Journal of Advances in Medical and Pharmaceutical Sciences
7(4): 1-10, 2016, Article no.JAMPS.24196
ISSN: 2394-1111

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Monosodium Glutamate Induced Hepatotoxicity and the Possible Mitigating Effect of Vitamin C and Propolis

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Authors' contributions

This work was carried out in collaboration among both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMPS/2016/24196
Editor(s):
(1) Claudia Borza, Department of Pathophysiology, "Victor Babes" University of Medicine and Pharmacy, Romania.
Reviewers:
(1) Ioana Stanciu, University of Bucharest, Romania.
(2) Azhari Hamid Nour, University Malaysia Pahang, Malaysia.
Complete Peer review History: http://sciencedomain.org/review-history/14270

Original Research Article

Received 8th January 2016
Accepted 31st March 2016
Published 20th April 2016

ABSTRACT

Monosodium Glutamate (MSG) has been recognized as a relish additive that adversely affects liver function's parameters. The aim of this study was to assess the effects of MSG on the liver enzyme markers, lipid profile and antioxidant system and also speculated the ameliorating effects of Vitamin.C (Vit.C) and propolis in the rat liver tissues. Mature male rats (weighing 150-200 g and each group of seven animals) were given MSG (60 mg/Kg) and/or Vit.C (200 mg/Kg) and/or Propolis (200 mg/Kg) extract daily via gavage for 4 weeks. In the present study, MSG exposure resulted in an increase in the TBARS level and a decrease in the SOD, CAT, GPX activities in liver homogenates, with respect to the control. Supplementations of Vit.C and/or propolis to MSG treated group induced decrement in the level of MDA, increased SOD, CAT, GPX activities. As a result, MSG afforded hepatotoxicity, which is reduced by administration of Vit.C and/or propolis to a great amplitude by the entire recovery of the liver function markers and the antioxidant status.

Keywords: Monosdium glutamate; vitamin. C; propolis; oxidative stress; liver functions.

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ABBREVIATIONS

MSG: Monosodium Glutamate, Vit.C: Vitamin C, SOD: Superoxide dismutase, CAT: Catalase, MDA: Malondialdhyde, GPX: Glutathione peroxidase.

1. INTRODUCTION

Eating behavior plays an essential role in metabolis and diet-related disturbances. Some mechanisms for food desiring may benefit mammals from the scarcity of nutrients, otherwise overfeeding of tasty and savoury food may cause an imponderable intake of nutrients. Monosodium glutamate (MSG) is one of the most plentiful naturally occurring non-essential amino acids and MSG-treatment is able to produce metabolic changes, which can further result in intense and intractable bodily disorders [1].

Glutamate is one of the most plenty amino acids in nature. Glutamate, which is a subaltern of l-glutamic acid is reported to be a natural nutrient in many foods. Today it is increasingly used (in the form of its salt MSG (monosodium glutamate) in food processsing and home cooking all over the world aims to upgrade the savory of the food for humans. It gives a particular taste named umami is now renowned as the fifth basic taste. Recently it was known as a triggering neurotransmitter in mammalian nervous system [2].

During intestinal absorption, a large quantity of glutamic acid is transmitted and eventually alanine levels in the blood of portal veins are elevated. If large amounts of glutamate are absorbed, portal glutamate levels will elevate, this elevation result in an increase hepatic metabolism of glutamate, leading to the release of glucose, lactate, glutamine, and other amino acids, into systemic circulation [3].

Antioxidants have been reported to perform a significant role in the protection against lipid peroxidation [4]. Some investigators reported that antioxidants inhibit chemicals causing cancerous diseases when the antioxidants are administered either prior or during carcinogenic changes [5].

Antioxidants as vitamins, can prevent the ultimate non-controlled formation of free radicals, or inhibit their reaction with biological sites, also the destruction of most free radicals rely on the oxidation of endogenous antioxidants mainly by scavenging and reducing molecules [6]. Vitamin C, as a water soluble antioxidant is reported to equalize ROS and minimize oxidative DNA damage and hence genetic mutations [7].

