Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2022; 11(6): 14-20
Received: 10-07-2022
Accepted: 14-08-2022

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Anti-oxidative and hepatoprotective effects of the red alga *Falkenbergia rufolanosa* against methyl thiophanate induced liver damage in adult rats

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Abstract
Hepatotoxicity occurs as a result of deleterious effects of some xenobiotics on the liver. Methyl thiophanate (MT), used as a fungicide, is an environmental poison established to induce organ toxicity. The current study reported the protective effect of red marine macro-alga *Falkenbergia rufolanosa* against MT-induced hepatotoxicity in adult Wistar rats. The animals were divided into four groups: group 1 used as a control group, group 2 received MT (300 mg/kg) by intraperitoneally injection, group 3 received MT (300 mg/kg) along with alga methanolic extract (150 mg/kg *via* their diet), and group 4 received only the algal extract (150 mg/kg *via* their diet). Up to MT treatment, results showed in increased levels of malondialdehyde, advanced oxidation protein product, and protein carbonyl groups, as well as disruption of the antioxidant defense status. In addition, significant perturbations in morphological parameters followed by a disruption in plasma biomarkers, lipid profile and histological observations in hepatic tissue were depicted, after MT injection. Nevertheless, co-treatment with algal extract appeared to be effective against MT-induced hepatotoxicity as shown by an improvement of the oxidative stress biomarkers, plasma biochemical parameters and histological injuries.

Keywords: *Falkenbergia rufolanosa*, methyl thiophanate, hepatotoxicity, oxidative stress

Introduction
Modern agricultural production in major agriculture countries is based on the use of pesticides. Fungicides represent about 20% of all pesticides used [1]. The interest in the impact of fungicides is commonly associated to their toxicity. Due to the overuse of these pesticides, they can affect the environment and aquatic ecosystems as well as human health [2]. Methyl-thiophanate (MT) is a benz imidazole fungicide generally applied to control some important fungal diseases of crops [3]. Many previous findings established that MT could affect the cell division mechanism by inhibiting DNA synthesis, stopping some metabolizing enzymes and disrupting biological cell activities [4]. Further, the highest residual levels of this chemical compound was mostly occurred in the liver, thyroid, and kidneys [5]. Some previous research declared that MT had the potential to induce perturbations in blood, liver and kidney histo-morphology [4-6], as well as to provoke toxicity in reproduction and immunity systems [7]. In fact, several investigations had reported the critical role played by oxidative stress in MT induced toxicities [8]. According to Braud *et al*. [9], reestablishment of the oxidative balance could be realized through using herbal antioxidants derived extracts. Currently, seaweeds represent one of the most promising candidates used in traditional medicine, pharmacology and food. Actually, phytochemical analyses delivered their richness in bioactive compounds including phenolics and flavonoids [10]. These bioactive substances present a large specter of biological activities such as antioxidant, anticancer, antimicrobial, antifungal, antiviral and anti- inflammatory activities [10-11]. Interestingly, the red macro-alga *Falkenbergia rufolanosa* was used to quench or alleviate the MT's free radicals damages. To the best of our understanding, no systematic study has been carried out to demonstrate the protective effect of *Falkenbergia rufolanosa* against MT induced oxidative stress, histological and biochemical liver injuries.

Materials and Methods
Plant material
The red marine seaweed *Falkenbergia rufolanosa* was collected from Sfax City (south of Tunisia), in December 2019. The leaves were transferred to our laboratory and washed with Tap water many times, then with distilled water to remove impurities and air-dried in shade

(25 ±2 °C) away from sunlight for 15 days. Then, the cleaned algal materials were ground to powder using a blender and stored in plastic bags at room temperature in a dry dark place before use.

The air-dried leaf powder (30 g) was extracted by maceration. Firstly, 30 g of crude extracts of dried alga powder was added with 200 ml of methanol solvent. Secondly, the mixture was incubated for 24 h with stirring at room temperature. After filtration with Whatman filter paper, the solution was evaporated using Rotary evaporator. As the final step, the dried precipitate was stored at 4 °C.

