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In vitro evaluation of antioxidant defense mechanism and hemocompatibility of mauran

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ABSTRACT

Mauran (MR), a highly polyanionic sulfated exopolysaccharide was extracted from moderately halophilic bacterium; *Halomonas maura* and characterized using X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy. Purified MR was evaluated for antioxidant defense mechanisms under *in vitro* conditions using L929, mouse fibroblast cell line and mice liver homogenate. It was demonstrated that MR could impart protective effect against oxidative stress in both cells and tissue up to a concentration of 500 μg, which is found to be safe under laboratory conditions. Various enzymatic and non-enzymatic parameters of antioxidant mechanisms were evaluated and concluded that MR has the tendency to maintain a balance of antioxidative enzymes with in the test systems studied. Also, hemocompatibility assay performed revealed that MR has a lesser hemolytic index and exhibited a prolonged clotting time, which shows both antihemolytic, and antithrombogenic nature respectively. Furthermore, absorption studies performed using fluorescent-labeled MR confirmed that MR accumulated within the cell cytoplasm neither induced cellular lysis nor affected the cell integrity.

1. Introduction

Extreme habitats are well known for its endless source of industrially important molecules (Galinski & Tindall, 1992). Microorganisms that are able to thrive under extreme stress conditions are studied utmost in the field of modern pharmaceutical research. *Halomonas* are a class of moderately halophilic bacteria that can with stand 5–25% of salt concentration (Llamas et al., 2006; Mata et al., 2006; Argandona et al., 2005). *Halomonas maura* can produce a versatile, polyanionic, sulfated exopolysaccharide called mauran (MR) (Bouchotroch, Quesada, Moral, Llamas, & Bejar, 2001; Arco et al., 2005). This acidic polysaccharide has unique physicochemical properties like stable structure, composition, fluid dynamics, extreme stability, biodegradability and biocompatibility (Arias et al., 2003); they are widely exploited in modern biotechnology, material science and more recently in nanotechnology (Raveendran, Dhandayuthapani, et al., 2013). Owing to their exceptionally best viscoelastic properties and fluid dynamics, mauran has been successfully employed in the nanoparticle synthesis and application for sustained drug delivery, cancer chemotherapy and bioimaging (Raveendran, Poulose, Yoshida, Maekawa, & Kumar, 2013). MR based tissue-engineering nanofiber scaffolds were investigated for enhanced cell proliferation and migration under *in vitro* conditions using various mammalian cell lines (Raveendran, Dhandayuthapani, et al., 2013). As a widely accepted biomolecule, with a lot of potential applications in the biomedical and pharmaceutical industries, mauran can be studied as a vital candidate biodrug for evaluation of various bioactivities (Llamas et al., 2006).

Polysaccharides with high sulfate contents are generally known for various biopharmaceutical activities like anticancer, antiviral, antiparasitic, anticoagulant, and antioxidant properties (Kodali, Perali, & Sen, 2011; Raveendran, Yoshida, Maekawa, & Kumar, 2013; Toshihiko, Amornrut, & Robert, 2003). However, evaluation of a bacterial polysaccharide for specific bioactive mechanisms are always challenging due to their immunogenic nature. In spite of being a polysaccharide of bacterial origin, MR has an excellent biocompatibility and biodegradability with enhanced cell proliferation effect. Furthermore, MR has been widely employed in the commercial level for industrial, food and pharmaceutical applications. Medically they are found important since they posses'

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immunomodulating and antiproliferative effect on cancer cells (Llamas et al., 2006). Enhanced accumulation of free radicals with in the cells causes abnormal necrosis and cell death. The phenomenon of an excess free radical generation and related abnormal tissue mechanisms causes oxidative stress (Surendran, Geetha, & Mohanan, 2012). As a result, various macromolecules like DNA, proteins and lipids are damaged nonspecifically (Anjana, Tinu, Geetha, & Mohanan, 2012; Mohanan & Yamamoto, 2002). Natural antioxidant defense mechanism can be restored and ameliorated with the help of various biologically active polysaccharides irrespective of their source of extraction (Guerra Dore et al., 2013; Kodali et al., 2011 Silva et al., 2005; Wang, Wang, Liu, Yuan, & Yue, 2013; Yan et al., 2012). The major enzymes that play a vital role in antioxidant defense mechanisms by catalyzing various free radicals include glutathione reductase, glutathione peroxidase and superoxide dismutase. These enzymes differ in their structure, tissue distribution and cofactor requirement (Arun, Silija, Sheeja, Geetha, & Mohanan, 2012).

In the present study, we are evaluating the protective antioxidant effect of MR by analysis of various parameters responsible for oxidative stress in mammalian cells and tissues separately. Apart from that hemocompatibility and absorption studies were also concentrated.

2. Materials and methods

2.1. Chemicals and reagents

Thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), glutathione reduced (GSH), glutathione oxidized (GSSG), dithio-bis-2-nitrobenzoic acid (DTNB), hydrogen peroxide (H_2O_2), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and ethylene diamine tetra acetic acid (EDTA) were procured from Sigma–Aldrich, USA and BCA reagent kit (Qiagen, Germany). Sypro-Ruby was purchased from Invitrogen, USA. All other chemicals and reagents used were of analytical grade.