Propolis has caught awareness of researchers because of its various biological activities and therapeutic properties. Propolis is a viscid, resinous product that is produced by honeybees for the construction ,conservation and servicing of their hives. It is made by blending the honeybees own waxes with the resins that are accumulated from various plants. Propolis contains a variety of chemical compounds, such as polyphenols (flavonoid aglycones, phenolic acids and their esters, and phenolic aldehydes), alcohols and ketones, sesquiter-penequinones, coumarins, steroids, amino acids, and inorganic compounds [8].

One of the biological performances of propolis, its antibacterial and antifungal activities of propolis is the most inclusively investigated [9]. Its pharmacological activities, such as anticancer [10], anti-inflammatory [11], antibiotic [12], antioxidative [13], antiviral [14] anaesthetic and immunostimulant [13] have been described to the ethanolic extracts of propolis.

In the last few years, There are many solicitude about the adverse effects and toxicity of MSG, with few number of literature concerning the dangerous biochemical or severe histological effect of MSG on the hepatic toxicity of animals treated with MSG. So, the present study was designed to elucidate the effects of MSG on the male rats hepatic tissues. In addition, to the best of our knowledge, no comprehensive study concerning the protective effect of Vit.C and propolis on MSG-induced hepatic dysfunction. The main objective of the present work is to study the effect of propolis or Vit.C each alone or their combination and their role in the amelioration of the hepatic toxicity induced by MSG.

2. MATERIALS AND METHODS

2.1 Chemicals

Monosodium glutamate ($C_5H_9NO_4$.-Na) Purity 99% NT, it was sold in an extermly open market under the license of Ajinomoto Co. INC. Tokyo, Japan. A stock solution was prepared by dissolving 60 g of MSG crystals in 1000 ml of distilled water. The dose schedule was so adjusted that the amount of MSG administration per animal was as per their respective weight.

The utilized doses were chosen according to [15]. Vitamin (C) was supplied by Merck (Germany) and it was dissolved in dist. water and administered orally for 30 successive days at a dose 200 mg/Kg [16].

Commercially obtainable diagnostic UV kits were used to deduct alanine transferase (ALT), lactate dehydrogenase (LDH) and total proteins (Human diagnostic worldwide, Germany). Moreover, total cholesterol and high density lipoprotein-cholesterol (HDL-c) were bought from Bio-diagnostic, Egypt.

2.2 Propolis Preparation

Fifty grams of the resinous material of Egyptian propolis were split into small pieces and extracted with 600 ml of 80% (v/v) ethanol at 60℃ for 30 min. After extraction, the mixture was centrifuged and the supernatant was evaporated to complete dryness under vacuum at 40℃ [17]. It was preserved at 4℃ for further use. A propolis suspension was prepared in 1% gum acacia suspension, and orally administered to the animals at a dose of 200 mg/kg [18].

2.3 Animals and Experimental Design

Seventy male rats (Weighing 150-200 g), were used in all experiments of this study. They were obtained from the Animal House of Faculty of Pharmacy , Zagazig University , Zagazig, Egypt. The animals were kept on solid-bottom shoe box, type polycarbonate cages with stainless steel wire-bar lids, using a wooden dust free litter as a bedding material. Animals were allowed free access to diet and water in good air conditioned room and were allowed free access and tap water for two weeks before starting the experiment. We have followed the European community Directive (86/609/EEC) and national rules on animal care. One group served as control. Animals were weighed and randomly allocated into 7 groups (7 rats each) as following:

Group 1 – control rats treated with 1 mg/kg BW corn oil per day; Group 2 – MSG–treated rats (60 mg/Kg BW per day in distilled water) [15]; Group 3– Vit.C treated rats (200 mg/kg BW per day in corn oil) [16]; Group Group 4 – Propolis treated rats (200 mg/kg BW per day in distilled water) [18]; Group 5– MSG plus Vit.C-treated rats. Group 6 – MSG plus propolis. Group 7– MSG treated rats followed by Vit.C and propolis daily for successive 30 days.The doses were administered in the morning (between 9.30 and 10.30 h) to non-fasted rats. The first day, when the animals were treated was considered experimental day 0. At the end of the 30 days of treatment, all animals were scarified.