Animals and experimental design

Adult Wistar rats, weighing 180±4 g, obtained from the Central Pharmacy (SIPHAT, Ben Arous, Tunisia), were housed in plastic cages under standard conditions with a constant light/dark cycle at a temperature of 22±2 °C and 40% of humidity.

The LD50 of MT was evaluated (1000 mg/kg) in our laboratory by Ben Amara *et al.* [4].

Rats were randomly, divided into four groups of 8 animals each:

- Group 1 (control group) rats received oil corn injection, used as vehicle;
- Group 2 received by intraperitoneal a single injection of 300 mg/kg of MT;
- Group 3 received daily both MT (300 mg/kg) by intraperitoneal injection and alga methanolic extract (150 mg/kg of the algal extract) via the alimentation;
- Group 4 received only alga (150 mg/kg of the algal extract) added to their diet.

The treatment was carried out for a period of 7 days. The treatment period and the dose of MT were selected on the basis of previous studies to be toxic but not lethal [4]. The dose of alga methanolic extract was shown in previous studies to induce benefic effects without being toxic, referring to Jaballi *et al.* [8].

The experimental procedures were carried out according to the Natural Health Institute of Health Guidelines for Animal Care and approved by the Ethical Committee of Sfax Sciences Faculty. All animal procedures were conducted in strict conformity with the "Institute ethical committee guidelines" for the Care and Use of laboratory animals [10].

During the treatment period, food (g/day/rat), water intake (ml/day/rat) and body weight (g) of the animals were monitored daily.

After 7 days, Wistar rats were sacrificed and blood samples were collected in heparin tubes, which served to determine biochemical parameters. Livers were immediately dissected out, cleaned and weighed. Some samples were rinsed and homogenized and supernatants were used for oxidative stress markers. Other samples of the liver were fixed in 10% formalin solution and embedded in paraffin for histological studies.

Protein quantification

Protein content in liver was determined according to Lowry *et al.* [11] using bovine serum albumin as a standard.

Oxidative stress markers

Determination of lipid peroxidation assay

The lipid peroxidation level in liver homogenate was assayed spectrophotometric ally by measuring thiobarbituric acid reactive substances (TBARS), expressed in terms of malondialdehyde content (MDA), as reported by Draper & Hadley [12]. The absorbance was calculated at 532 nm and MDA levels were expressed in nmoles of MDA / mg protein.

Advanced oxidation protein product (AOPP) levels were measured spectrophotometric ally at 340 nm referring to the method of Witko *et al.* [13]. The concentration of AOPP was determined using the extinction coefficient of 261 cm-1 mM-1 and expressed as µmoles/mg protein.

Protein carbonyls (PCOs) were determined as described by Reznick & Packer [14]. The absorbance was calculated at 370 nm and the carbonyl contents were expressed as µmoles/mg protein.

Determination of enzymatic and non-enzymatic antioxidants

Superoxide dismutase (SOD) activity was estimated according to the method of Beauchamp & Fridovich [15]. The absorbance was measured at 580 nm and the activity was expressed as U/mg protein in liver.

Glutathione peroxidase (GPx) activity was determined as described by Flohé & Günzler [16]. The absorbance was recorded at 340 nm and the GPx enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

Reduced glutathione (GSH) in hepatic tissue was evaluated using Ellman‘s method [18] modified by Jollow *et al.* [19]. The absorbance was measured at 412 nm and the total GSH content was expressed as mmol / mg protein.

Catalase (CAT) activity was determined according to the method of Aebi [20]. Changes in absorbance due to H2O2 degradation were calculated spectrophotometric ally at 240 nm. The limit of detection of the method is 0.98 mmoles. The enzyme activity was expressed as µmol H2O2 consumed/min/mg of protein.

