

## Protective Effects of Rosemary Extract Against Microcystin-Lr-Induced Liver Injury in Balb/c Mice

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**Abstract:** The aim of this study was to investigate the potential benefits of dietary supplementation of rosemary (*Rosmarinus officinalis*) extract (ROE) as antioxidant on microcystin-LR toxicity in mouse liver and kidney. The LD<sub>50</sub> value for MC-LR, measured intraperitoneally (i.p.) in Balb/c mice was found to be 56 µg/kg mouse body weight. Mice were pretreated orally for two weeks with ROE (400 mg/kg mouse body weight / day). The results showed that ROE completely abolished the lethal effects of MC-LR toxin and significantly decreased the levels of serum enzymes, alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and inhibited protein phosphatase (PP1), lipid peroxidation (LPO), protein oxidation and DNA-protein cross links. Also ROE reactivated catalase (CAT) and superoxide dismutase (SOD). Therefore, dietary supplementation of rosemary may have protective effects against chronic exposure to MC-LR.

**Key words:** Rosemary • Chemoprotectants • Microcystin • Toxicity

### INTRODUCTION

Contamination of natural waters by cyanobacterial blooms is a worldwide problem, causing water pollution and public health hazard to humans' livestock [1-2]. Blooms of cyanobacteria in ponds and water reservoirs have been associated with acute, usually lethal toxicity in various species of domestic animals and wildlife in addition to human due to the entrance of the toxins in the aquatic environment after cell lysis [3-4].

Microcystins (MCs) are the most commonly found group of cyanotoxins and more than 80 variants are known [5-6] many of which are potent hepatotoxins, with microcystin-LR (MC-LR) being one of the most abundant and toxic variants in blooms [7]. Acute toxicity of potent MCs variants in mammals includes liver necrosis and MCs act as tumour promoters [8-11] and the exact mechanisms by which microcystins induce hepatotoxicity and tumour promotion has not been fully elucidated. It is well recognized that they are potent inhibitors of protein phosphatases PP1 and 2A [12] leading to increase protein phosphorylation which is directly related to their cytotoxic effects and tumour-promoting activity [4,12-14]. Some evidences suggest that oxidative stress may play a significant role in the pathogenesis of MC-LR toxicity

[15-16], in mammals, being a component of the pathologic changes brought about by prolonged sub lethal exposure to MC-RL in rats [17]. Furthermore, the formation of free radical species, possibly derived from oxidative lipid alterations, was demonstrated as a result of *in vivo* cyanobacterial toxin-induced hepatotoxicity in rats [18]. To date, however, there is still no effective clinical treatment to the MC-LR induced acute liver injury, therefore, pretreatment with antioxidant could effectively attenuate MC-LR induced liver injury suggesting that antioxidants might serve as the potential therapeutics [19-21].

*Rosmarinus officinalis*, belonging to family labiatae, commonly called as rosemary, is widely found along the north and south coasts of the Mediterranean's sea [22]. Rosemary has been used as an antispasmodic in renal colic and dysmenorrheal and in relieving respiratory disorders. It has also been used as an analgesic, antirheumatic, carminative, cholagogue, diuretic, expectorant and antiepileptic, but its antioxidant against reactive oxygen species (ROS)-induced by MC-RL are not known. Therefore, the present study has been targeted on liver and kidney to evaluate protective potential of ROE in MC-LR toxin-treated Balb/c mice.

## MATERIALS AND METHODS

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100.$$

**Reagents:** All reagents used in this study were of analytical grade and purchased from Sigma Chemical Inc (USA). MC-LR was obtained from Carmichael Lab (USA).

**Mice:** Male Balb/c mice 6-7 weeks old, (average body weight was 30 g) were used in this study. Mice were kept on standard laboratory diet and tap water ad libitum throughout all these experiments. Ten animals were housed stainless metal cages under 12 h light-dark cycle and room temperature of 22-26°C.