2.4 Biochemical Analyses

2.4.1 Lipid profile

Triglycerides, cholesterol and high density lipoprotein-cholesterol (HDL-c) were determined using the commercial kits. Low density lipoprotein-cholesterol (LDL-c) levels were calculated by using the following formula of Muruganandan et al. [19] LDL-c = total cholesterol—(HDL-c + triglycerides)/5. Volatile low density lipoprotein-cholesterol (VLDL-c) levels were calculated by using the following formula : VLDL-c = triglyceride/5.

2.4.2 Hepatic markers

Serum aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) enzyme activities were determined with kits from Human diagnostic worldwide, Germany.

2.5 Tissue Homogenates Preparation and Estimation of Antioxidant Capacities Parameters

The excised liver tissues were washed with distilled water for the removal of blood, and later the fatty parts were removed. Tissues were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The supernatant was separated by means of centrifugation at 5000 rpm for 20 min at 4℃. The supernatant was used for the analyses of all biochemical parameters.

2.5.1. Lipid peroxidation assay

TBARS content was evaluated by using the thiobarbituric acid (TBA) test as substantive by Ohkawa et al. [20]. After incubation of liver homogenates with TBA at 95℃, TBARS reacts to form a colored complex. Absorbance was measured spectrophotometrically at 532 nm to determine the TBARS content. The level is expressed as nmol/mg protein.

2.5.2 Measurement of superoxide dismutase (SOD)

SOD activity was measured according to the method described according to Misra and

Fridovich [21] by assaying the auto oxidation of pyrogallol at 440 nm for 3 min. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol autooxidation inhibition. A blank without homogenate was used as a control for non-enzymatic oxidation of pyrogallol in Tris–EDTA buffer (50 mM Tris, 10 mM EDTA, pH 8.2). The SOD activity is expressed as U/mg protein.

2.5.3 Measurement of catalase (CAT)

CAT activity was measured according to the method described by Aebi [22] by assaying the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25℃. Before determination of the CAT activity, samples were diluted 1:9 with 1% (v/v) Triton X-100. CAT activity is expressed as mmol/mg protein.

2.5.4 Measurement of glutathione peroxidase (GPx)

GPx activity was measured using H_2O_2 as substrate according to the method described by Paglia and Valentinen [23]. The reaction was monitored indirectly as the oxidation rate of NADPH at 240 nm for 3 min. A blank without homogenate was used as a control for non-enzymatic oxidation of NADPH upon addition of hydrogen peroxide in 0.1 M Tris buffer, pH 8.0. Enzyme activity was expressed as nmol/mg protein.

2.6 Statistical Analysis

Data were collected, arranged and reported as mean ± standard error of mean (SEM) of twelve groups, and then analyzed using the computer program (SPSS/version 15.0). The statistical method was one way analyzes of variance ANOVA test, and if significant differences between means were found, Duncan's multiple range test (Whose significant level was defined as $P < 0.05$) according to [24] to estimate the effect of different treated groups.

3. RESULTS

3.1 Biomarkers of Liver and Lipogram Assessment

All the parameters of the lipid profile (TG, TC, LDL-c and VLDL-c) were increased except the HDL-c was decreased when the rats exposed to the dose of MSG (Table 1). Administration of Vit. C or/and propolis to the MSG-treated group restored most normal values of the parameters cited above (Fig. 1). Serum ALT activity of Vit.C and propolis groups elicited non-significant changes when compared with the control group. In the MSG-treated group, the ALT was significantly increased by 78 fold when compared to control group (Table 2). The group treated with MSG (60 mg/Kg) in combination with Vit.C and propolis induced decrement in the activity of ALT more than the MSG combination with each compound separately.The same observation has been noticed in the AST activity that increased by administration the dose of MSG and decreased by the treatment of each antioxidant separately and the most increment was recorded in the combination of both antioxidants (Vit.C and propolis) with MSG (Table 2). LDH enzyme highly increased in MSG-treated group by 1890 fold when compared with the control group, but increased by 543 and 545 fold in the groups treated with the MSG and Vit.C or MSG and propolis, respectively (Table 2). The best combination group that highly decreased LDH value was recorded in the group treated with a combination of Vit.C and propolis with MSG as LDH was decreased by 1550 fold as compared to MSG treated group.