Biochemical assays

Asparate aminotransferase (AST, Ref. 20012), alanine aminotransferase (ALT, Ref. 20043), Gamma-glutamyl transferase (GGT, Ref. 1001186) and total bilirubin (Ref. 20102) were used as biochemical markers for the hepatic damage using commercial kits (Biomaghreb, Tunisia) on an automatic biochemistry analyzer in the Hospital Habib Bourguiba of Sfax, Tunisia.

Plasma lipid parameters such as total cholesterol (CT, Ref. 20111), triglycerides (TG, Ref. 20131) and high-density lipoprotein cholesterol (HDL, Ref. 20113) levels were determined by enzymatic methods using commercial kits from Biomaghreb (Ariana Tunis, Tunisia). The low- density lipoprotein cholesterol (LDL) fractions were determined according to the equation of Friedewald *et al.* **[21]** as follow:

Triglyceride5 LDL = Total cholesterol – HDL

Histological studies

Pieces of liver from different groups of rats were fixed in a 10% formalin solution for 48 h. The fixed tissues were embedded in paraffin and cutted in 5 µm thick sections. Different sections were then stained with hematoxylin-eosin and visualised under a Motic AE2000 light microscope.

Statistical analysis

Statistical analyses were performed with SPSS ver. 17.0, professional edition using ANOVA analysis at a p level=0.05. A standard deviation at the 95% confidence level was used to compare all parameters.

Results

Effects of treatment on morphological parameters

In the present study, rats did not show any important behavioural changes such as irritation, respiratory distress, abnormal locomotion or catalepsy, in all treated groups either immediately or during the post-treatment period. Additionally, no mortality was observed until the end of the experiment.

The effects of different treated groups on body and liver weights are shown in Table 1. In fact, a significant depletion in body weight in the MT-treated groups (233±2.98 g) during the experimental periods (of 7 days) was noted compared to the control (260±3.01 g) (p<0.001). Co-treatment with alga extract led to the amelioration in body weights, when compared to MT- treated rats.

Additionally, MT induced a significant decrease (p<0.05) in absolute and relative liver weights up to 30% and 20% respectively, compared to the controls (Table 1). This result are followed by a significant decrease (p<0.05) in food and water consumption by MT treated rats. However, co-treated groups with alga extract displayed significant improvement in their body weights, when compared to MT-treated rats (Table **1**).

Oxidative stress marker

Our results revealed, in MT-treated rats, a significant increase in lipid peroxidation level in the liver. In fact, a marked increase in MDA levels (30%) as end product of lipid peroxidation was found in the hepatic tissue homogenate of MT intoxicated experimental rats, when compared to the control group (Fig. 1A). Conversely, co-treatment with alga extract modulated significantly the MDA level in the liver, compared to the MT-treated group.

In addition, a slight increase in AOPP and PCO levels in liver homogenates was observed in MT-treated rats by 5% and 10% respectively, when compared to controls (Fig. 1A-B). However, supplementation of the algal extract to the animal diet improved protein oxidative damages by decreasing the AOPP and PCO levels in liver tissue. No significant difference was noted in these two parameters, in liver, upon alga treatment alone.

A significant reduction (p<0.05) in GSH content by 25% was observed in hepatic tissue of MT-treated rats, when compared to the control group (Table 3). Nevertheless, co-administration with algal extract restored significantly GSH levels, reaching normal values. No significant differences in GSH levels were noted for only alga treated group (Table 3).

Antioxidant enzymes

Administration of MT induced significant increase (p<0.05) in GPx, SOD and CAT activities by 47%, 35% and 65% respectively in hepatic tissue homogenate, as compared to the control group (Table 3). However, supplementation with alga methanolic extract uplifted significantly these enzymes activities, compared to the MT treated group.

