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Biochemical Screening of Hesperidin and Naringin Against Liver Damage in Balb/c Mice Exposed to Microcystin-LR

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Abstract: The cyclic heptapeptide hepatotoxin microcystin-LR (MC-LR) from the cyanobacterium *Microcystis aeruginosa* (*M.aeruginosa*) was suspected to generate oxidative stress and induces rapid and characteristic deformation of isolated mice hepatocytes. The influence of hesperidin and naringin, the main flavonones of orange and grapefruit, were investigated on MC-LR induced hepatocellular injury in mice. Two groups of Balb/c mice were pre-treated for ten days with hesperidin / naringin (300 mg of hesperidin or naringin /kg mouse body weight given orally once a day for 10 days), before an intraperitoneal injection (i.p) with 200µg toxin/kg mouse body weight of MC-LR (according to LD_{50} value). The potential benefits of hesperidin and naringin were evaluated based on alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT) in the serum. Protein phosphatase, methylglyoxal, lipid peroxidation (LPO) as MDA amount in the liver homogenate. Furthermore, Jordanian citrus (hesperidin and naringin) showed a strong protective effect against chronic exposure to MC-LR. It was concluded that hesperidin and naringin extracted from orange and grapefruit peels have a high contents of polyphenols and thus, acting as a potent antioxidants.

Key words: Hepatoprotective antioxidants · Microcystin-LR · Hesperidin · Naringin

INTRODUCTION

Toxic cyanobacterial blooms in freshwater supplies are an increasing threat to human health [1]. Anthropogenic activities have contributed to increasing the formation of toxic cyanobacterial blooms all over the world [2]. MC-LR is a naturally occurring monocyclic heptapeptide hepatotoxin, produced principally by strains of cyanobacteria (blue-green algae) belonging to the species *M. aeruginosa* [3]. MC-LR is the most frequently occurring and widespread of the cyanotoxins [4]. The specific inhibition of serine/ threonine protein phosphatases (PP1 and PP2A) by MC-LR results in an increase of the phosphorylation of proteins in the liver cells, affecting several processes like metabolism, cell contractility, membrane transport, secretion, cell division and gene transcription and translation, leading to complete disruption of the liver architecture and rapid death of the animal [5, 6].

Recent evidence suggested that generation of reactive oxygen species (ROS) and oxidative stress also played a role in the MC-LR toxicity [3]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [2, 7]. Several citrus fruits were widespread for their many therapeutic, antioxidant and pharmaceutical virtues. These benefits provide form their big content on bioactive compounds mainly flavonoids [8, 9]. In grapefruit, naringin is the major flavonoid glycoside [14] while hesperidin is found in great quantity in oranges [15]. In addition to a number of other health benefits, these antioxidants can act as free radical scavengers [9, 10].

The increase in occurrence of cyanobacterial toxic blooms in King Talal Reservoir (one of the main source of drinking and recreation water and is a largest surface water in Jordan). The aim of this work was to investigate the beneficial effect of hesperidin and naringin extracted from grapefruits and orange species from Jordan on liver damage in Balb/c mice against MC-LR.

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MATERIALS AND METHODS

Chemicals: All chemicals used in this study were of analytical grade and purchased from Sigma Chemical Co. USA.

Samples: Samples of *M. aeruginosa* cells were collected according to Al-Jassabi and Khalil [16], from selected sites of KTR in Jordan during blooming season (July, August and September, 2007). Microcystin was extracted from the lyophilized cells of *M. aeruginosa* according to Lawton *et al.* [17] and the LD_{50} of the extracted toxin was determined according to the up-down method described by Fawell *et al.* [18].

Hesperidin Extraction: Identified oranges species (Moro blood, Washington navel and Valencia) which were obtained from a farm in Shubak (Jordan). Hesperidin was extracted according to the method recommended by Lombardo *et al.* [19].

Naringin Extraction: Identified grapefruits species (Kara, King and Fortune) were obtained from a farm in Shunna (Jordan). Naringin was extracted according to the method recommended by Markham [8].

Treatment Schedule: Male Balb/c mice 6-7 weeks old (average body weight 30 g) were used in this study. Mice were obtained from the Animal House/ Yarmouk University and were maintained on standard laboratory diet and tab water *ad libitum* throughout the experiments. Ten animals were maintained in each cage (stainless) under a 12-12h light-dark cycle and room temperatures of 23-26°C.