3.2 Assessment of Enzymatic Antioxidants and Non-enzymatic Lipid Peroxidation as Oxidative Biomarkers

As shown in Table (3) and Fig. (2), the data showed that treatment with MSG caused a significant ($p < 0.05$) decrease in the activity of SOD level in MSG treated group as compared to control group. Meanwhile, the administration of Vit.C alone and propolis alone to rats elicited non-significant changes as compared to a normal control group. Administration of Vit.C in combination with propolis exhibited slight significant decrease in SOD activity with respect to control group by 27.35%. In addition, a significant recovery relating to CAT and GPX was observed in response to the presence of Vit.C with propolis in treated rats with MSG. Treatment of the rats with MSG alone decreased the CAT and GPX activities. However, Co administration of Vit.C and propolis with MSG increased the CAT and GPX activities as compared with the MSG group.

The treatment of the rats with MSG alone induced a highly significant increase in MDA

level (non-enzymatic antioxidant) as MDA is the final product marker of lipid peroxidation.The treatment of rats with Vit.C and propolis in combination with MSG afforded a significant reduction in MDA as compared to MSG treated group alone by 53.2%.

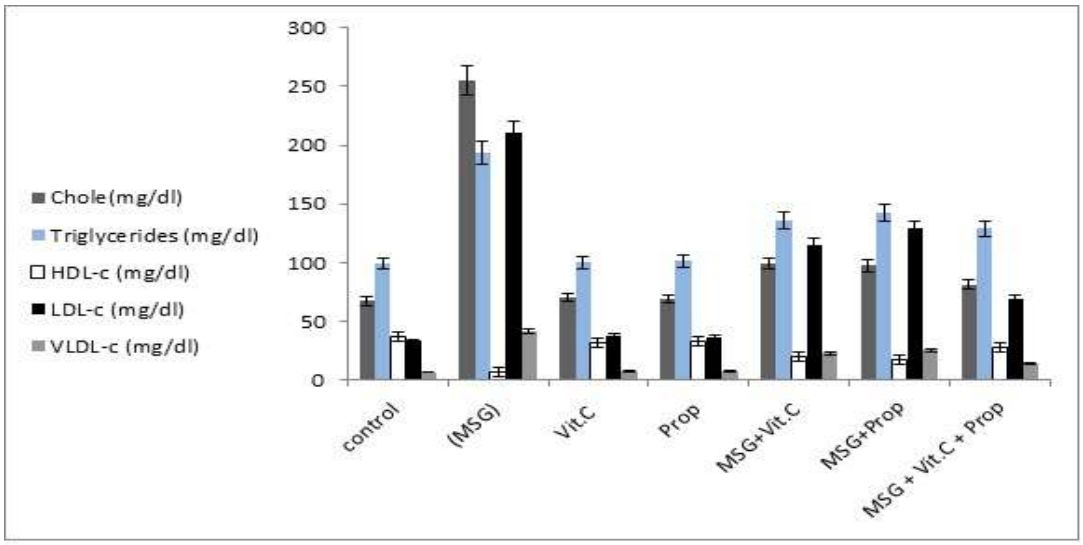

Fig. 1. Effects of treatment of monosodium glutamate, Vitamin C, propolis and their combinations with MSG on lipid profile parameters

Table 1. Effects of treatment of monosodium glutamate, Vitamin C, propolis and their combinations with MSG on lipid profile parameters

Groups	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL-c (mg/dl)
1- control group	67.22±4.57^{g}	98.99±4.77^{f}	36.78±3.62^{ab}	33.24±2.52^{f}	6.64±2.45^{e}
2-Mono sodium Glutamate (MSG)	255.14±22.1^{a}	193.62±9.54^{a}	7.25±2.42^{g}	210.23±12.45^{a}	42.04±4.21^{a}
3-Vitamin C	70.11±2.15^{ef}	100.25±10.25^{ef}	31.20±5.24^{c}	37.20±5.42^{ef}	7.44±3.52^{de}
4-Propolis	69.11±2.15^{fg}	101.63±8.65^{ef}	33.25±3.24^{bc}	36.52±3.52^{ef}	7.30±5.75^{de}
5-MSG+Vit.C group	99.41±6.75^{bc}	135.74±6.48^{c}	20.34±3.21^{e}	115.32±4.36^{c}	23.06±6.75^{b}
6-MSG+Propolis	97.52±3.54^{c}	142.03±7.25^{bc}	17.28±3.21^{f}	128.98±6.38^{b}	25.79±3.68^{b}
7- MSG+Vit.C + Propolis	81.08±4.12^{d}	129.24±9.45^{d}	27.36±2.75^{d}	69.21±4.12^{d}	13.84±3.96^{c}