2.2. Equipments

Rotor stator homogenizer at 1000 rpm, Refrigerated centrifuge (Himac, CF12RX), UV–Visible spectrophotometer (CU730, Beckman Coulter) and Confocal laser scanning microscope (Olympus 81 under DU897 mode).

2.3. Bacterial culture and MR production

The strain was grown in MY medium as mentioned elsewhere (Arias et al., 2003). Briefly, the growth medium composition: NaCl, 51.3 g; $MgCl_2{\cdot}6H_2O$, 9 g; $MgSO_4{\cdot}7H_2O$, 13 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; KCl, 1.3 g; $NaHCO_3$, 0.05 g; NaBr, 0.15 g; $FeCl_3{\cdot}6H_2O$, traces; Glucose, 10 g; Yeast extract, 3 g; Malt extract, 3 g; Proteose peptone, 5 g; Trace salt solution, 0.00325 g. Bacto agar (2 g/L) was added for the preparation of solid medium. Liquid medium was prepared, sterilized and inoculated with 1 ml of 48 h culture grown in the same medium (OD_{520} = 2.5) and incubated at 32 °C in a rotary shaker at 110 rpm for 15 days. Bacterial growth and EPS production were monitored in batch cultures of 500 ml Erlenmeyer flasks with 100 ml of medium in each. At the end of incubation culture was centrifuged using an ultracentrifuge, Himac, CF12RX at 12,000 rpm for 1 h at 4 °C. Supernatant was precipitated out with cold ethanol and again centrifuged. Pellet was dissolved in ultra pure distilled water and purified using dialysis against distilled water (3–4 exchanges) for 48 h using Snakeskin pleated dialysis tubing from Thermo scientific of 10,000 MWCO. Purified MR was freeze dried and subjected to evaluate the antioxidant activity.

2.4. Electron microscopy of H. maura

Bacterial cells were taken from mid exponential phase culture of *H. maura* for electron micrographic study of the EPS layering the bacterial cell. Ultra thin sectioning was performed as mentioned elsewhere (Bouchotroch et al., 2001) with slight modifications in the proto-col and viewed through transmission electron microscope (TEM) (JEOL, JEM-2200FS) and images were recorded.

2.5. Preparation of mammalian cell and tissue homogenates

L929, mouse fibroblast cell line homogenate and mice liver homogenates were used for testing the *in vitro* antioxidant activity of MR. L929 cells were cultured and maintained using DMEM, supplemented with 10% of FBS at 37 °C in a 5% CO_2 atmosphere. Cells were trypsinized after attaining confluent growth and collected separately. L929 cells were diluted using normal saline, in an order of 2×10^5 cells/ml and mixed with various concentrations of MR. Liver homogenate was prepared using fresh liver isolated from rat. 10% liver homogenate were made in ice-cold normal saline using a glass-homogenizing vessel in a rotor stator homogenizer at 1000 rpm. The suspended mixture was maintained in an ice bath until used for various antioxidant assays and total protein estimation. Standard protocols with slight modifications were used for assaying antioxidant enzymes. Effect of oxidation stress and the amount of its inhibition by MR was evaluated using optical density (OD) measurement by UV–visible spectrophotometer.

2.6. Total protein assay

Determination of total protein concentration in cell and liver homogenates were performed using bicinchonic acid assay method (Smith et al., 1985) with bovine serum albumin as standard and expressed in mg/ml.

2.7. Lipid peroxidation (LPO) assay

Cell membrane damage is characterized with the oxidation of the lipids, released from the tissue or cells. As a result of free radical generation, electrons will be removed from the cellular membrane, causing a rupture that result in the lipid peroxidation. LPO assay characterizes the degradation of the TBA and the concentration of melondialdehyde reacted *in vitro* to give a fluorescent product that can be measured at 532 nm. LPO assay or thiobarbituric acid reactive substances (TBARS) assay were performed using L929 cell and mice liver homogenate, as described by Ohkawa, Ohishi, and Yagi (1979). Briefly, 1.5 ml of 0.8% of TBA was mixed with 0.2 ml of 8.1% of SDS and 1.5 ml of 20% acetic acid. Above mixture was added with 0.2 ml of test sample and volume was adjusted using deionized water. The reaction mixture was kept in a boiling water bath for 1 h and cooled using tap water. 1 ml of deionized water was added and centrifuged at 3500 rpm for 10 min. Supernatant was collected to measure the OD value.

2.8. Reduced glutathione (GSH) assay

Moron et al., method was used to assay the GSH level present in the cell and tissue homogenate with a slight modification (Moron, Depierre, & Mannervik, 1979); DTNB was used to react with GSH to form a spectrophotometrically detectable product. Briefly, 0.5 ml of the supernatant of cell and tissue homogenate obtained after centrifugation at 3500 rpm for 10 min at 4 °C were mixed with 4 ml of 0.2 M Phosphate buffer solution (PBS), respectively and the total volume was adjusted using deionized water. 0.5 ml of DTNB was added to the mixture, prior to the measurement of absorbance at 412 nm. The change in absorbance is a linear function of the GSH

concentration in the reaction mixture. The amount of GSH was expressed in nmol/mg of protein.