**Preparation of Rosemary Extract:** Rosemary leaves were collected from north area of Jordan, washed in tap water 3 times, then by distilled water. Leaves were dried in oven at 50°C for 5 days, ground in multimill and passed through a 0.5 mm mesh sieve to obtain a fine powder. 0.5 g of this powder was dissolved in 10 ml of cold distilled water then homogenized using IKA Ultra Turrax homogenizer for 5 min, then centrifuged at 10,000 xg for 15 min, the supernatant was filtered through whatman No.1 paper. The extract then was lyophilized to dryness and stored in dark bottle at -22°C for further tests.

**Determination of Total Phenolic Compounds and Reducing Power of ROE:** The total phenolic content of ROE was determined using the method recommended by McDonald *et al.* [23]. The reducing power of ROE was measured by the procedure described by Benzie and Stain [24] using the butylated hydroxytoluene (BHT) as standard antioxidant. The principle of this method is based on the reduction of a ferric-tripyridyltriazine ( $\text{Fe}^{3+}$ -TPTZ) complex to its ferrous coloured form in the presence of antioxidant.

**Evaluation of Antioxidant Activity of ROE:** The antioxidant activity of rosemary extract was measured by DPPH assay according to Turkoglu *et al.* [25]. Briefly, the hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple coloured methanol solutions of 2,2-Diphenyl-1-picrylhydrazyl (DPPH). 1 ml of ROE in ethanol was added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I%) was calculated in the following way:

Where  $A_{\text{blank}}$  is the absorbance of the control reaction (containing all reagents except the test compound) and  $A_{\text{sample}}$  is the absorbance of the test compound according to the procedure described by Turkoglu *et al.* [25] Tests were carried out in triplicate.

**Toxicological Studies:** The intraperitoneal  $\text{LD}_{50}$  value of microcystin-LR in Balb/c mice was determined by using up-down method [26]. Forty male Balb/c mice were divided into 4 groups (10 mice each). The control group (C), the mice of this group did not receive toxin or ROE supplementation. The toxin control group received toxin i.p. (according to  $\text{LD}_{50}$  value). Mice of this group were killed 24 h after the injection. Group 3 was ROE control group (ROEC), mice of this group were supplemented orally with 400 mg/kg bwt daily for 14 days. The fourth group was ROE-toxin group (ROET), mice of this group were supplemented with 400 mg/kg bwt orally, daily for 14 days then injected i.p. with 56  $\mu\text{g}/\text{kg}$  bwt of MC-LR and sacrificed 24 h after the injection.

The blood (about 0.7 ml per mouse) was collected by extirpation the eyeball and allowed to coagulate for 2h on ice. Serum was isolated as a supernatant fraction following centrifugation at 2000 xg for 20 min and stored at -70°C. Liver and kidneys were removed immediately and perfused with Hank's buffered saline to remove excess blood, then homogenized using (IKA Ultra-Turrax homogenizer) in homogenizing buffer (0.01 M Tris-HCl, pH 7.8; 0.2 mM DTT and protease inhibitors: 7.5 mM PMSE, 2.5 mM EDTA, 3.25 mM bestatin, 2.5 mM leupeptin and 0.75 mM aprotinin).

**Reactive Oxygen Species Determination in Mice Liver and Kidney Cells:** By using oxidant sensitive probe dihydrorhodamine 123 (DHR), we detected the ROS concentration in both liver and kidney homogenates. As an oxidative sensitive indicator, DHR can be oxidized to the positive charged rhodamine 123 localized in mitochondria and therefore has been suggested to be effective probe to measure ROS production [27].

**Serum ALT and GGT Enzymes Levels Determination:** Serum Alanine transaminase (ALT) and gamma glutamyl transferase (GGT) levels were determined according to the procedure described by Wahsha [28].

**Protein Phosphatase 1 (PP1) Activity Measurement:**

Protein phosphatase 1 (PP1) activity in hepatocytes homogenate was determined by measuring the rate of colour production from dephosphorylation of  $\rho$ -nitrophenyl phosphate (PNPP) substrate as a function of time using microtitre plate reader according to An and Carmichael's [29] method.