Means within the same column in each category carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range tests, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically

Table 2. Effects of treatment of monosodium glutamate, Vitamin C, propolis and their combinations with MSG on liver function parameters

Groups	ALT (U/L)	AST (U/L)	LDH(U/L)
1- control group	13.00±0.56^{ef}	12.20±0.54^{de}	310.66±20.63^{d}
2-Mono sodium Glutamate (MSG)	91.00±1.18^{a}	177.80±7.39^{a}	2200.40±25.37^{a}
3-Vitamin C	12.35±1.42^{f}	11.98±0.23^{e}	341.30±23.10^{cd}
4-Propolis	13.01±1.35^{ef}	12.24±1.01^{de}	345.69±17.33^{cd}
5-MSG+Vit.C group	70.45±1.18^{c}	109.36±5.36^{b}	853.12±23.04
6-MSG+Propolis	78.80±1.82^{bc}	107.80±6.02^{b}	855.40±25.37^{b}
7- MSG+Vit.C + Propolis	20.40±0.30^{d}	55.40±1.12^{c}	650.21±18.23

Means within the same column in each category carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range tests, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically

Table 3. Effects of treatment of monosodium glutamate, Vitamin C, propolis and their combinations with MSG on antioxidant parameters capacities in liver homogenates

Groups	MDA (nmol/mg protein)	CAT (nmol/mg protein)	SOD (U/mg protein)	GPx (nmol/mg protein)
1- control group	0.24±0.01^{b}	0.86±0.12^{a}	9.65±0.36^{ab}	6.67±0.63^{ab}
2-Mono sodium glutamate (MSG)	0.92±0.03^{a}	0.25±0.10^{e}	2.63±0.45^{e}	1.10±0.85^{f}
3-Vitamin C	0.25±0.02^{b}	0.85±0.23^{a}	9.30±0.96^{b}	6.55±0.74^{b}
4-Propolis	0.26±0.02^{b}	0.87±0.15^{a}	9.38±0.42^{b}	6.50±0.36^{b}
5-MSG+Vit.C group	0.63±0.01^{d}	0.43±0.18^{d}	5.55±0.36^{d}	3.79±0.10^{cd}
6-MSG+Propolis	0.76±0.04^{d}	0.49±0.20^{cd}	5.58±1.20^{d}	3.87±0.57^{c}
7- MSG+Vit.C+ Propolis	0.43±0.03^{c}	0.65±0.35^{b}	7.01±1.11c	4.36±0.85^{e}

Means within the same column in each category carrying different litters are significant at (P ≤ 0.05) using Duncan's multiple range tests, where the highest mean value has symbol (a) and decreasing in value were assigned alphabetically