2.9. Glutathione reductase (GR) assay

As an important cellular antioxidant, GSH is formed during the reduction of glutathione disulfide to its sulfhydryl form. One mole of NADPH is required for every single reaction that converts oxidized glutathione (GSSG) to its reduced form of GSH. In GSH assay, the conversion of NADPH to $NADP^+$ is measured spectrophotometrically at 340 nm. Mize and Langdon method was followed to evaluate the reduction process (Mize and Langdon, 1962). Here, 0.5 ml of 0.5 mM EDTA and 0.15 ml of 20 mM GSSG was mixed with 2 ml of 0.1 M PBS and incubated at 37 °C for 10 min. At the end of incubation, 0.15 ml of 20 mM NADPH and 0.1 ml of sample were added to the reaction mixture and OD was measured at 0, 1, 2, and 3 min.

2.10. Glutathione peroxidase (GPx) assay

GPx activity was evaluated using the method described by Rotruck, Pope, Ganther, Hafeman, & Hoekstra (1973). GPx plays an important role in preventing oxidative damage by converting free H_2O_2 to water and to reduce the lipid peroxides to its corresponding alcohols forms. The GSH remaining after the enzyme-catalyzed reaction was complexed with DTNB, which has an absorption maximum at 412 nm. Enzyme activity was expressed as μg of GSH consumed/min/mg of protein.

2.11. Superoxide dismutase (SOD) assay

SOD assay was performed using L929 cells and liver tissue homogenate using modified pyrogallol autooxidation method. The assay measures the amount of inhibition of autooxidation of pyrogallol by SOD activity with in the cells and tissue homogenate, which is read spectrophotometrically at 420 nm (Nandi & Chatterjee, 1988).

2.12. Hemolytic assay

This is a colorimetric assay for determining the plasma free hemoglobin when blood is exposed to polysaccharide, MR. In this assay heparinized human blood is used to check the hemolytic property of MR. Hemolysis test is considered as a very simple and reliable measure of analyzing the blood compatibility of materials (Shelma & Sharma, 2011). Hemolysis is damage to red blood cells (RBCs), which results in the release of the hemoglobin into the plasma. It is a potentially life-threatening condition, as it may result in anemia and jaundice. Due to the unique physicochemical properties and bioactive nature of MR, their interaction with RBC may differ from other toxic materials. The method used for this experiment is cyanomethaemoglobin method (Shelma & Sharma, 2011). 40% poly ethylene glycol (PEG) is used as negative control and 10% solution of Triton X 100 in water is used as positive control. To evaluate total Hb, 5 ml of cyanomethaemoglobin reagent was mixed with 20 μl of blood and incubated for 3 min and absorbance was measured at 540 nm. To measure the plasma Hb, 3 ml of blood was centrifuged at 800 g for 15 min and 1 ml of plasma was mixed with 1 ml of cyanmethemoglobin reagent. The mixture was incubated for 3 min and OD was measured at 540 nm. Hemolytic assay was performed in a microplate. 100 μl of various MR concentrations were mixed with equal volume of blood and 600 μl of PBS and incubated at 37° C for 3 h. The mixture was centrifuged at 800 g for 15 min and 100 μl of the supernatant was mixed with 100 μl of cyanomethaemoglobin reagent in a microplate. After 3 min incubation the plate was read at 540 nm. Percentage of hemolysis was calculated using the following formula:

$$\%\text{hemolysis} = \frac{\text{Supernatant Hb released} \times 100 \times 8(\text{dilution factor})}{\text{Total Hb conc.of dil. blood}}$$

2.13. Whole blood clotting time

In vitro examination of blood clotting ability is widely used for diagnosing clotting disorders in the medical field (Shelma & Sharma, 2011). Human blood collected in 3.8% of sodium citrate (1:9) was incubated with different concentrations (equal volume) of MR and incubated at 37 °C for 15 min. 50 μl of this mixture was mixed with 25 μl of 50 mM calcium chloride. The time at which first clot appears was noted.

2.14. MR absorption studies – confocal microscopy

MR was fluorescently tagged with Sypro ruby, biofilm matrix stain for investigating the adhesion and absorption of MR by mammalian cell line using confocal microscopy (Raveendran, Dhandayuthapani, et al., 2013). Glass base confocal plates were used for imaging the cells treated with fluorescent labeled MR. L929, cells were used for demonstrating the adhesion and absorption of MR. Approximately $5–6 \times 10^3$ cells were seeded in the confocal plates and incubated for attaining a confluent growth. Fluorescent tagged MR was added to culture plate and incubated for 24 h. At the end of incubation unbound and free MR was washed with 1× PBS. Cells were then treated with DAPI and mitotracker green for 30 min; excess stains were removed using PBS. Finally, 300 μl of PBS was added to the cells to prevent drying and viewed through confocal laser scanning microscope (Olympus 81 under DU897 mode) using 488 nm laser line for SR, 490 nm for mitotracker green and 405 nm for DAPI.

