPSs are high molecular weight polymers composed of homo- or hetero-carbohydrates. They are produced by a different microorganism such as bacteria, fungi and yeasts, where they are secreted by these microorganisms to the external environment (Selim et al., 2018). It was known that EPSs are bioactive natural products used in different applications such as medical, the biochemical also pharmacological application as a result of their specific biological activities (Ye et al., 2012; Selim et al., 2018).

In the present study, a heterogeneous exopolysaccharide will be obtained and identified from bacterial isolate. The chemical characteristics of EPS will be determined while the hepatic and renal toxicity will be induced by INH/RFP and this model will be used to reveal the protective effect of bacterial EPS.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 150-200 g were housed in the animal house, pharmacology department, National Research Centre Experimental protocol was approved (Number: 16-438) and met the Guidelines for Animal Experimentation of the Ethical Committee of the National Research Centre which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).

Chemicals, Drugs and Diagnostic Kits

All chemicals used in casein hydrolysate glucose broth medium, chemical analysis of EPS and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma, USA. Other chemicals and their sources were: paracetamol was obtained from (EIPICO, Egypt), silymarin (SEDICO, Egypt) and isoniazid/rifampicin (Sandoz Pharmaceutical Co., Germany). Diagnostic kits for ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were obtained from Biodiagnostic Co., Egypt.

Methods

Isolation of bacteria

The serial dilution method was applied for isolation of bacteria, the sample (sediment of the shore of Ageeba beach (the Mediterranean Sea, Marsa Matrooh governorate) was diluted to 10−8 and plated on marine nutrient agar medium (Hayakawa and Nonomura, 1987) then incubated at 37°C for 48 h. Smooth, roppy and mucous colonies from each plate were selected.

Production and isolation of exopolysaccharide (EPS) from liquid culture

Pure colonies were screened for EPS production by inoculation (107/mL) of cells into 250 mL Erlenmeyer flasks containing 50 mL of casein hydrolysate glucose broth (Al-Nahas et al., 2011) consisting of (g/L): Casein hydrolysate 2.5; K2HPO4 4.0; MgSO4·7H2O 0.7; MnSO4·7H2O 0.05; and glucose 30.0, dissolved in 75% seawater, pH 7.0. The fermentation cultures were incubated at 37°C under shaking at 120 rpm for 3 days. The culture medium was centrifuged at 5000 rpm for 10 min (Sigma-Laborzentifugen, 2K15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight at 4°C and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH (0.1M) solution and dialyzed three times against distilled water using dialysis tubing (MWCO 2000). The supernatant was completed in four volumes with ethanol 95% and left overnight at 4°C. The precipitated EPS were separated by centrifugation at 5000 rpm, for 15 min, redissolved in distilled water, dialyzed with distilled water and fractionation by precipitation using 1, 2, 3 and 4 volumes of chilled absolute ethanol collected, washed by acetone, diethyl ether and dried at 60°C until constant weight (Shene et al., 2008), the main fraction was designated as EPSMSHN.

Identification of bacteria

Strain number 5, which produces elevated EPS levels, was identified on the basis of morphological, physiological, and biochemical characteristics (Cappuccino and Sherman, 2004) combined with 16S rRNA sequence analysis. The universal primers delineated by Weisburg et al. (1991) particularly ITS1 (5′-TCCGTAAGGTAGTTGTG G C-3′) and ITS4 (5′-TCTCCGCTTATGATATGC-3′), were used to amplify the 16S rRNA gene sequence. A single, discrete, polymerase chain reaction amplicon was resolved on agarose. Sequencing products were resolved on an Applied Biosystems (Foster City, CA, USA) model 3730 XL, automated DNA sequencing system. Data were submitted to GenBank and the sequence compared with the GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST (Tamur et al., 2007).

Chemical analysis

Sulfate was determined using the turbidimetric method (Dodgson and Price, 1962). Uronic acids were determined at 525 nm by the m-hydroxybiphenyl colorimetric method (Filisetti-Cozzi and Corpita, 1991). The monosaccharide composition was determined by HPLC on shim pack SCR-101N column (Shimadzu) with water deionized as the mobile phase at 0.5 mL/min (Sudhamani et al., 2004; El-Sayed, et al., 2005).

