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Hepatoprotective Impact of Vitamin C against Mitochondrial Dysfunction Induced by Malathion on Rat Liver

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ABSTRACT

The present study is designed to investigate the possible hepatoprotective effect of vitamin C on liver of rats. A total number of 32 female albino rats. Animals were divided into four groups; **Group 1:** Negative control group **Group 2:** Positive control group, animals in this group were administrated with Malathion (50 mg/kg/day) intraperitoneally, **Group 3:** Animals in this group were orally administrated with vitamin C (100 mg/kg/day) and **Group 4:** Animals in this group were administrated with malathion (50 mg/kg/day) intraperitoneally with vitamin C (100 mg/kg/day) orally. The blood samples were centrifuged in cooling centrifuge to separate serum to measure aspartate amino transferase (AST) and alanine amino transferase (ALT) activity, total protein and albumin. Tissues of the liver was homogenized to estimate the content of reduced glutathione (GSH), malondialdehyde (MDA) and the activity of copper-zinc superoxide dismutase (SOD). Also another portion of the liver tissue was homogenized to isolate mitochondria to estimate the content of GSH, MDA and the activity of SOD, nuclear factor-erythroid 2-related factor2 (Nrf2) and kelch like ECH associated protein 1(keap1). Histological examination of the tissues was conducted after removal of liver tissues from rats. The results indicated a significant increase MDA level in the liver and mitochondria homogenates of malathion-treated rats compared to the negative control ones. Malathion also resulted in a significant decrease in liver and mitochondrial GSH content. ALT & AST activities significantly increased while protein and albumin significantly decreased compared to the negative control. The obtained results illustrated that rats treated with Malathion for 30 days showed a significant decrease in Nrf2 while Keap1 decreased significantly compared to the negative control group. Animals treated with malathion+ vitamin C showed an improvement in all these parameters and the histological study confirmed these result. In a conclusion, vitamin C can ameliorate the hazardous effects of Malathion.

Keywords: Nrf2**,** Keap1, histology, Malathion, hazardous

1. Introduction

The liver is one of the organs rich in mitochondria. Hepatic mitochondria have unique features compared to other organs' mitochondria, since they are the hub that integrates hepatic metabolism of carbohydrates, lipids, and proteins. Thus, correct functioning of hepatic mitochondria is essential to prevent liver disease (Esposti *et al.,* 2012). Mitochondria, an abundant source of intracellular reactive oxygen species (ROS), has been implicated as the major target for oxidative damage. As reported by Mannam *et al.* (2014). Mitochondria, the principal source of cellular adenosine triphosphate, are dynamic organelles that undergo delicate fusion and fission cycles to maintain their functions when cells experience metabolic or environmental stress (Youle and Bliek, 2012).

Pesticides are an important class of environmental chemical pollutants. Their steady and continuous use for more than half a century has now become a major public health problem. Thus,

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some studies suggest that the exposure to a wide range of pesticides could result in the production of free radicals and the inactivation of components of the mitochondrial respiratory chain (Bettiche, 2017). Malathion is an organophosphorus pesticide that is widely used in agricultural and household applications to control pests (Helal *et al.,* 2016).

Vitamin C (Ascorbic acid) is the most important vitamin in fruits and vegetables, and has been regarded as the most potent natural antioxidant and thus, vitamin C has become an essential dietary component for human survival (Ambali *et al.,* 2011). Vitamin C has anti-inflammatory effects, prevents endothelial dysfunction and apoptosis, and reduces the risk of arteriosclerosis, cardiovascular disease and some forms of cancer (Razaa *et al.,* 2015). Oxidative stress occurs when the generation of reactive oxygen species in the body exceeds the ability of the body to neutralize and eliminate them (Sharma *et al*., 2012). In agriculture, pesticide has been used to enhance food production by eradicating unwanted insects and controlling disease vectors. Pesticides are found as common contaminants in soil, air, water and on non-target organisms in our urban landscapes. Once there, they can harm plants and animals ranging from beneficial soil microorganisms and insects, non-target plants, fish, birds, and other wildlife (Toualbia *et al.,* 2017). This study will clarify the hepatoprotective effect of vitamin C on liver of rats treated by Malathion.

Experimental design:

A total number of 32 female albino rats (180-200 g) obtained from the laboratory stock colony of National Organization for Drug Control and Research (NODCAR) were used in the present study. The animals were kept under normal environmental conditions for two weeks before the initiation of the experiment. The animals get a free access of water and fed on a standard diet. The local ethics committee of NODCAR approved this study with an approval number, NODCAR/II/5/2020.