3. Results and discussions

In the present study, MR was extracted from moderately halophilic bacterium, *Halomonas maura* and assayed for potential biological properties including antioxidant, antihemolytic and anticoagulant activities. Electron microscopy studies of *H. maura* revealed the adherence of MR as an external sheath to the cell wall of bacteria giving a beaded blanket like appearance. Fig. 1 shows the TEM image of ultra thin section of 4 days old *H. maura* with MR surrounding the bacteria. MR extracted using cold ethanol method was then subjected to purification *via* dialysis and dried using lyophilization. Freeze dried MR was used for characterization as well as for performing various assays.

On chemical analysis using colorimetric reactions, it was found that the total carbohydrate concentration in MR extracted was $73 \pm 0.02\%$ and protein content was about $2.9 \pm 0.5\%$. As already reported by Arias et al. (2003), MR is an anionic sulfated polysaccharide (SPS) of average molecular weight of 4.7×10^6 Da with high uronic acid content. MR produced under optimal conditions posses' glucose, mannose, galactose and galacturonic acid as four constituent sugar components (Arias et al., 2003). It also has a high sulfate content of 6.5% w/w that contributes to its polyanionic nature. Furthermore, MR also contains 1.3% of phosphate residues (Arias et al., 2003). Fig. 2A shows the characteristic ESCA peaks of elements in MR. The presence of S and P was confirmed by elemental analysis of MR using XPS. The peak showing at 168 eV depicts sulfate peak of MR. Polysaccharide structure and the presence of sulfate moiety was estimated using FTIR spectra of MR in Fig. 2B. As common to all polysaccharides two characteristic peaks

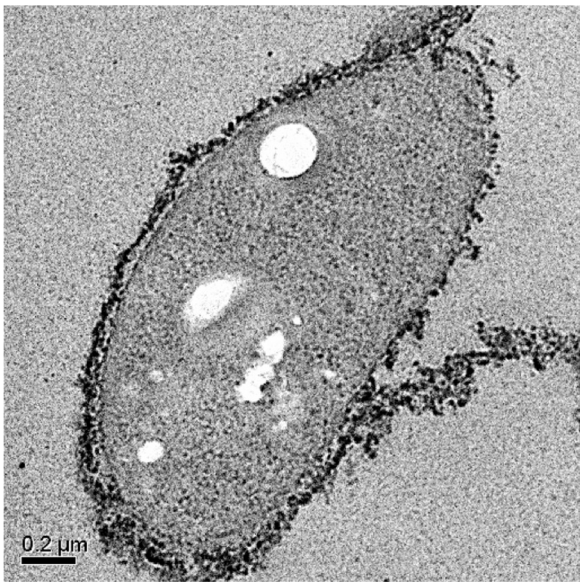

Fig. 1. TEM image of ultra thin section of *Halomonas maura* showing the accumulation of MR surrounding the cell wall.

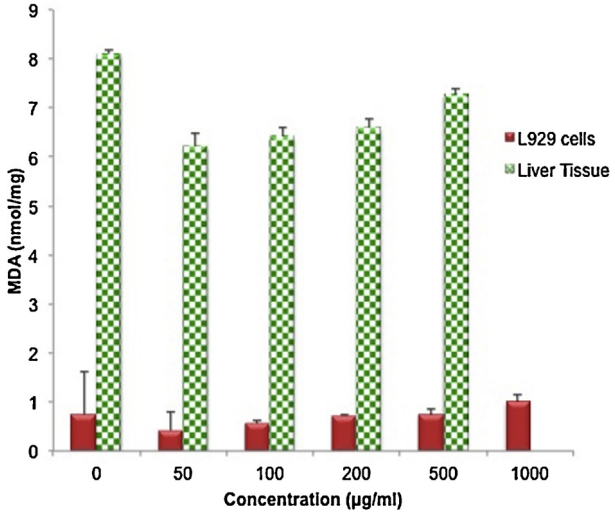

Fig. 3. Levels of malondialdehyde (MDA) in L929 cell lines and liver homogenate incubated with MR at different concentrations. Values are expressed as mean: SE.

appeared at 3432 and 2932 cm^{-1} assigned to O—H and C—H stretching vibrations respectively. Similarly, strong absorption bands at 1131 and 1057 cm^{-1} may be assigned to C—O and C—C stretching in the pyranoid ring. In addition, the band at 979 cm^{-1} can be due to C—O—C stretching of glycosidic linkages. Two strong peaks at 1739 and 1650 cm^{-1} can be assigned to carbonyl groups in two respective forms, carboxylic ester (C=O) form and carboxylate anionic (COO^-) form. These carboxylic stretching vibrations can be from the uronic acid residues in MR. Spectral vibration at 1419 cm^{-1} may be assigned to C—OH deformation vibration of carboxylate symmetric stretching. The sharp peak at 1261 cm^{-1} and its shoulder vibration at 1225 cm^{-1} confirm the presence of sulfate ester groups (S=O), which is a characteristic component of MR (Raveendran, Dhandayuthapani, et al., 2013; Gomez-Ordonez & Ruperez, 2011; Dev et al., 2010).