Fourier-transform infra-red (FT-IR)

The FT-IR of the EPS was measured on a Bucker scientific 500-IR Spectrophotometer (Billerica, MA, USA). The sample was mixed with KBr powder, ground and pressed into a 1 mm pellets for FTIR measurements in the range of 400-4000 cm−1 (Ray, 2006).

Determination of the molecular weight of EPS

The molecular weight of EPS was determined to an Agilent 1100 HPLC with a refractive index detector (RID), Water Company Ireland according to Jun et al. (2006). The polydispersity index (PI) calculated from the Mw/Mn magnitude relation (You et al., 2013).

In-vitro antioxidant activity using DPPH

Free radical scavenging activity was measured against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals using the method of Asker and Shawky (2010). DPPH ethanol solution (5 mL, freshly prepared) was added to 1 mL of EPS solution with deferent (Sigma-Laborzentifugen, 2K15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight at 4°C and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH (0.1M) solution and dialyzed three times against distilled water using dialysis tubing (MWCO 2000). The supernatant was completed in four volumes with ethanol 95% and left overnight at 4°C. The precipitated EPS were separated by centrifugation at 5000 rpm, for 15 min, redissolved in distilled water, dialyzed with distilled water and fractionation by precipitation using 1, 2, 3 and 4 volumes of chilled absolute ethanol collected, washed by acetone, diethyl ether and dried at 60°C until constant weight (Shene et al., 2008), the main fraction was designated as EPSMSHN.
concentrations (30-200 μg/mL) in water. Solutions were mixed vigorously and incubated at room temperature in the dark for 60 min. Supernatant absorbance was measured at 517 nm. A lower absorbance indicates higher free radical scavenging activity, as determined by graphing inhibition percentage plotted against compound concentration. All experiments were carried out in triplicate and averaged.

The scavenging activity was calculated as follows:

\[
\text{Scavenging ability (\%)} = \frac{(A_{517} \text{ of control} - A_{517} \text{ of sample})}{A_{517} \text{ of control}} \times 100.
\]

The EC$_{50}$ value is the effective concentration of EPSMSHN at which the DPPH radicals were scavenged by 50%.

**Acute toxicity study**

EPS was dissolved in distilled water then given orally to rats of both sex in graded doses up 4 g/kg. The control group received the same volumes of distilled water. The percentage mortality was recorded 24 hours later. Observation of rats for 14 days, for any changes in the skin and fur, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behavior pattern (Desoukey et al., 2016).

**Studying the hepatoprotective effects of EPS**

**Experimental design**

The animals were randomly allocated into 5 groups (6 rats in each), and treated as follow: Group I, the negative control, received 10 ml/kg of normal saline/day; group II, the positive control, received i.p. daily injection of isoniazid/rifampicin (INH/RIF) formulation at a dose of 50/100 mg/kg (Shih et al., 2013), respectively; group III, received oral doses of silymarin, the standard hepatoprotective drug (50 mg/kg/day), with the daily doses of INH/RIF as in group II; group IV and V treated with the investigated polysaccharide, administered orally by gavages tube at doses of 50 and100 mg/kg, with the daily doses of INH/RIF as in group II. All treatments lasted for 28 days. After 24 h last drug administration, the animals were sacrificed and blood samples were obtained from the retro-orbital plexus in a plain tube. Samples were left to clot and centrifuged for 5 minutes at 5000 x g using a cooling centrifuge (Sigma and Laborzentrifugen, 2k15, Germany) to obtain the serum.

**Biochemical analysis**

**Liver and kidney related parameters**

Aspartate transaminase (AST), alanine transaminase (ALT), total, direct bilirubin serum creatinine and blood urea nitrogen (BUN) were measured in serum.

**Oxidative stress biomarkers**

Tissue samples of liver and kidney were quickly homogenized for determination of lipid peroxides (Ohkawa et al., 1979) and reduced glutathione (GSH)(Ellman, 1959) contents.

**Histopathological study**

The kidneys and livers were harvested from the sacrificed rats after dissection, fixed in 10% buffered formalin (pH 7.4). The fixed specimens were sliced, processed, and embedded into paraffin blocks. The blocks were cut into 4 μm paraffin sections by a rotator microtome. The section was stained with Hematoxylin and Eosin (H&E) and with Van Gieson stains (Bancroft and Gamble, 2008).