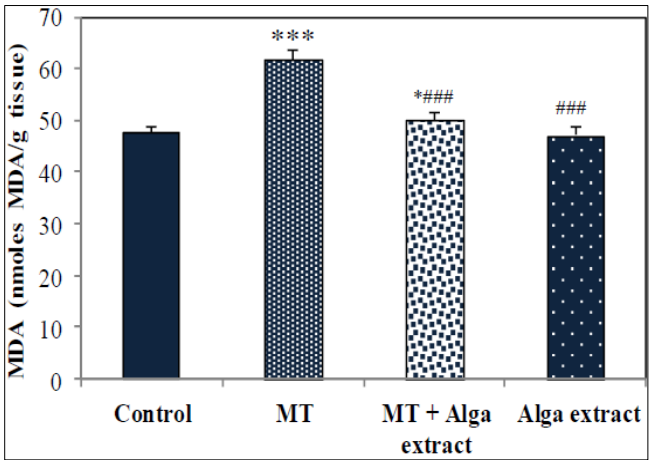
Plasma biomarkers levels

As shown in Table 2, the plasma AST, ALT, GGT and bilirubin were significantly elevated (p<0.05) by 50%, 20%, 45% and 28% respectively in MT-intoxicated group compared to the control group. However, co-treatment with alga methanolic extract significantly (p<0.05) recovered the level of plasma enzymes like AST (15%), ALT (22%), GGT (13%) and bilirubin (15%), respectively.

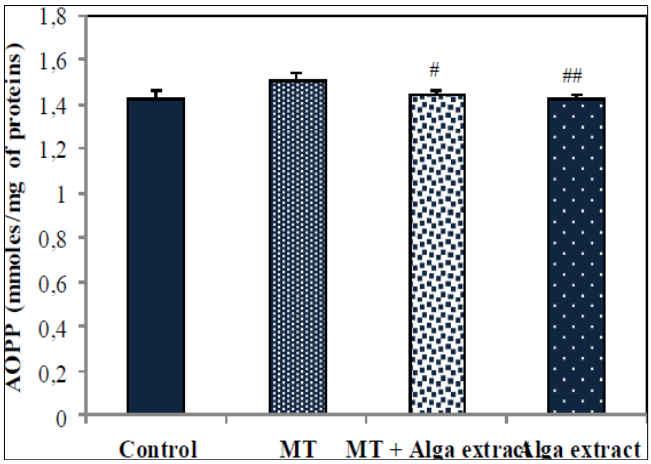
Besides, hepatic lipid levels (TC and TG) and low-density lipoprotein levels (LDL) were increased by 31%, 30% and 63% respectively, while the HDL levels were decreased by 45% in MT-treated groups, when compared with that in the control group (Table 2). Supplementation of alga methanolic extract produced significant recovery in the above-mentioned perturbations reaching the control rates, compared to the MT-treated group.

Histological studies

Light microscopic examination of the slides of MT-treated group showed severe and numerous abnormalities (Fig. 2). Liver controls' pictures indicated normal cellular architecture evidenced by distinct hepatic cells, sinusoidal spaces, and a central vein (Fig. 2A). In contrast, the exposure of rats to MT induced serious degenerative changes in liver histology (Fig. 2B) characterized by an infiltration of inflammatory leucocytes localized around the central vein, apoptosis, hepatic steatosis, and hepatocyte vacuolization with dilated sinusoidal spaces. However, the co-treatment with alga methanolic extract induced significant improvement on histopathology, evidenced by the diminution of necrotic zones and the absence of steatosis and vacuolization (Fig. 2C). The hepatic architecture was almost similar to the normal hepatic structure.



a)



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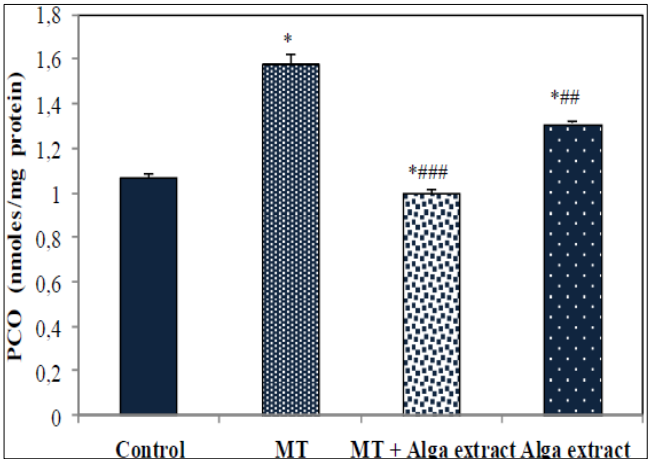

c)