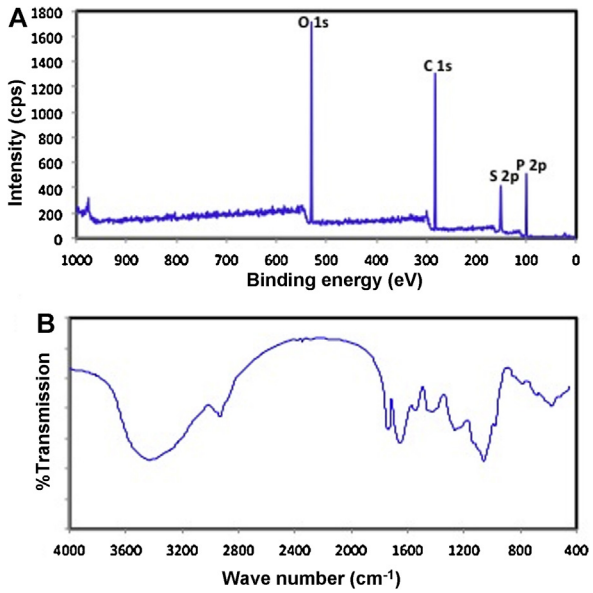

Fig. 2. (A) XPS spectrum of MR showing various elemental composition. (B) FTIR spectrum of MR.

Ability of MR to exhibit antioxidant defense mechanism was evaluated using lipid peroxidation (LPO), reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) and super oxide dismutase (SOD) assays. The amount of LPO occurred in case of both L929 cells and liver tissue homogenate can be compared to each other though there is noticeable difference in the amount of MDA formation. Incubation of L929 cells with five different concentrations of MR revealed that the level of MDA formed were lesser than the control, which is around 0.746 nmol/mg. However, there was a slight increase in the case of 1 mg of MR concentration where the MDA content raised to 1.007 ± 0.133 nmol/mg showing a significant level of LPO. Where as in the case of liver tissue homogenate, the values were well correlated with the control value showing that there is a significant amount of free radical scavenging by MR up to the highest test concentration; that is 1 mg/ml. Fig. 3 depicts the concentration of MDA formed during TBARS assay using L929 cells and liver homogenate. It is confirmed from the graph that MR induces antioxidant property by preventing the LPO and free radical generation. However, the difference observed in the cells and the tissue homogenate may be due to the inconsistency of the two different test systems (Weydert & Cullen, 2010). It was well known that LPO is generated naturally in small amounts in the body, mainly by the effect of several reactive oxygen species or by the action of several phagocytes (Surendran et al., 2012; Arun, Silija, & Mohanan, 2011). It was established from the present study that, the test sample MR does not induce reactive oxygen species, which may attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids if happened by the action of MR, the end products of such LPO reactions are dangerous for the viability of cells or tissues and lead to the pathogenesis of several disease. This reaction was not happened when MR was treated with cell lines or tissue homogenate, which clearly indicates the cytocompatibility of MR under *in vitro* conditions.

Fig. 4 shows the levels of GSH produced in cell line and liver homogenate. The concentration of GSH in the control of L929 cells was found to be 0.157 ± 0.07 nmol/mg. Thus, the GSH concentration in all test concentrations was below the control level showing a minimal oxidation of GSH. However, in 1000 µg/ml concentration of MR, the GSH level is 0.124 ± 0.008 nmol/mg, indicating a tendency to reach the control limit. In case of liver homogenate, the test values were significantly higher than the control, indicating a progressive antioxidant effect as the concentration of MR increases.

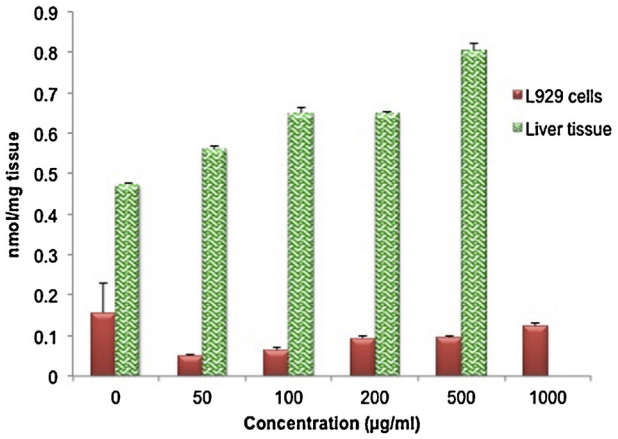

Fig. 4. Levels of reduced glutathione in L929 cell lines and liver homogenate incubated with MR. Values are expressed as mean: SE.