**Morphometric measurements**

**Detection of fibrotic area**

The morphometric analysis was carried out on Van Gieson stained slides. This area was determined as an area per field in micrometer square. The results appeared automatically on the monitor in the form of a table with the total, mean, standard deviation, standard error, the minimum area and the maximum area was measured. The area of fibrosis measured in 10 fields in each slide.

**Counting of the inflammatory cells**

The morphometric analysis was carried out on H and E stained slides. First, detection and counting of the target cells (inflammatory cells) were performed. The average of counted inflammatory cells was determined by counting in 10 fields in each slide. The morphometric analysis was performed at the Pathology Department, National Research Center using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,) which consists of Leica DM-LB microscope with JVC color video camera attached to a computer system Leica Q 500IW.

**Statistical analysis**

All the results were expressed as mean ± SEM (standard error of the mean). Analyses were processed using Graph Pad Prism software for Windows (version 6.0, Graph Pad Software, Inc., San Diego, CA). The significance of difference among the studied groups was determined using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Values with P < 0.05 were considered significant. Statistical analysis for histopathological studies was performed using SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows.

**RESULTS**

**Isolation, screening, and identification of the EPS-producing bacteria**

Eighteen isolates have been isolated from the sediment of the shore of Ageeba beach (MarsaMatrooh governorate); nine isolates only produced EPS. Strain number five was selected for further studied due to its highest production of EPS (8.25 g/L). The strain showed mucous appearance on solid medium, aerobic, Gram-positive, motile, spore-forming, halophilic, catalase and oxidase positive. The partial 16S rDNA sequence was resolved from the investigated isolate, administered orally by gavages tube at doses of 50 and100 mg/kg, with the daily doses of INH/RIF as in group II. All treatments lasted for 28 days. After 24 h last drug administration, the animals were sacrificed and blood samples were obtained from the retro-orbital plexus in a plain tube. Samples were left to clot and centrifuged for 5 minutes at 5000 x g using a cooling centrifuge (Sigma and Laborzentrifugen, 2k15, Germany) to obtain the serum.

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with respect to closely related sequences available in GenBank databases was seen in (Fig. 1) So the produced EPS was named EPSMSHN.

**Antioxidant properties of EPSMSHN**

Natural antioxidants assume principle defensive parts against various infections and maturing forms because of their capacity of scavenging free radicals and binding transition metal ion catalysts and also their reductive action and ability to avert chain start. EPSMSHN exhibited the scavenging activity toward DPPH radicals in a concentration-dependent manner, with an EC_{50} value of 77 μg/mL (Fig. 2) These outcomes showed that the EPSMSHN noticeably affected scavenging free radical, particularly at high concentration. It is notable that reactive oxygen species (ROS), for example, hydroxyl radicals, superoxide anion, and hydrogen peroxide, are identified with the pathogenesis of different ailments.

**Acute toxicity study**

The results exhibited no mortality after 24 h of oral administration of EPSMSHN in rats of both sex at graded doses up to 4 g/kg. 1/80 and 1/40 (50 & 100 mg/kg) of the maximum dose (4 g/kg) were chosen to be used for the pharmacological investigation throughout the study.

**Effect on liver-related parameters**

Intraperitoneal injection of INH/RIF (50/100 mg/kg) over a period of 4 weeks significantly (p < 0.05) increased all biochemical parameters of liver function as activities of ALT and AST as well as total and direct bilirubin levels compared to the normal control. Treatment with both doses of EPS significantly decreased serum ALT and AST activities as well as total and direct bilirubin levels compared to INH/RIF control. It is worthy to mention that, the effect of EPSMSHN on the hepatotoxicity parameters was nearly similar to that of silymarin (Table 1).

**Effect on blood urea nitrogen (BUN) and serum creatinine**

INH/RIF injection induced a significant (p < 0.05) increase in BUN level and creatinine level as compared to the normal group indicating kidney injury. Treatment with EPSMSHN could normalize BUN level and reduced serum creatinine level as compared to INH/RIF control. The levels of both BUN and serum creatinine in the EPSMSHN treated groups were lower compared to that of silymarin (Table 2).