Animal Grouping:

The animals were divided into four groups:

Group 1: Negative control group.

Group 2: Positive control group, animals in this group were administrated intraperitoneally with Malathion (50 mg/kg/day) (Ranjbar *et al.,* 2014).

Group 3: Animals in this group were orally administrated with vitamin C (100 mg/kg/day) (Ambali *et al.,* 2011**)**.

Group 4: Animals in this group were intraperitoneally administrated with Malathion (50 mg/kg/day) with vitamin C (100 mg/kg/day) orally.

Duration of the experiment is 30 days. At the end of the experiment, rats were fasted overnight and blood sample was collected from retirobulbar venous plexus by fine capillary tubes. Blood then centrifuged and the obtained serum was used for biochemical analyses. After that, rats were euthanized by decapitation for the isolation of liver.

Sampling:

The blood samples were centrifuged at 4000 rpm for 15 minutes at cooling centrifuge to separate serum to measure aspartate amino transferase (AST) and alanine amino transferase (ALT) activity. Total protein and albumin. Tissues of the liver was homogenized to estimate the content of reduced glutathione (GSH), malondialdehyde (MDA) and the activities of copper-zinc superoxide dismutase (SOD), Also another portion of the liver tissue was homogenized to isolate mitochondria to estimate the content of GSH, MDA and the activity of SOD**,** Nrf2 and keap1.

Isolation of liver mitochondria

The liver tissue was distanced with small cutters in a cold mannitol solution containing 0.225 mmol D-mannitol, 75mmol sucrose, and 0.2 mmol EDTA. The minced liver was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700 r.p.m for ten minutes at 4°C to remove nuclei, unbroken cells, and other non-subcellular tissues. The supernatants were centrifuged at 7000 r.p.m for 20 minutes. These second supernatants were taken as the crude microsomal part and the pale loose upper layer of sediments, which was rich in swollen or broken mitochondria, lysosomes and some microsomes, was washed away. The dark packed lower layer (heavy mitochondrial fraction)

was resuspended in the mannitol solution and centrifuged twice at 7000 r.p.m for 20 minutes. Then weighty mitochondrial sediments were floated in Tris solution buffer (pH, 7.4) according to Ramasarma, (1982) and Ghazi *et al.,* (2006).

2. Materials and Methods

2.1. Biochemical analysis:

Aspartate amino transferase (AST) and alanine amino transferase (ALT) activities were estimated by using an enzymatic kit (Randox Laboratories, Crumlin, UK) according to the manufacturer's instructions. Total protein and albumin were measured colorimetrically by using Biodiagnostic kits according to the manufacturer's instructions. Nrf2 and keap1 determined by ELISA according to the manufacturer's instructions. The concentration of protein in liver tissues and mitochondrial fraction were determined according to method of Lowry (1951). GSH content was determined according to Beutler method (Beutler *et al.,* 1963), estimation of MDA according to the method of Buege and Aust (1978). The activity of SOD was determined according to the method of Marklund and Marklund (1974) with some modification according to Nandi and Chatterjee (1988).

2.2. Histopathological Assessment:

Liver of rats collected for histopathological examination and fixed in 10% buffered formalin overnight, then embedded with paraffin. All paraffin-embedded tissue was sectioned at 4µm, deparaffinized in xylene, dehydrated by ethyl alcohol in a decreasing concentrations (100%, 95%&70%), and stained with haematoxylin and eosin stain. These specimens were examined under bright field optical microscopy using a light microscope and 40×magnification powers. Corresponding digital images were captured for later analysis **(**Banchroft *et al.,* 1996**)**.

2.3. Statistical Analysis:

Statistical analysis was performed using SPSS for Windows version 17.0. Data was given in the form of arithmetical mean values ± standard error (S.E). Differences between groups were evaluated by one-way ANOVA according to $P<0.05$ and post–hoc Duncan test (Dawson and Trapp, 2004).

3. Results

3.1. Effect of Malathion and Vitamin C on Oxidative Strees:

The results indicated that in the liver and mitochondria homogenates of malathion-treated rats, MDA level was significantly increased. While GSH content was significantly decreased when compared to controls (figures 1A&B). Malathion also resulted in a significant reduction in SOD activity in tested liver and mitochondria (figure 1C). Whereas animals treated with malathion+ vitamin C showed significant ameliorative changes in all the tested parameters in liver and mitochondria**.**

3.2. Effect of Malathion and Vitamin C on Biochemical parameters

Animals treated with malathion showed a significant increase in ALT and AST while they showed a significant decrease in protein and albumin compared to the negative control ones (figure 2A, B, & C). However, animals treated with malathion+ vitamin C showed an improvement in all these parameters**.**