It is well known that glutathione protects cells from the free radicals produced through oxidation. GSH is an antioxidant found in every cell in the body and is widely known for controlling free radicals (Anjana et al., 2012). The majority of glutathione present in the body is in its reduced form, so that it is readily available to neutralize free radicals (if any) by bonding with them and converts to its oxidized form. The results of GSH assay shows that MR is not influencing the action of GSH in cells, whereas it shows some protective action on oxidative injury in tissue homogenate. This deviation may be due to cell specific reactions and reduced amount of protein in cell line homogenate.

GR is a homodimeric flavoprotein that is required for the conversion of GSSG to GSH. In the majority of eukaryotic cells, GSR upholds the ratio of GSH/GSSG, and take part in the detoxification of reactive oxygen species as well as protein and DNA biosynthesis (Anjana et al., 2012). Fig. 5 shows the level of GR in the control and various test concentrations of MR incubated with cell line and liver tissue homogenate. The results of the study suggests that the concentration of GR was not exceeding the control value, which is 0.009 ± 0.0002 and 0.089 ± 0.003 units/mg of protein, in both cases of cell line and tissue homogenate respectively. However, the highest concentration of MR in cell line homogenate showed a slight increase in the GR level compared to the control. Whereas in all other concentrations, the values were found insignificant compared to control level. The results indicated that the test sample MR

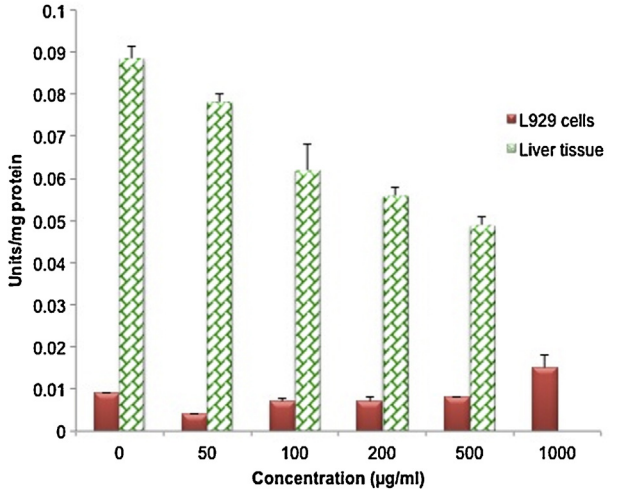

Fig. 5. Levels of glutathione reductase in L929 cell lines and liver homogenate incubated with MR. Values are expressed as mean: SE.

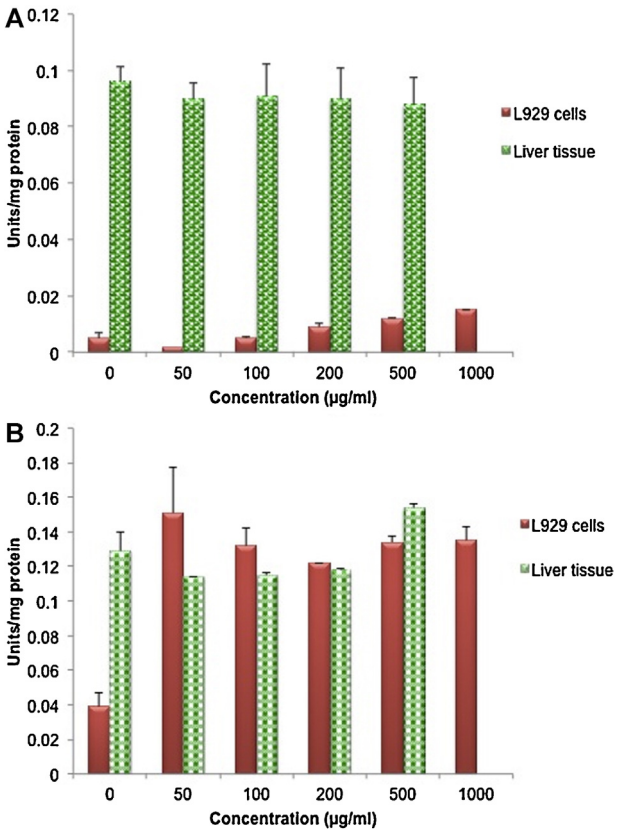

Fig. 6. (A) Levels of glutathione peroxidase in L929 cell lines and liver homogenate incubated with MR. (B) Levels of SOD in L929 cell lines and liver homogenate incubated with MR Values are expressed in mean: SE.

is not inhibiting the conversion of GSSG to GSH up to the concentration of 500 µg in both cell and tissue homogenates.

It was well established that the GPx functions in the scavenging and inactivating of hydrogen and lipid peroxides, thereby protecting the body against oxidative stress. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Anjana et al., 2012; Surendran et al., 2012). Fig. 6A demonstrates the level of GPx in MR treated cell lines and tissue homogenate. It is evident from the figure that there is an insignificant level of increase till 100 µg/ml concentration, beyond which there is gradual increase in the GPx concentration in cells. Where as in the case of liver homogenate it was observed that the MR polysaccharide could positively maintain a decreased level of GPx in all test concentrations below the control value of 0.096 ± 0.005 units/mg protein. Hence the results of the study cleared that test sample MR is not influenced on the action of GPx. However, the inconsistency observed in the case of cell line may be due to low protein content (Weydert & Cullen, 2010).