![Fig. 1: Phylogenetic tree of the partial sequence of 16S rDNA of the local isolate (Bacillus sp. MSHN2016) with respect to closely related sequences available in GenBank databases.](image)

**Fig. 1:** Scavenging effects of EPSMSHN during DPPH test and measured by changes in absorbance at 517 nm.

![Fig. 2: Scavenging effects of EPSMSHN during DPPH test and measured by changes in absorbance at 517 nm.](image)

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**Isolation and chemical structure of EPSMSHN**

The maximum yield of EPSMSHN from Bacillus sp. MSHN2016 was 8.25 g/L after 96 h. It showed up as a smooth powder, with a negative reaction to the Bradford test. The way that no retention was recognized by the UV spectra at both 260 and 280 nm showed the nonattendance of protein and additionally nucleic acids. EPSMSHN also contained 14.35% uronic acid and 18.99% sulfate. Examination by HPLC demonstrated that EPSMSHN was made out of arabinose, xylose, glucose and glucuronic acid with a relative ratio 1: 1: 2: 1, respectively. The (Mw) and (Mn) of EPSMSHN was determined to be 5.50 × 10^5 and of 3.45 × 10^5 g/mol, respectively and (P/I) was 1.59. The FT-IR has been accounted for to be a tool for observing the structural components of polysaccharides. The general appearance of the FT-IR range of EPSMSHN was common of those from polysaccharides. The EPSMSHN contained a significant number of OH groups, which may be associated with the glucuronic acid and internal hydrogen bonds. The absorption band at 1420 cm⁻¹ indicated the presence of sulfate group at 1237 cm⁻¹. The absorption at 874 cm⁻¹ indicated the α-glycosidic linkages of the fraction.

Liver damage was induced by daily injection of INH/RIF (50/100 mg/kg; I.P.) for 4 weeks. Tested agents were orally administered daily for 28 days in consonant with an injection of INH/RIF. Data were expressed as mean ± SE (n = 6). Data were analyzed by one-way ANOVA followed by Tukey comparison test. *vs normal control. **vs INH/RIF control at p < 0.05. EPSMSHN: exopolysaccharide; INH/RIF: isoniazide + rifampicin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen.
Table 2: Effect of polysaccharide on liver and kidney parameters.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Total Bilirubin (mg/dl)</th>
<th>Direct Bilirubin (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.51 ± 0.02</td>
<td>0.37 ± 0.02</td>
<td>33.68 ± 0.61</td>
<td>1.23 ± 0.01</td>
</tr>
<tr>
<td>INH/RIF</td>
<td>1.60 ± 0.09</td>
<td>1.17 ± 0.07</td>
<td>38.65 ± 0.52</td>
<td>1.71 ± 0.06</td>
</tr>
<tr>
<td>Silymarin (25 mg/kg)</td>
<td>0.78 ± 0.04</td>
<td>0.57 ± 0.02</td>
<td>36.44 ± 0.37</td>
<td>1.51 ± 0.05</td>
</tr>
<tr>
<td>EPSMSHN (50 mg/kg)</td>
<td>0.97 ± 0.03</td>
<td>0.71 ± 0.02</td>
<td>33.82 ± 0.51</td>
<td>1.45 ± 0.07</td>
</tr>
<tr>
<td>EPSMSHN (100 mg/kg)</td>
<td>0.83 ± 0.03</td>
<td>0.61 ± 0.02</td>
<td>34.76 ± 0.31</td>
<td>1.48 ± 0.07</td>
</tr>
</tbody>
</table>

Kidney damage was induced by daily injection of INH/RIF (50/100 mg/kg; I.P.) for 4 weeks. Tested agents were orally administered daily for 28 days in concomitant with an injection of INH/RIF. Data were expressed as mean ± SE (n = 6). Data were analyzed by one-way ANOVA followed by Tukey comparison test. *vs normal control. a vs INH/RIF control at p < 0.05.

**Effect on oxidative stress biomarkers**

Hepatic and renal Glutathione (GSH) contents were significantly decreased in rats treated with INH/RIF by about 1.6 and 2.7 folds, respectively. Conversely, the levels of (MDA), which is an index of lipid peroxidation, significantly (p < 0.05) increased in the livers and kidneys of rats treated with INH/RIF to about 1.7 and 2.7 folds as compared to the normal group. The study groups treated with EPSMSHN at both doses showed a significant (p < 0.05) increase in INH/RIF-induced hepatic and renal GSH depletion. Furthermore, EPSMSHN prevented INH/RIF-induced lipid peroxidation in liver and kidney tissues. The effect of 100 mg/kg EPSMSHN on hepatic and renal MDA contents was better than that of silymarin, the standard hepatoprotective and antioxidant drug (Fig. 3).
Histopathology of liver tissue and kidney