Fig 1: (A) Malondialdehyde (MDA), **(B)** advanced oxidation protein products (AOPP) and **(C)** protein carbonyl (PCO) levels in the liver of different treated groups

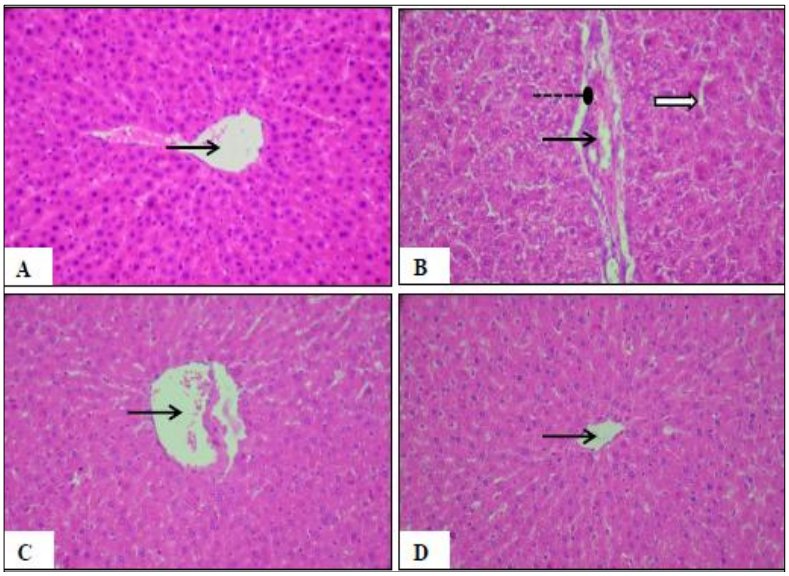

Fig 2: Histological changes of the hepatic tissues stained with Hematoxylin–Eosin at magnification (x400). **(A)** Control group; **(B)** MT treated group; **(C)** MT co-treated group at the dose of 300 mg/kg of body weight; **(D)** Only alga treated group at the dose of 150 mg/kg of body weight

Arrows indicate the following:

Leucocyte inflammatory cells

Congested central veins

Hepatocyte vacuolization

Table 1: Initial and final body weights, absolute and relative liver weights, daily food and water consumption by control and treated rats with MT and MT co-administrated with alga extract during 7 days.

Parameters	Treatment groups			
	Control	**MT**	**MT + Alga**	**Alga**
Initial body weights (g) Final body weights (g)	253±2.82	251±2.78	256±2.91	261±3.12
	260±3.01	233±2.98***	244±2.95*	268±3.03###
Change in body weights (%) Absolute liver weights (g) Relative liver weight (g/100 g BW) Food consumption (g/day/rat) Drinking water intake (ml/day/rat)	+3	-7	-4	+3
	0.721±0.132	0.57±0.061***	0.675±0.181	0.671±0.110
	0.275±0.01	0.242±0.02*	0.283±0.03#	0.248±0.07
	17.141±0.561	13.111±1.63**	16.004±0.431#	16.066±1.082#
	15.985±0.664	12.240±0.664***	13.961±1.015*	14.850±1.431#

Values are expressed as means ± S.D. For eight animals in each group. Comparisons are made between two groups in two cases:*p<0.05; **p<0.01; *** p<0.001, treated groups (MT, MT + alga, Alga) versus controls; # p<0.05; ### p<0.001, MT + alga and alga-treated group versus MT-treated group

Table 2: Effect of the treatment on enzymatic antioxidant activities in the different treated