Fig. 6B shows the units of SOD per mg of protein content. SOD is an enzyme found in all living cells that speeds up certain chemical reactions and catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. SOD can cause mutations in DNA or attack enzymes that make amino acids and other essential molecules (Nandi & Chatterjee, 1988). Thus, they are an important antioxidant defense in all cells exposed to oxygen. It helps break down potentially harmful oxygen molecules in cells, which might prevent damage to tissues. The results indicated that, there was a slight increase in the SOD production, when MR was exposed to cell lines. However, the enzyme level was comparable to control values when MR exposed to tissue homogenates. A slight increase was

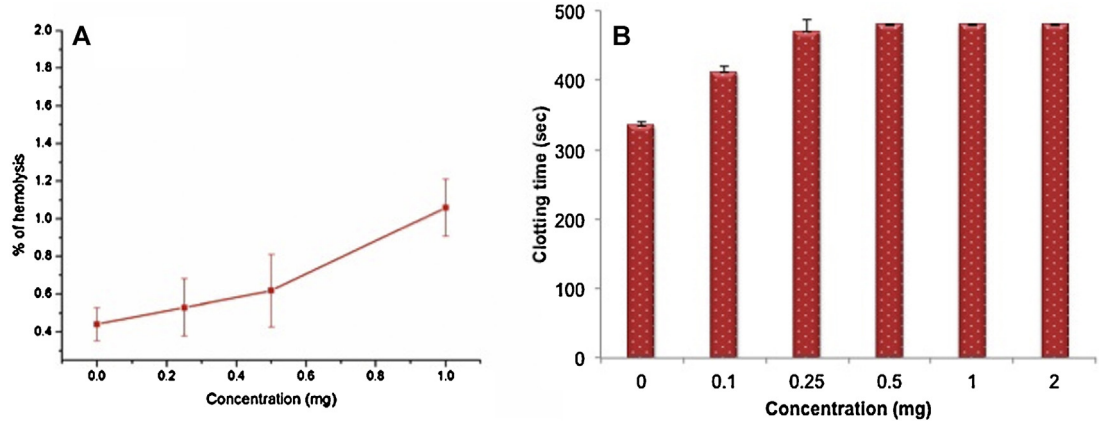

Fig. 7. (A) Percentage of hemolysis exhibited by MR, compared with the positive control value of 78.994 ± 0.274. Standard acceptable range is 5%; (B) Whole blood clotting time exhibited by MR; normal clotting time: 4–8 min (240– 480 s).

Table 1
Whole blood clotting time shown by various concentrations of MR in comparison with control.

MR concentration						
	Control	2 mg/ml	1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.1 mg/ml
Seconds	330	>480	>480	>480	450	420
	335	>480	>480	>480	480	405
	340	>480	>480	>480	480	410
Mean	335	>480	>480	>480	470	411.66
SD	5	0	0	0	17.32	7.63

100 μg/ml (0.1 mg/ml) normal. From 0.25 mg/ml (250 μg/ml) it is interfering in clotting time (prolonged clotting time).

observed at 500 μg concentration with liver homogenate, which is considered to be insignificant in nature. The fluctuation observed in the SOD values of the test concentrations may be due to cell line specificity.

Percentage of hemolysis was measured to demonstrate the hemocompatibility of the MR when treated with mammalian blood. Fig. 7A depicts the hemolytic index of the MR at different concentrations. It is evident from the curve that the highest test concentration, 1 mg/ml shows only 1.059 ± 0.263%, which is far below the acceptable standard range of 5% (ASTEM, 2008). Thus, it is confirmed that MR does not induce any rupturing of red blood cells rather keeps the integrity of the cells. Similarly, the antithrombogenic property of the MR was evaluated using human whole blood (Fig. 7B). Table 1 shows the time taken for whole blood clotting in the presence of various concentrations of MR. It was shown from the result that MR is not interfering with the thrombin to prothrombin activity to a concentration up to 0.1 mg/ml. However, a prolonged clotting time was observed from the concentration 0.25 mg/ml onwards.

MR absorption studies were demonstrated using confocal microscopy. Fluorescent-tagged MR was treated with L929 cells for 24 h and images were recorded. Fig. 8 shows that L929 cells