Examination of H&E stained sections from the control group (Normal control) showed the liver to have a normal histological structure of the hepatic tissue showing the normal hepatic architecture in form of hepatic lobules with the average normal thickness of hepatic cords which radiating from centrally located central vein to portal tract peripherally (Fig. 4A), the group which received INH/RIF their hepatic cells has been injured in different forms as ballooning degeneration or apoptosis and some cells appear with pyknotic nuclei most of them losing their cellular wall, Also there is portal tridaitis; inflammatory cells around portal tract with dilated congested hepatic sinusoids (Fig. 4B), On the other hand, the third group which received (silymarin 25 mg/kg) has improved in their architecture also the hepatic cells retune their normal features with moderate infiltration of inflammatory cells mainly around the portal tract (Fig. 4C), In the fourth group and fifth group which received EPSMSHN 50 mg/kg & EPSMSHN 100 mg/kg respectively looking to be improved much better than the third group as the hepatic cells mostly retune their cellular lining with minimal inflammatory cells infiltration (Fig. 4D & E).

Fig. 4: A photomicrography for five studied groups of the hepatic tissue: (C.V .); Central vein, (P.T.); Portal tract, (Straight Arrow); Inflammatory cells infiltration, (Arrow Head); Dilated congested sinusoids, (Curved Arrow); Thrombichepatic vessels, (Green Arrow) Ballooning degeneration with others showing pyknotic nuclei (H&E 200). (A): Normal group, (B): INF/RIF, (C): silymarin (25 mg/kg), (D): EPS (50 mg/kg), (E): EPS (100 mg/kg) (Stained Van Gieson X 200).

Examination of H&E stained sections from the control group (normal control) showed the renal tissue with normally appeared glomeruli and average wall thickness of the tubules lined by well-formed epithelial lining (Fig. 5A), On the other hand, examination of second group INH/RIF showed congested glomerular capillaries with shrunken glomerular tufts some of them have almost lost all their capillaries, the tubules showing edema and shedding of their epithelial lining with signs of degeneration in the form of pyknotic nuclei, vacuolation, many of tubules with intratubular debris within congested interstitial tissue and foci of fibrosis (Fig. 5C). According to the fourth group & fifth group which treated by EPSMSHN 50 mg/kg & EPSMSHN 100 mg/kg respectively the renal tissue have the evidence of normal appearance of the glomeruli also the tubules are well improved (Fig. 5D & E).

Morphometric analysis of liver and kidney tissue

The fibrosis area percentage and inflammatory cells of liver tissue are significantly increased in all other groups of rats in comparison with control group which were $3.766 \pm 0.20$ and $14.5 \pm 1.027$ respectively, on another hand its noticed that the second
group who taken INF/RIF gives the highest readings; 24.72 ± 0.15 for the fibrotic area % and 67 ± 1.107 for the inflammatory cell count denoting the degree of destruction. The third group which treated by silymarin has significantly decreased from the second group in relation to the inflammatory cell count 30.3 ± 2.80, while there was non-significant difference in the percentage of the fibrotic area from INF/RIF group; the positive control group 23.303 ± 1.41. According to the treated groups by EPSMSHN group 4 and 5; both has highly significant decline in fibrotic area percentages and the counting of inflammatory cells, which were 29.7 ± 0.42 for EPS50 mg/kg group and 25.4 ± 0.54 for EPSMSHN 100 mg/kg group in comparison with the positive control INF/RIF group also with our reference treatment silymarin group (Fig. 6 & 7). To be noticed that the fifth group EPSMSHN 100 mg/kg has better improvement, especially in fibrotic area% which was 9.063 ± 0.15, significant improvement than fourth group EPSMSHN 50 mg/kg 14.02 ± 0.26 (Table 3).