Parameters	Treatment groups			
	Control	MT	MT + alga	Alga
GPx (nmoles of GSH/min/mg protein)	7.10±0.06	10.44±0.4***	8.12±0.15***###	8.07±0.25***###
GSH (mg/g tissue)	60.83±0.87	47.33±0.74***	52.75±0.86*###	53.11±0.72##
Catalase	7.34±0.47	9.69±0.49**	8.16±0.69##	8.09±0.51#
SOD (U/mg of protein)	14.47±1.53	24.12±1.49**	17.05±1.05##	15.70±1.23##

Values are expressed as means ± S.D. For eight animals in each group. Comparisons are made between two groups in two cases: *p<0.05; **p < 0.01; *** p<0.001, treated groups (MT, MT + alga, Alga) versus controls; # p<0.05; ##p<0.01; ### p<0.001, MT + alga and alga-treated group versus MT-treated group

Table 3: Effect of the treatment on plasma biochemical parameters in the different treated groups

	Control	MT	MT+ alga	Alga
ALT (UI/l)	28,37±2,56	53,81±2,13**	42,2±17,25*##	41,2±12,44#
AST (UI/l)	118,25±12,61	141,25±15,30***	124,45±14,35**###	119,12±25,12##
AST/ALT	4,16±0,82	2,68±1,28***	5,14±0,53**###	2,89±2,02#
GGT (UI/l)	2,68±1,28	3,90±1,06***	3,40±2,53**#	2,6±0,42#
Bilirubin (mg/l)	1,56±0,42	2,00±0,12**	2,05±0,78*#	1,99±1,12
CT (mmol/l)	2,08±0,08	2,72±0,27***	2,4±0,96**#	2,11±0,41##
TG (mmol/l)	1,41±0,03	1,83±0,1***	1,61±0,05**#	1,43±0,08#
HDL (mmol/l)	0,42±0,01	0,23±0,03***	0,36±0,03**##	0,24±0,09
LDL (mmol/l)	1.02±0,01	1.66±0,02***	1.31±0,11**##	1.22±0,01#

Values are expressed as means ± S.D. for eight animals in each group. Comparisons are made between two groups in two cases: *p<0.05; **p<0.01; ** p<0.001, treated groups (MT, MT + alga, Alga) versus controls; # p<0.05; ##p<0.01; ### p<0.001, MT + alga and alga-treated group versus MT-treated group

Discussion

The liver is a vital organ in the human body, responsible for metabolism, detoxification, bile production, vitamin storage, immunity, and other important functions [22]. The unique physiology of the liver is essential to perform a wide range of functions needed to maintain normal physiology. However, with external factors, the liver function can be extensively endangered, leading to life-threatening conditions. In this fact, Methyl Thiophanate (MT) is widely used in worldwide and is a typical example of pesticide residues. Some previous research declared that MT had the potential to induce perturbations in liver histo-morphology [6-22]. Nowadays, natural antioxidants have shown a rise in treating hepatic failure involved due to severe oxidative stress [7]. The latter was generated when the balance between reactive oxygen species (ROS) and antioxidants is disrupted, leading to various pathological conditions [23].

MDA, as an important end product of lipid peroxidation (LPO), could effectively reflect the degree of hepatic oxidative damage [24]. In the current study, MT treatment in the experiment resulted in a significant increase in the level of MDA in liver. The increase in hepatic MDA and LPO contents suggested the elevation of peroxidation, resulted in the failure of the antioxidant defence mechanism to prevent the formations of excessive ROS and thereby the damage of tissue [25]. Besides, the increased free radicals generation could lead to protein– protein cross linkages formation and protein backbones oxidation, giving rise to protein fragmentation and/or a reduction of sulphhydryl groups in amino acids chains [4]. Our data revealed an increase in PCO and AOPP levels, markers of protein oxidative injury, by ROS probably generated by MT treatment, which induce protein oxidative damages in the liver tissue. However, the addition of alga methanolic extract could prevent lipid peroxidation and protein oxidation in rats and reduced the MT-induced liver damage. This result affirmed that the free radicals generated in the liver were efficiently scavenged or the oxidative chain reaction was effectively blocked by alga addition.