**Liver and Kidney Superoxide Dismutase and Catalase Activities Determination:**

The CAT and SOD activities in liver and kidney cells homogenates were assayed according to the method described by Moreno *et al.* (2005).

**Lipid Peroxidation (LPO) Determination:**

The Malondialdehyde (MDA) as a marker of lipid peroxidation was measured in the homogenates of liver and kidneys according to the method described by Hosseinzadeh *et al.* [30].

**Protein Carbonyl Contents Determination:**

Protein carbonyls content in the liver and kidney tissues was determined by the method described by Galazyn-Sidorczuk *et al.* [31]. The liver or kidney homogenates were incubated with streptomycin sulphate to remove nucleic acid for overnight. The homogenates then treated with DNPH and HCl and finally with guanidine hydrochloride. Assessment of carbonyl formation was done on the basis of formation of protein hydrazone by reaction with DNPH. The absorbance was measured at 370 nm. Protein carbonyl content was expressed as nmol of carbonyl / mg protein.

**DNA Protein-Cross Links Assay:**

The extent of DNA-protein cross-links were assayed according to the method described by Zhitkovich and Costa [32].

**Statistical Analysis:**

All values were expressed as mean  $\pm$  SEM from ten mice per group. One way analysis of variance (ANOVA) followed by Dunnett's t-test was used to determine the significance of the difference between the groups. Significant were declared when p-value was less than 0.05.

**RESULTS**

We adopted the up and down method for the evaluation of i.p. LD<sub>50</sub> value of MC-LR in our laboratory conditions, in order to establish adequate dose of MC-LR

Table 1: Reducing power of rosemary extract (ROE) and butylated hydroxytoluene (BHT)

BHT (mg)	Absorption at (700 nm)	ROE (mg)	Absorption at (700 nm)
1.0	0.02	1.0	0.01
2.5	0.33	2.5	0.27
4.0	0.51	4.0	0.41
5.5	0.66	5.5	0.52

Table 2: Scavenging effect of rosemary extract (ROE) on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical.

ROE (mg)	Scavenging effect (%)
0.75	18
1.25	38
1.75	44
2.25	52
2.75	61
3.25	68
3.75	76
4.25	90
4.75	90
5.25	90

to investigate its effect on the liver and kidney damage of male Balb/c mice. The MC-LR LD<sub>50</sub> was found to be 56 $\mu$ g/kg body weight.

**Total Phenolic Compounds, Reducing Power and Antioxidant Activity of ROE:**

The total phenolic concentration in ROE was found to be 8.58 mg/g powder. The reducing power of ROE (as indicated by the absorbance at 700 nm) correlated well with increasing concentration. However, as anticipated, the reducing power of BHT (a known standard antioxidant) was relatively more pronounced than that of ROE (Table 1). Total DPPH scavenging potential of the freeze-dried extract of rosemary at varying concentrations were measured and the results are depicted in Table (2). Significant DPPH radical scavenging activity was evident at all tested concentrations of ROE. The scavenging effect increased with increasing ROE concentration up to certain extent (4.25 mg) and then leveled off with further increase.

**Protection Effects of ROE on Cell Damage:**

MC-RL treated mice (TC group) showed increased liver: body mass ratio due to massive intrahepatic haemorrhage and pooling of blood in the liver, the livers of the control mice (C group) were an average of 5% of the total body weight, while it was about 8.8% in toxin-treated mice (TC group) and it was about 5.6% in ROE and MC-RL treated groups (ROET group). Livers of mice received ROE only (ROEC) were within the normal value. There was no any change in

Table 3: Protection effects of ROE on cell damage on Balb/c mice receiving single dose of MC-LR (24ug/Kg body weight).