Table 3: Morphometric parameters of all studied groups of liver & kidney tissue.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liver tissue</th>
<th>Kidney tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrosis area%</td>
<td>Inf. cells count</td>
</tr>
<tr>
<td>Normal</td>
<td>3.77 ± 0.20</td>
<td>14.5 ± 1.027</td>
</tr>
<tr>
<td>INF/RIF</td>
<td>24.72 ± 0.15*</td>
<td>67.0 ± 1.107*</td>
</tr>
<tr>
<td>Silymarin (25 mg/kg)</td>
<td>23.30 ± 1.41*</td>
<td>30.3 ± 2.80**</td>
</tr>
<tr>
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<td>14.02 ± 0.26*</td>
<td>29.7 ± 0.42**</td>
</tr>
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<td>EPSMSHN (100 mg/kg)</td>
<td>9.06 ± 0.15*</td>
<td>25.4 ± 0.54**</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SE (n = 6). Data were analyzed by one-way ANOVA. *vs Normal Control. **vs INH/RIF Control at p < 0.05. EPSMSHN: exopolysaccharide; INH/RIF: isoniazide + rifampicin.

Fig. 5: A photomicrography for five studied groups of the renal tissue: (G); Normal appeared glomeruli, (G*); Congested glomeruli capillaries, (G**); Glomeruli capillaries mostly are lost, (Black Arrow); Normal appearance of tubules, (Yellow Arrow); Tubules with edematous shedded epithelial lining, (Green Arrow); Intratubular debris, (F); fibrosis, (Black Star); Congestion in interstitial tissue (H& E200). (A): Normal group, (B): INF/RIF, (C): Silymarin (25 mg/kg), (D): EPS (50 mg/kg), (E): EPS (100 mg/kg) (Stained Van Gieson X 200).
Fig. 6: The print screens of the morphometric measurement of fibrotic area in all studied groups of both liver and kidney tissue. L: Liver tissue, K: Kidney tissue, (A): Normal group, (B): INF/RIF, (C): silymarin (25 mg/kg), (D): EPS (50 mg/kg), (E): EPS (100 mg/kg) (Stained Van Gieson X 200).
According to the fibrosis area percentage and inflammatory cell count of kidney tissue the control negative group were 3.697 ± 0.62 and 4.2 ± 1.03 respectively which were significantly decreased than all other four groups (Fig. 6 & 7). While the second group which received INF/RIF gives the highest readings; in both parameters; 39.23 ± 1.15 for fibrotic area percent and 59.27 ± 1.14 for the inflammatory cell count. The third group treated by silymarin has significantly decreased from the second group in relation to the inflammatory cell count 20.2 ± 0.44 while there was non-significant difference in the percentage of the fibrotic area 32.58 ± 0.93 from the positive control INF/RIF group (Table 3). According to the treated groups by polysaccharides group 4 and 5; both have a high significant decline in fibrotic area % 17.40 ± 0.01 and 12.45 ± 0.86, respectively also it happened in the counting of inflammatory cells 15.7 ± 1.88 for fourth group EPSMSHN 50 mg/kg and 6.3 ± 2.62 for the fifth group EPSMSHN 100 mg/kg in comparison with the positive control INF/RIF group also with our reference treatment silymarin group.

**DISCUSSION**

In present work the isolated *Bacillus* sp. MSHN2016 produced EPSMSHN with a very good yield, also EPSMSHN is contained functional groups like SO$_3^−$ and COO$^-$. The $\text{Mw}$ of EPSMSHN was $5.50 \times 10^5$ g/mol. All the previous results indicate the protective role of EPSMSHN against INH/RFP-induced hepatic and renal toxicity. It was found by many authors that marine bacteria consider very good sources of EPSs if compared with the terrestrial sources (Jensen and Fenical, 1994; Laurienzo, 2010). The majority of EPSs produced by marine bacteria are heteropolysaccharides where the hydrolysis of the EPSMSHN by HPLC indicates the presence of four different monosaccharides, while monosaccharides of EPS from marine *Bacillus subtilus* were five (Ghoneim et al., 2016).

EPSMSHN is also contained different functional groups like SO$_3^−$ and COO$^-$. The percentage of sulfate and uronic differ from EPS to another wherein marine *Bacillus subtilus* the percentage of SO$_3^−$ was lower than that found in EPSMSHN, while the uronic was higher in case of *Bacillus subtilus* due to the different origin of both organisms (Casillo et al., 2018). Some EPS are neutral macromolecules, yet the larger parts are polyanionic because of the nearness of uronic acids or ketal-connected pyruvate or inorganic residues, like PO$_4^{3−}$ and/or SO$_3^−$ (Zhang et al., 2016).