Enzymatic and non-enzymatic antioxidants play a major role in the defence against oxidative stress generated by excessive production of free radicals [26]. Our results displayed that the activity of SOD, CAT and GPx were significantly increased in hepatic tissues of MT- treated rats compared to controls, which may be related to response to increased oxidative stress. On the other hand, it is well established that GSH, one of the essential cell function-regulating compounds can modulate the depletion of lipid peroxides and some oxidized amino acid residues of proteins [27]. Under MT induction, the activity of GSH antioxidant enzymes was significantly reduced, as compared with the control group. Diminution in GSH activity might be related to the decreased availability of its substrate and the enhanced lipid peroxidation [26]. The obtained results are in agreement with the studies of Feki *et al*. [28]. However, in the case of rats co- treated with MT, these changes were significantly enhanced, which was marked by a decrease in lipid peroxidation level and a recovery of antioxidant status when compared with control group. The current data corroborate with previous studies of Chan *et al*. [29], who suggested that natural antioxidants derived from red alga are effective in reducing and repairing the damage affecting the liver. Thereby, it was interesting to conclude from our results that alga methanolic extract could prevent oxidative stress induced by MT intoxication generally related to their richness in active antioxidant compounds

Other biomarkers enzymes of liver toxicity like AST and ALT were also determined in the present work. The elevation of AST and ALT activities suggested their leakage from the liver to the blood stream following hepatocellular necrosis as reported by previous studies of Ben Saad, Kharrat, *et al*. [10] and Shao *et al*. [30]. In parallel, a marked increase in bilirubin level and in GGT was detected. Generally, the elevation of GGT emerge to reflect cholestatic injury [31], while increased level of bilirubin is suggestive of jaundice and might be due to metabolic problems in the hepatic tissue accompanied with reduced hepatocyte uptake [32]. However, co- treatment with *Falkenbergia rufolanosa* methanolic extract significantly attenuated MT-induced liver injury. This effect was shown by

the reduction in plasma aminotransferases and bilirubin levels.

The liver is also considered as the site of CT and TG synthesis. The lipid metabolism levels were considered as biochemical evidence for monitoring the progress of toxic damage especially the liver steatosis installation [33]. Our present results displayed that after 4 weeks of MT treatment, there was an increase in CT, LDL and TG levels, while HDL noted a significant decrease. Hypercholesterolemia plays a crucial role in atherosclerosis by inducing ROS overproduction and endothelial cell injury, which accentuate the atherosclerosis and/or hepatotoxicity installation [30]. The protective effect of alga extract was objectified by a decrease in CT, TG and LDL-cholesterol levels and an elevation of HDL. This could be explained by the richness of the red alga in polysaccharides and β-carotene, strongly liable to hypolip idemic effects [10].

The damage provoked by MT in the liver of adult rats was justified by histological changes, including a marked leucocyte infiltration, steatosis, and apoptosis. In fact, MT give rise to several liver histological injuries such as distortion in tissue histoarchitecture, congestion of the central vein, sinusoidal dilatation, generalized congestion, hemorrhage, and degenerative changes. The hepatic sections in control animals presented normal hepatocellular architecture along with lobular pattern and visible central vein with absence of sign of necrosis. Co-treatment of rats with alga extract might alleviate histological damages induced by MT due to its powerful antioxidant capacity.

According to previous data the alga extract was rich in polyphenols, flavonoids, and anthocyanins. These bioactive compounds are able to exert multiple biological effects, including antioxidant free radical scavenging ability and anti-inflammatory and antibacterial effects [34- 35].

Conclusion

To conclude, our study demonstrates, for the first time, that *Falkenbergia rufolanosa* ameliorates MT-induced hepatotoxicity in rats. This effect could be attributed to the antioxidant activities of this alga, and to the presence of large variety in antioxidant components such as polyphenols, flavonoids, polysaccharides and β-carotene.

Acknowledgments

This work was funded by the Ministry of Higher Education and Scientific Research-Tunisia.

Conflicts of interest

The authors declare that there are no conflicts of interest

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