	Control group	MC-LR treated group	Rosemary extract group	Rosemary extract and MC-LR treated group	
Serum	ALT (U/L)	55.26±2.18	266.6±4.08	53.3±1.92	110.2±3.11
	GGT (U/L)	39±3.6	230±8.06	32±2.12	67±5.66
Liver homogenate					
Protein phosphatase					
	(PP1) (U/mg)	0.585±0.18	0.1116±0.08	0.562±0.12	0.226±0.22

The results expressed in mean± standard error of the mean

Table 4: Antioxidative effects of Rosemary extract in Balb/c mice receiving single dose of MC-LR (24ug/Kg body weight)

	Control group	MC-LR treated group	Rosemary extract group	Rosemary extract and MC-LR treated group
Liver homogenate				
Superoxide dismutase (SOD) U/mg protein	10.2±2.97	3.96±1.49*	10.0±2.11	5.22±1.87*
Catalase (CAT) U/mg protein	220±33.1	41.0±2.21*	222±33.1	186±9.61*
Lipid peroxidation (MDA)	0.067±0.07	4.008±1.88*	0.053±0.02	0.545±0.142*
Protein carbonyl contents	1.61±0.08	29±3.66*	1.58±0.03	11.13±0.17*
DNA protein cross link%	0.9	9.7*	0.7	2.2*
Kidney homogenate				
Superoxide dismutase (SOD) U/mg protein	24.7±1.7	5.08±1.99*	23.8±2.19	9.6±1.07*
Catalase (CAT) U/mg protein	123±8.66	55.1±3.48*	123±8.02	88.2±6.11*
Lipid peroxidation				
(MDA)	0.055±0.002	1.71±0.31	0.091±0.001	0.161±0.2
Protein carbonyl contents	1.42±0.21	22±3.1*	1.33±0.11	6.22±0.76*
% DNA protein cross link	0.78	5.8*	0.81	1.66*

The results expressed in mean± standard error of the mean. \*significant if less P-value<0,05

kidneys weight or color. The ROS concentration in liver and kidney tissues of MC-LR-treated mice were about five-fold and three-fold higher than control mice respectively. These levels were attenuated to about normal in mice pretreated with ROE supplementation.

As shown in Table (3), MC-LR produced nearly five folds increase in serum ALT level compared to control mice, indicating severe liver injury. ROE pretreated could significantly antagonize the enhancement of serum level of ALT caused by MC-LR. Mice group which received ROE only showed that the level of GGT was within the normal value (32 U/L). Furthermore, MC-LR administration (56 µg/kg bwt) increased serum levels of GGT about 5.9 fold, while the increment was dramatically diminished by ROE pre-treatment as shown in Table (3). Results of spectrophotometric measurements of protein phosphatase activity of liver homogenate for all groups are presented in Table (3). PP1 activity was significantly inhibited in group MC-LR treated group; 80% inhibition has occurred for this group when compared with control group. Supplementation with ROE caused a partial protection of PP1 activity against the action of MC-LR of almost five-folds compared with the levels in MC-LR treated group.

**Antioxidative Effects of ROE:** The results showed a decrease in the activities of SOD (2.6 and 4.8 fold) and CAT (5.3 and 2.2 fold) in liver and kidneys of Balb/c mice subjected to acute exposure to MC-LR respectively when compared to control group as shown in Table (4). ROE supplementation attenuated this decrease in the activities of SOD into 1.9 and 2.6 fold in liver and kidney cells respectively and approximately eliminated the MC-LR effect on CAT activities. Control mice exhibited normal level of lipid peroxidation measured as amount of MDA, it was 0.067 µM in hepatocytes homogenate. There was a 60 fold increase in MDA level in liver homogenate of MC-LR treated groups when compared with those of control mice (p< 0.005) (Table 4). The mice receiving MC-LR and ROE supplementation revealed an increased MDA value by 8 folds only when compared to controls. Also it was 31 folds in the kidney homogenates of MC-LR treated group when compared with control and dropped into 3 folds only in MC-LR and ROE supplementation group (ROET).

Protein oxidation was examined by determining carbonyl group levels in amino acids. The carbonyl group levels (PPC) were 18 and 15.5 fold higher in liver and kidney tissues of MC-LR treated mice compared to control

group respectively. This increase was attenuated in liver and kidney tissues into 7 and 4.4 fold respectively when MC-LR treated mice were pretreated with ROE table (4). The percentage of DNA protein-cross links was increased by 10.8 and 7.4 in liver and kidney homogenates of MC-LR treated mice respectively. While the effect of MC-LR toxin on DNA protein-cross links was decreased into 2.4 and 2.1 in liver and kidney homogenates of ROE supplemented mice respectively as shown in table (4).