Ninety mice were assigned into nine groups (10 mice each), group 1 was the control group (C), without supplementation of antioxidant or treatment with toxin; Group 2 was the toxin control group (with 2 sub groups, 10 mice each) (T_6 and T_{12}), treated with toxin only, intraperitoneal injection (i.p) with 200 µg toxin / kg mouse body weight. 10 mice were killed after 6 h and 10 mice were killed after 12 h; Group 3 was the hesperidin control group (HC), supplemented orally with 300 mg hesperidin /kg mouse body weight daily for 10 days according to [46], then they were killed; Group 4 was the hesperidin and toxin (HT) with two sub group (10 mice each) supplemented orally with 300 mg hesperidin / kg mouse body weight daily for 10 days, then were injected i.p. with 200 µg toxin/kg mouse body weight. 10 mice were killed after 6 h (HT_6) and 10 mice were killed after 12 h (HT_{12}). Group 5 was the naringin control group (NC), supplemented orally with 300 mg naringin/ kg mouse body weight daily for 10 days according to Gonzalez *et al.*[14], then they were killed; Group 6 was the naringin and toxin (NT) with two sub group (10 mice each) supplemented orally with 300 mg naringin/ kg mouse body weight daily for 10 days, then were injected i.p. with 200 µg toxin/kg mouse body weight. 10 mice were killed after 6 h (NT_6) and 10 mice were killed after 12 h (NT_{12}).

Blood was collected immediately after sacrification of mice and serum was isolated and stored at -20°C for the biochemical tests. Livers were removed, immediately after death, perfused with normal saline containing heparin, weighed and homogenized with phosphate buffer saline (pH 7.2) using Ultra Turax homogenizer, centrifuged at 3000g for 30min. The supernatant was removed and stored at -20°C.

Cytotoxicity Assay

LDH: Serum lactate dehydrogenase (LDH) activity was measured according to Doyle and Griffiths [20].

Alanine Aminotransferase (ALT) Assay: Determination of ALT activity in the serum sample was measured according to the procedure recommended by Gehringer *et al* [6].

Gamma Glutamyl Transferase Assay (GGT): Determination of GGT activity in the serum sample was measured based on Szasz [21].

Protein Phosphatase (PP1) Assay: Depending on the procedure described by Yuan *et al.* [22], PP1 activity in liver homogenate was assayed by measuring the rate of formation of the yellow color of p-nitrophenol (*p*-NP) produced by hydrolysis of p-nitro-phenylphosphate (*p*-NPP) in an alkaline solution spectrophoto-metrically.

Lipid Peroxidation (LPO) Assay: The lipid peroxidation level of the hepatocyte was measured according to the method described by Hosseinzadeh [23].

Methylglyoxal Assay (MG): Methylglyoxal was determined from liver homogenate according to the method described by Ratliff [24].

Statistical Analysis: Results were expressed as mean±S.E.M. The intergroup variation was measured by one way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was considered at $p < 0.05$. The statistical analysis was done using Sigma Stat statistical software version 3.5.

RESULTS

In this study, no mortality associated with MC-LR and hesperidin and naringin administration was observed throughout the experimental period and the behaviour of the MC-LR-treated mice could be distinguished from that of the controls and antioxidants pre-treated mice by trend toward decreased gain in body weight followed by losing of their energetic activity, subsequently sufficient to cause detectable weight loss.

MC-LR treated mice showed significant ($P \leq 0.05$) differences in ratios of body weight to liver weight due to massive intrahepatic hemorrhage and pooling of blood in the liver, as shown in Table 1. The livers of the control mice were an average of 5% of the total body weight, while it was 6.7 and 9.1% in toxin-treated mice (T_6, T_{12} respectively). The livers of mice which received hesperidin or naringin only were within the normal value, but this value increased slightly after the exposure to the toxin.

As shown in Table 1, MC-LR alone produced after 6 h of exposure nearly three-fold and five-fold after 12 h increases in serum LDH level compared to control mice, indicating severe liver injury. Mice receiving toxin and antioxidant supplementation revealed an increased LDH value in sera by folds of 2.4 and 2.2 in HT_6 and NT_6 respectively. A further increase was shown as 2.9 and 2.7 folds for HT_{12} and NT_{12} respectively compared to controls.