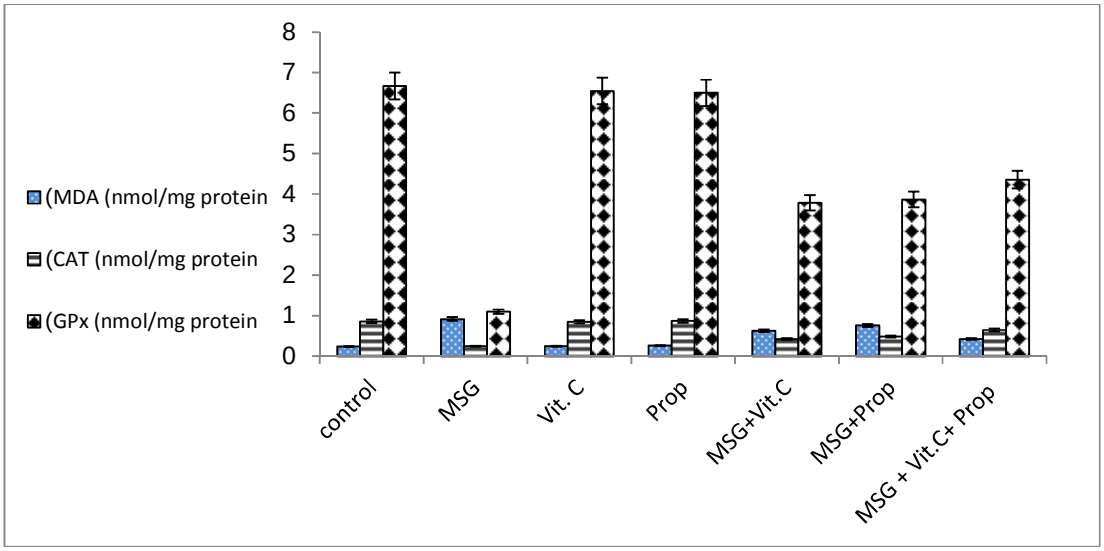

Fig. 2. Effects of treatment of monosodium glutamate, Vitamin C, propolis and their combinations with MSG on antioxidant parameters capacities in liver homogenates

4. DISCUSSION

The present study was conducted to elucidate the protective role of Vit.C or propolis separately or in combination against MSG-induced hepatic damage and oxidative stress in liver tissues of rats. There is no literature report available on the efficacy of Vit.C and propolis in combination in cases of MSG exposure in rats. In this respect, the present study bears originality.

Variation in total cholesterol levels were significantly within the groups; however, MSG treated group induced significant increase in cholesterol levels more than other groups treated with a combination of MSG and VitC and propolis, which suggest a direct correlation between MSG administration and the increment of cholesterol level and affecting on fat metabolism. Inuwa et al. [25] found that the potential explanation for MSG – obesity link lies in the alteration of regulatory mechanisms that affect fat metabolism.Also, another explanation in this study related to increased levels of TG, LDL-c and vLDL-c and decreasing HDL-c level suggests that MSG could be a risk factor or coronary heart disease [26].

According to our previous results recorded by Hamza et al. [27] as they reported the role of propolis in decreasing blood glucose levels in diabetic rats and thus enhancing lipid metabolism as it is known that liver is organ involved in glycogenesis, glycogenolysis, gluconeogenesis, and glycolysis and thus affecting by direct way on lipid metabolism and this confirmed our obtained results by decreased the levels of TC,TG,LDL-c and VLDL-c in groups treated by either propolis or combination between propolis and MSG.

Results of Elmazoudy et al. [28] supported our results as they clearly indicated that previous administration of the propolis induced marked change in lipoproteins and elicited marked fall in the total cholesterol and LDL-c levels in rats induced by CPF in the rat blood serum.

The elevated levels of ALT and AST levels could be an indication of hepatocellular damage induced by MSG [25]. Our findings are greatly supported by our previous results obtained by Diab et al. [29] as they showed that the AST activity determined to be higher in the group that was administered Chlorpyrifos and AST was demonstrated to be decreased in the groups that were administered Propolis in combination with Chlorpyrifos.

Ramadan et al. [30] reported that oral administration of Propolis for 70 days; decreased the activities of AST and ALT in plasma. Also, Sforcin et al. [31] reported that treatment of rats with Propolis does not induce any alteration in AST level.

Moreover, Mani et al. [32] found no alteration in AST value in the serum of Propolis treated rats for (30 or 90 or 150 days) at doses of (1, 3 and 6 mg/kg/day) and so all these findings supported our obtained results that propolis protect against hepatotoxicity induced by MSG administration.

It has been generally accepted that the infiltration of LDH enzyme corresponds well with the viability of the cell membrane, thus being a good marker of the damage of the plasmatic membrane and/or necrosis of hepatocytes [26]. The present significant elevation in liver enzymes AST and ALT in MSG treated group , thus may confirm that the hepatocytes are more affected by the exposure to MSG.