### **DISCUSSION**

The presented investigation examined the toxic effects of the MC-RL in hepatic and kidney cells of Balb/c mice after intraperitoneal route of exposure to the toxin. Besides we investigated the potential hepato- and kidney protective efficacy of ROE as naturally isolated antioxidant against MC-LR effects. The results in toxin groups indicate that severe liver damage accompanied by marked change in colour and weight can occur by intraperitoneal injections of LD<sub>50</sub> dose which in agreement with previous studies [4, 30]. Hepatocellular damage was first noticed by the increase in total liver size due to intrahepatic haemorrhage and accumulation of fluids caused by the action of MC-LR. Exposure to MC-LR causes a disturbance of cellular iron homeostasis as a result of ferritin inhibition [33]. Mackintosh *et al.* [34] reported that protein phosphatases are inhibited with high affinity MC-LR. Thus, MC-LR completely blocks access to catalyst [28], thereby causing hyperphosphorylation of the cell and a massive disruption of a number of important cellular mechanisms [4]. ROE in the mice pre-treated group could hinder partially the inhibition of PP1 binding to Fe<sup>+3</sup> [24]. Also, the results showed that there was a severe damage occurs to kidney cells as shown by results of LPO and carbonyl contents as well as antioxidant enzymes. ROE changes all these alteration because of the antioxidant property of ROE.

One important consequence of excessive free radical production after exposure to MC-LR is the toxin ability to attack many organic molecules, including polyunsaturated fatty acids in the cell membrane [5, 28] leading to lipid peroxidation [26]. Several studies reported that ROS can initiate lipid peroxidation through the action of hydroxyl radicals [35]. Our analysis of hepatocytes for lipid peroxidation showed that ROE could bring a decrease in the formation of lipid peroxidation through their ability to scavenge the hydroxyl radicals.

The induction of DNA-protein cross-linked has been proposed as an indicator of early biological effects due to the fact that known or suspected carcinogens induce an increased proportion of protein tightly bound to DNA [36]. Our presented observation exhibits that the level of DNA-protein cross-links increased in liver of MC-LR exposed mice. DNA-protein cross-links induced by MC-LR can be attributed to the free radicals produced during the destruction of cellular components. Free radicals generated by MC-LR can oxidize amino residues in the protein as evidenced by increased carbonyls and thereby lead to the formation of cross-links with DNA. Increased formation of DNA-protein cross-links reduces DNA-repair machinery, which is likely to enhance the genotoxicity and mutagenicity of other directly genotoxic compounds as part of a carcinogenic mechanism of action [28]. The incidence of DNA-protein cross-links has been found to be reduced in ROE-supplemented, MC-LR-treated mice. This antioxidant, non-vitamin protects cells against free radical-mediated damages by directly scavenging them before these radicals interact with the cellular components [23, 25]. It was found by other researchers [12] that administration of antioxidant to toxin-exposed rats replenishes the level of reduced glutathione through the recycling mechanism and thereby regulates the redox potential of the cell and maintains DNA and proteins in native form. Apart from these factors, the improvement in the activities of enzymatic antioxidants such as catalase and superoxide dismutase, ROE in MC-LR-treated mice could play an additional role in eliminating the deleterious radicals [25].

Various authors have reported a correlation between phenolic content and antioxidant activity [22,37-38]. Also, there are different reports in the literature on the amount of total phenolic compound in rosemary [39-40].

### **CONCLUSION**

The pretreated Balb/c mice with ROE showed a great protection against microcystin-LR- induced injury which significantly decrease the levels of serum enzymes, ALT, GGT and inhibit liver protein phosphatase (PP1). This protection effect most probably due to the antioxidant effects of ROE as lipid peroxidation, protein oxidation and DNA-protein cross links were significantly decreased and antioxidant scavenger enzymes CAT and SOD were significantly reactivated.

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