Treatment with MC-LR resulted in a significant ($P \leq 0.05$) increase in levels of ALT activity compared to the saline-treated group (Table 1). The elevated levels of serum ALT was significantly reduced in mice received toxin and hesperidin or naringin supplementation. An increased ALT value was revealed in sera by folds of 1.8 and 1.7 in HT_6 and NT_6 respectively. A further increase was shown as 2.3 and 2.2 folds for HT_{12} and NT_{12} respectively compared to controls.

Mice groups which received antioxidants only show levels of GGT which are within the normal value (39±4U/mg). Furthermore, MC-LR administration (200µg toxin/kg mouse body weight) of 6 and 12 h increased serum values of GGT about 3.4 and 5.8 fold, respectively, compared with control mice ($P<0.05$), while we found that the increase was dramatically diminished by hesperidin and naringin in the pre-treated groups and it could significantly inhibit the increase of GGT induced by MC-LR as shown in Table 1.

Results of spectrophotometric measurements of protein phosphatase activity of liver homogenates for all groups are presented in Table 1. As shown, PP1 activity was significantly ($P \leq 0.05$) inhibited in the groups which received the toxin without being pre-treated with antioxidants: 0.46% inhibition has occurred for those of 6 hrs and 72% for those of 12 hrs when compared with controls. Supplementations with antioxidants caused a partial protection of PP1 activity against the action of MC-LR: it was almost three-fold compared with the levels in mice groups without antioxidant supplementation.

Analysis of liver tissue for LPO products showed that control mice exhibited normal levels of LPO measured as amount of MDA and it was 0.067iM in hepatocytes homogenate. MC-LR challenge caused a marked lipid peroxidation in liver by 16 fold in case of T_6 and 34 fold in case of T_{12} when compared with those of control mice ($P<0.05$), as reflected in the elevation of TBA values which appear time-dependent (Table 1). The mice receiving toxin and antioxidant supplementation revealed an increased TBA value by 6.2 and 5.3 folds in HT_6 and NT_6, respectively revealed further increase shown as 8.6 and 15.8 fold for HT_{12} and NT_{12} respectively in compared to controls.

Under the conditions of these experiments, the MG concentrations in the liver homogenates were determined at 6 and 12h after i.p injection of MC-LR and results were compared with controls as shown in Table 1. It was clearly that the effect of MC-LR on MG was time dependent; for mice receiving toxin and antioxidant supplementation the results showed decreased MG concentrations in liver homogenates by 10.2 and 9.5 folds in HT_6 and NT_6 respectively compared to controls. On the other hand an increase in MG concentrations was shown in liver homogenates when compared to those mice which were killed after 6 hrs. A decrease in MG concentrations is shown as 10.8 and 10.5 folds for HT_{12} and NT_{12} respectively comparing to control.

Table 1: Summary of results of the effect of hesperidin / naringin supplementation on mice receiving 200µg/Kg of MC-LR

	C	T_6	T_{12}	HC	HT_6	HT_{12}	NC	NT_6	NT_{12}
Livers weight (g)	1.47±0.02	2±0.057	2.74±0.06	1.35±0.01	1.6±0.02	2.04±0.09	1.46±0.044	1.71±0.022	2.11± 0.16
LDH (U/mg)	987±207	3352±123	4868 ±46	825.086±9.2	2368.236±60	2829.582±65	900.322±11	2188.746±39	2710.699±80
ALT (U/L)	574.6±6.95	1649±43	2461±51	641.784±5.9	1028.976±13	1331.304±38	601.120±3.2	974.168±1.9	1281.8±38
GGT (U/mg)	39±4	132±5	226±7	37.656±8	101.507±6	173.872±4	35.364±3	99.870±1	169.615±4
PP1 (U/mg)	0.583±0.01	0.316±0.01	0.165± 0.002	0.508±0.03	0.341±0.01	0.278±0.01	0.613±0.01	0.356±0.01	0.255±0.01
LPO (µM)	0.067±0.01	1.3±0.04	2.292±0.04	0.029±0.001	0.417±0.02	0.577±0.01	0.016±0.01	0.353±0.02	1.058±0.04
MG (µM)	1.61±0.075	29.03±0.39	41.94±0.797	1.43±0.0125	16.45±0.232	17.42±0.165	1.49±0.008	15.32±0.071	16.77±0.131