Oxidative stress refers to the disruptance of the redox equilibrium between the production of free radicals and the ability of cells to protect against damage caused by these species. Defense against oxidative stress are preserved by using several mechanisms which include antioxidant defense system. The lipids are among the first cellular components which are susceptible to damage by free radicals, proteins, carbohydrates and nucleic acids; this in turn can spoil cellular structure and function [33].

It has been indicated that the LPO is one of the molecular mechanisms involved in pesticide-induced cytotoxicity [34]. These findings support the presence of oxidative stress in the present study induced by MSG. The toxic appearance induced by MSG may be associated with the enhanced production of ROS or the increase in MDA levels [35].

Among the antioxidant enzymes, SOD, CAT and GPx are the first line of defense against oxidative injury. SOD is the primary step of the defense mechanism in the antioxidant system against oxidative stress by catalyzing the dismutation of superoxide radicals (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [36]. H_2O_2 is neutralized by the combined action of CAT and GPx in all vertebrates [37]. These enzymes act in coordination and the cells may be pushed to oxidative stress state, if any,change occurs in the levels of enzymes.

Our obtained data were confirmed by the results obtained by Hamza and AL-Harbi, 2014 who reported that MSG caused increasing in the level of lipid peroxidation parallel with significant decline in SOD, CAT as well as GPx activities in hepatic tissues.

The present data indicated that MSG-induced reduction in the activities of the antioxidant enzymes (SOD, CAT and GPx) and the content of the final product of lipid peroxidation (MDA). This effect might be due to the increased production of H_2O_2 and ROS triggering which in turn stimulates oxidative stress.

In the present study, a significant decline was recorded in the specific activity of SOD in the hepatic tissues was observed in MSG-treated group, indicates an increased superoxide radical production and other ROS thereby induce oxidative stress [38].

GPx is the common name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biological function of GPx is to minimize lipid hydroperoxides conversion to their congruent alcohols and to reduce free H_2O_2 reaction [39].

In our present study, a significant decrease in GPx activity was observed in MSG-treated group. This observation may be due to enhanced, free radical production (as evidence by increase LPO) and apart from CAT also involved in the elimination of H_2O_2. H_2O_2 generated due to MSG toxicity, Hence, the GPx level diminished after MSG administration.

It was found that the administration of Vit. C and/or propolis to MSG-treated rats restored SOD, CAT and GPx activity to a nearly normal control level. However, MDA level was declined significantly after Vit. C administration as emulated to MSG. Therefore, the present decrease in GPx activity and restoration of SOD activity to nearly control level, in addition to the decrease in MDA content in the hepatic tissues, could indicate the antioxidant properties of Vit.C. It also increased the antioxidant enzymatic activity.

In full agreement with the obtained results, the mild oxidative state observed in the hippocampus due to MSG administration may be due to the implicit high content of Vit.C in this brain area as well as our observations in the group treated with Vit.C either alone or in combination with MSG. The exhaustion of Vit.C is useful for the antioxidant effect as it offers an effective and safe way of increasing body immune system against free radicals and, at the same time, keeps the oxidative stress in a state of equilibrium [40].

The results indicate the ameliorative effect of Vit. C and/or propolis in combating oxidative stress. In addition, the high antioxidant activity was found in the rat treated with Vit.C alone and in combination with propolis as reported before by Hashim [41] and Diab et al. [29]. This result indicates that vit C and propolis are a strong scavenger of free radicals.

5. CONCLUSION

In conclusion, the present findings strongly indicate that hepatic damage, hyperlipidemia and oxidative stress resulting from MSG in dose dependent manner is involved in toxicity mechanisms of liver of rat. MSG increased the enzymes leakage of LDH due to increase of the generation of free radicals as hepatic MDA increased. MSG decreased the enzymatic and non-enzymatic antioxidants. These results suggest that these MSG-induced metabolic disorders associated with oxidative stress while,Vit.C and propolis exhibited strong antioxidant activities. The results emphasize the importance of reassessment of MSG toxicity, a substance widely used in food industry. Protection can be derived from treatment of the rats with antioxidant Vit.C and propolis that increased the stability of membrane and decreased the liberation of cellular enzymes. Treatment with Vit.C and propolis after treatment with the MSG restored the same efficiency of the antioxidant enzymes, and they plays a preventive role against MSG-induced cellular oxidative stress.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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