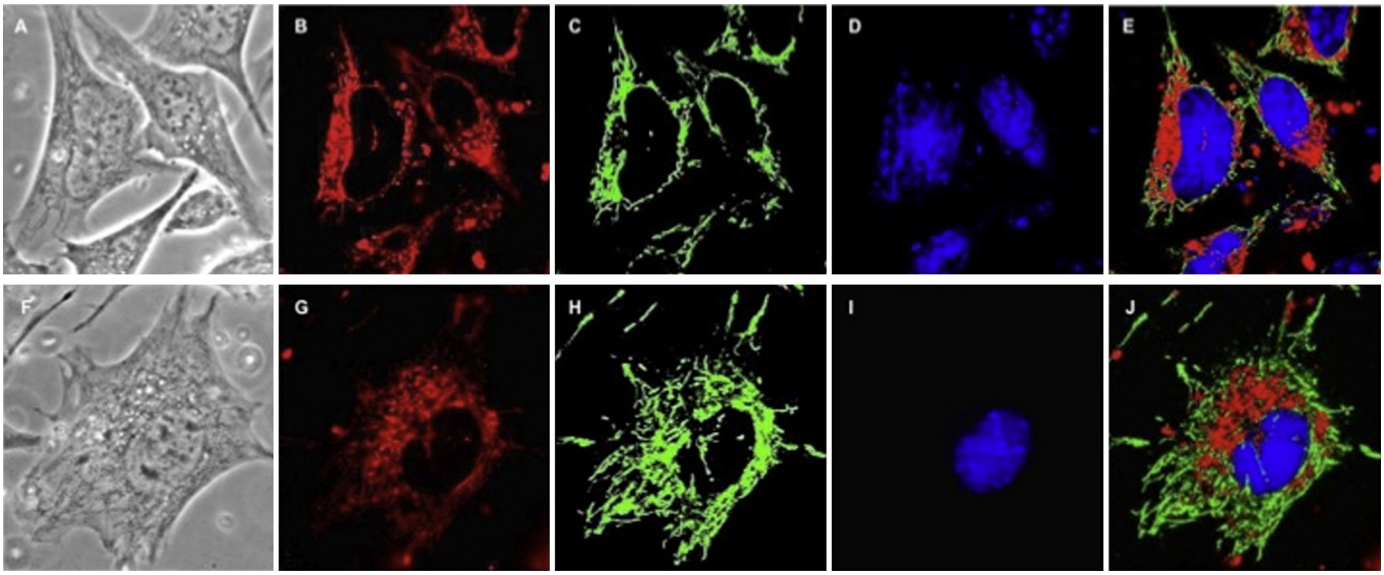

Fig. 8. MR absorption studies using confocal microscopy. (A and F) Bright field images of L929 cells. (B and G) Sypro-ruby tagged MR accumulation in cells. (C and H) Mitotracker green stained cells. (D & I) DAPI stained nucleus. (E and J) Merged images.

have effectively absorbed MR. Fig. 8A and F shows the bright field images of L929 cells. Fig. 8B and G depicts the presence of sypro-ruby tagged MR within the cell body. Whereas, C & H as well as D and I shows the mitotracker and DAPI stained images respectively. Finally the Fig. 8E and J shows the merged images. It is evident from the confocal studies that MR is capable of maintaining the cell integrity even after 24 h of incubation with mammalian cell line. This supports the antioxidant results of MR. Under laboratory conditions, MR is not inducing any oxidative damage to the cells, and they maintain the cell viability and structural integrity (Raveendran, Dhandayuthapani, et al., 2013).

Cellular oxidative stress occurs due to the imbalance in the generation of free radicals and lack of antioxidant defense mechanisms. As a result, cellular and molecular level damages arise leading to enormous necrosis and cell death. Hydroxyl radicals are most devastating and hepatic tissue damage is mainly associated with lipid peroxidation. Other than that glutathione plays a critical role in maintaining the equilibrium of oxidative defense mechanisms (Anjana et al., 2012). Here it is well understood that the action of MR at various concentrations are capable of providing defense against oxidative stress under laboratory conditions within cells as well as in tissue homogenate. Also, antihemolytic and antithrombogenic effect of MR was demonstrated and confirms that it is safe up to the highest dosage level demonstrated in this study; *in vivo* studies are required to confirm the efficacy and toxicity of MR to compare with other commercially available sulfated polysaccharides. However, the preliminary results obtained through the *in vitro* studies clearly demonstrate that MR, sulfated polysaccharide from *H. maura* would be highly efficient in supporting the antioxidant defense mechanisms.

4. Conclusion

In conclusion, it was investigated that the MR, polysaccharide extracted from *H. maura* was characterized and has been evaluated for antioxidant defense mechanism along with hemocompatibility studies. Results suggest that the application of MR does not induce excessive generation of free radicals in cell line and tissue homogenate, compared to the control of our study. It was observed that there were no significant variations in the levels of both enzymatic and non-enzymatic antioxidants under various test concentrations of MR. However, the slight fluctuations observed while comparing the cell and tissue homogenate may be due to the inconsistency of the two test systems. Cell viability and absorption studies was performed using confocal imaging that supports the antioxidant results, showing that MR is not inducing any oxidative damage to the cells. Hence we can conclude that MR can effectively contribute to antioxidant defense mechanism and it was found to be safe up to a concentration of 500 μg under laboratory conditions. Percentage of hemolytic index and prolonged clotting time suggests that MR posses a dose dependent effect on antihemolytic and antithrombogenic properties. Hereby, it is expected that MR based derivatives can be ideally developed for various antioxidant biodrugs after specific *in vivo* evaluation.

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