Data are expressed as mean±S.E.M. Statistical analysis by one way analysis of variance (ANOVA) followed by Tukey's test

DISCUSSION

M. aeruginosa dominate the phytoplankton community of freshwater ecosystem in KTR during the blooming season (specifically, from June to October [25]. Our methods of sample identification proved that the major cyanobacterial species found in the collected samples was *M. aeruginosa* in agreement with our previous study [16]. The phosphatase inhibitory activity showed the bioactivity of the toxin while the spectrophotometric analysis proved that the extracted toxin was MC-LR [26, 27].

The present study was conducted to investigate the effect of hesperidin and naringin as naturally isolated antioxidants against MC-LR effects. The LD_{50} concentration of MC-LR by i.p route was determined as 200 µg toxin/kg mouse body weight by a modified Fawell's up and down method [18] which is at variation with some earlier reports [19, 28]. A wide range of LD_{50} values have been reported for MC-LR by administration in mice.

In agreement with previous studies [5, 29, 30] the results of toxin group indicate that severe liver damage accompanied by marked change in colour and weight can occur by i.p injection of LD_{50} dose. 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 [31].

The amount of serum LDH as well as GGT represents an index of membrane cellular integrity [20, 32]. To assess whether the MC-LR-induced cytotoxicity have a potential cytotoxic effect on liver cells that could explain the increase in serum LDH in T_6 and T_{12} respectively. Based on this, LDH released was assessed as a strong cytotoxicity index induced by MC-LR.

One significant mode by which MC-LR can perturb cellular homeostasis is by modification of the phosphorylation-based signalling [33]. Mackintosh *et al.* [34] reported that MC-LR is a very potent inhibitor of the ubiquitous serine/threonine protein phosphatases PP1 and PP2A. There are many potential targets [35, 36], which are controlled by reversible phosphorylation within the cell including ion channels, metabolic enzymes, controllers of the cell cycle [37, 38] cytoskeletal proteins [39]. Hesperidin and naringin in the mice pre-treated groups could hinder partially the inhibition of PP1 binding to Fe^{+3} [40].

An additional finding of this study was a time-dependent increase in levels of MDA after exposure to MC-LR induced hepatocellular over production of free radicals and oxidative injury. Thus, increase the ability to attack many organic molecules, including polyunsaturated fatty acids in the cell membrane [41, 42], leading to lipid peroxidation through the action of hydroxyl radicals [43]. Our analysis of hepatocytes for lipid peroxidation showed that hesperidin and naringin could decrease the formation of MDA through their ability to scavenge the hydroxyl radicals. The activity of hesperidin and naringin as scavenger of free radicals has been described by others [10, 11, 12].

Methylglyoxal is a toxic metabolite unavoidably produced in mammalian systems as a by-product of glycolysis. [13].

Sato *et al.* [44] proposed that MG interacts with highly active sulfhydryl groups that may participate in regulation of cell division in tissues and that this MG-SH complex can arrest cell division in rapidly dividing cells. The cytotoxicity associated with the characteristic accumulation of MG after exposure to MC-LR is due to the inhibition of glyoxalase I and the depletion of the action of the antioxidant defense system found in the liver [25]. The increase in MG levels in the liver caused significant generation of free radicals which might further strengthen the damage and affect the hepatocyte function [13]. This was noticed in the toxin group which received the toxin only as shown in the results. Our results suggested that in hesperidin and naringin pre-treated groups performed a significant decrease in the accumulation of MG.

MC-LR-induced toxicity is mediated through the inhibition of cellular protein phosphatase 1 and 2A. To date, there are no known inhibitors of the interaction of MC-LR with protein phosphatases. Any potential chemoprotectant should have the ability to either prevent microcystin-protein phosphatase interaction or inhibit the uptake of the toxin into hepatocytes [2, 45]. The protective action of hesperidin and naringin might possibly be due to its high affinity of preserving and bind the free radicals. In conclusion, this study clearly demonstrates that hesperidin and naringin may play an important role in lowering the toxic effect of MC-LR in mice.

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