It was mentioned that most polymers are linear, with an average $\text{Mw}$ ranging from $1 \times 10^3$ to $3 \times 10^4$ Da (Casillo et al., 2018). The physical properties of polysaccharides are influenced by the way and aggregation the monosaccharides. The biological activities of the EPSs based on the chemical structure and the molecular weight (Laurienzo, 2010).

Albeit many known marine bacteria can deliver EPSs, few them are of biotechnological significance (Zhang et al., 2016; Kichemazova et al., 2017).

So, in the current study, EPSMSHN could ameliorate liver and kidney damage induced by INH and RIF by decreasing serum levels of ALT, AST, total and direct bilirubin, BUN and creatinine. Hepatotoxicity arises from long-term and clinical use of INH or RIF in (TB) treatment (Nanashima et al., 2012), different metabolic and morphologic aberrations were detected after administration because of their detoxification in liver (Santhosh et al., 2007). INH/RIF combination has been evolved as an experimental model of hepatic injury. Metabolism of INH occurs through acetylation and hydrolysis and the acylated metabolites are hepatotoxins (Steele et al., 1991). Hydrazine metabolite induces cytochrome P450 (CYP450) and promotes the production of more hepatotoxins (Vuilleumier et al., 2006). Several studies have proven that RIF elevates INH toxic metabolite (hydrazine) formation. Inhibition of biliary secretion, as well as lipid peroxidation in the liver tissue, is aggravated by this combination (Zhang et al., 2012). In the present study, INH and RIF injection produced signs of hepatotoxicity as indicated by increased ALT, AST, total and direct bilirubin. These findings are in accordance with previous data (Santhosh et al., 2006; Obogwu et al., 2014; Kim et al., 2017).

INH/RIF combination also elevated renal function tests, BUN and serum creatinine. Experimental nephrotoxicity was not previously documented except in the study by Hashmi et al. (2013), who found that INH/RIF induced significant renal damage to rabbits as evidenced by elevated concentrations of the BUN and serum creatinine. Renal toxicity of RIF has been reported sporadically, however, some authors have reported clinical cases...
occurring during continuous RIF therapy (Muthukumar et al., 2002; Yanardag et al., 2005). Oxidative stress has been implicated in the INH/RIF hepatotoxic effect (Cederbaum, 2006) via endogenous lipids peroxidation with the subsequent cell membrane destruction (Santhosh et al., 2007). In the current study, the combined anti TB drugs increased lipid peroxidation and decreased GSH (the endogenous antioxidant) contents in the liver, this is in agreement with other results (Tasduq et al., 2005; Saad et al., 2010), and for the first time in kidney tissues.

Treatment with EPSMSHN prevented the incidence of hepatocellular apoptosis and degeneration and fibrosis in many tubules also promoted tissue recovery due to liver and kidney injury. Li et al. (2006) studied the mechanism of polysaccharides from Cordyceps sp. resistance on dimethyl-induced liver fibrosis through elevation of liver Kupffer cell function, toxic substances removal as well as reducing the immune and inflammatory response (Fang et al., 1997).

Bioactivities of EPSs were found to be ascribed to their individual structure, such as the type of glycosyl units, the configuration of glycosidic bonds, and the substituent of the EPSs. Also, these biological effects are closely related to the spatial structure and molecular weight (Tsiaipali et al., 2001). Therefore, the anti-oxidative activity of the EPS may not be a result of a single factor, but a combination of different factors, most importantly variation in monosaccharide composition, structure configuration, and mode of attending glycosidic bonds and other structural characteristics of the EPS (Moure et al., 2006).

In the present work, EPSMSHN could ameliorate liver and kidney lipid peroxidation and increased GSH contents induced by INH and RIF. In addition, EPSMSHN could attenuate liver inflammation and kidney fibrosis as shown in our histopathological study compared to the control group and better than silymarin.

CONCLUSION

EPSMSHN produced from marine Bacillus sp. MSHN2016 at both doses protected from hepatic and renal toxicity induced by INH/RFP through its antioxidant and antifibrotic influence; therefore, it can be used as neutraceutical for treatment of hepatic and renal diseases.

CONFLICT OF INTEREST

None declared.

REFERENCES


Nanashima K, Mawatari T, Tahara N, Higuchi N, Nakaura A. Genetic variants in antioxidant pathway: risk factors for hepatotoxicity in
Selim et al. / Journal of Applied Pharmaceutical Science 8 (08); 2018: 001-011

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