3.3. Effect of Malathion and Vitamin C on Nrf2 and Keap1

The obtained results illustrated that rats treated with Malathion for 30 days showed a significant decrease in Nrf2 while there is an elevation in the level of Keap1 when compared to the negative control group. However, treatment with Malathion plus vitamin C ameliorate these parameters

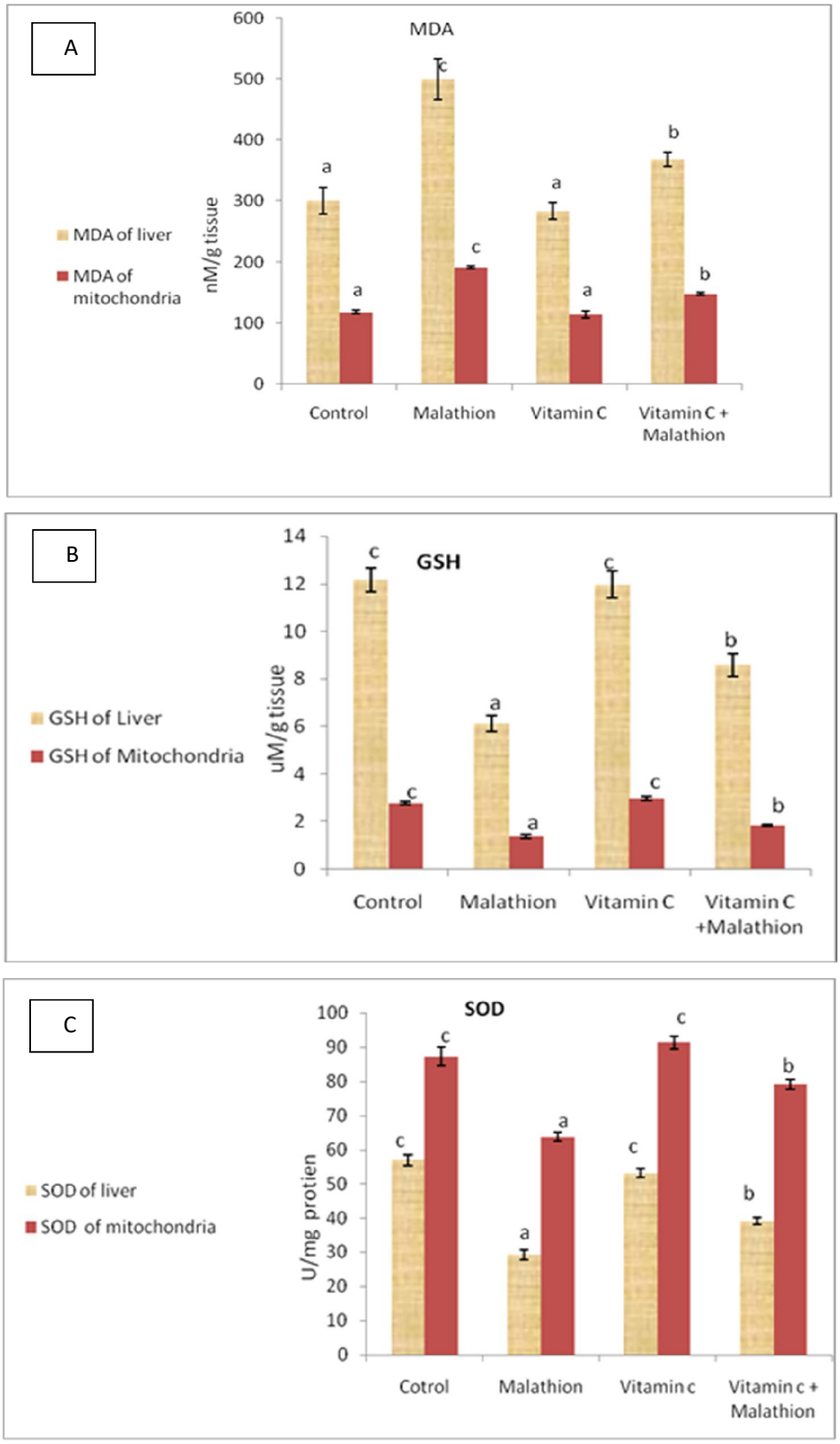

Fig. 1: Effect of malaththion and vitamin C on MDA,GSH and SOD of all studied groups

Each bar represents a mean value ± SE (n=6). The presence of different letters on each bar means significant differences between groups. ANOVA test followed by multiple comparisons between groups at $p < 0.05$.

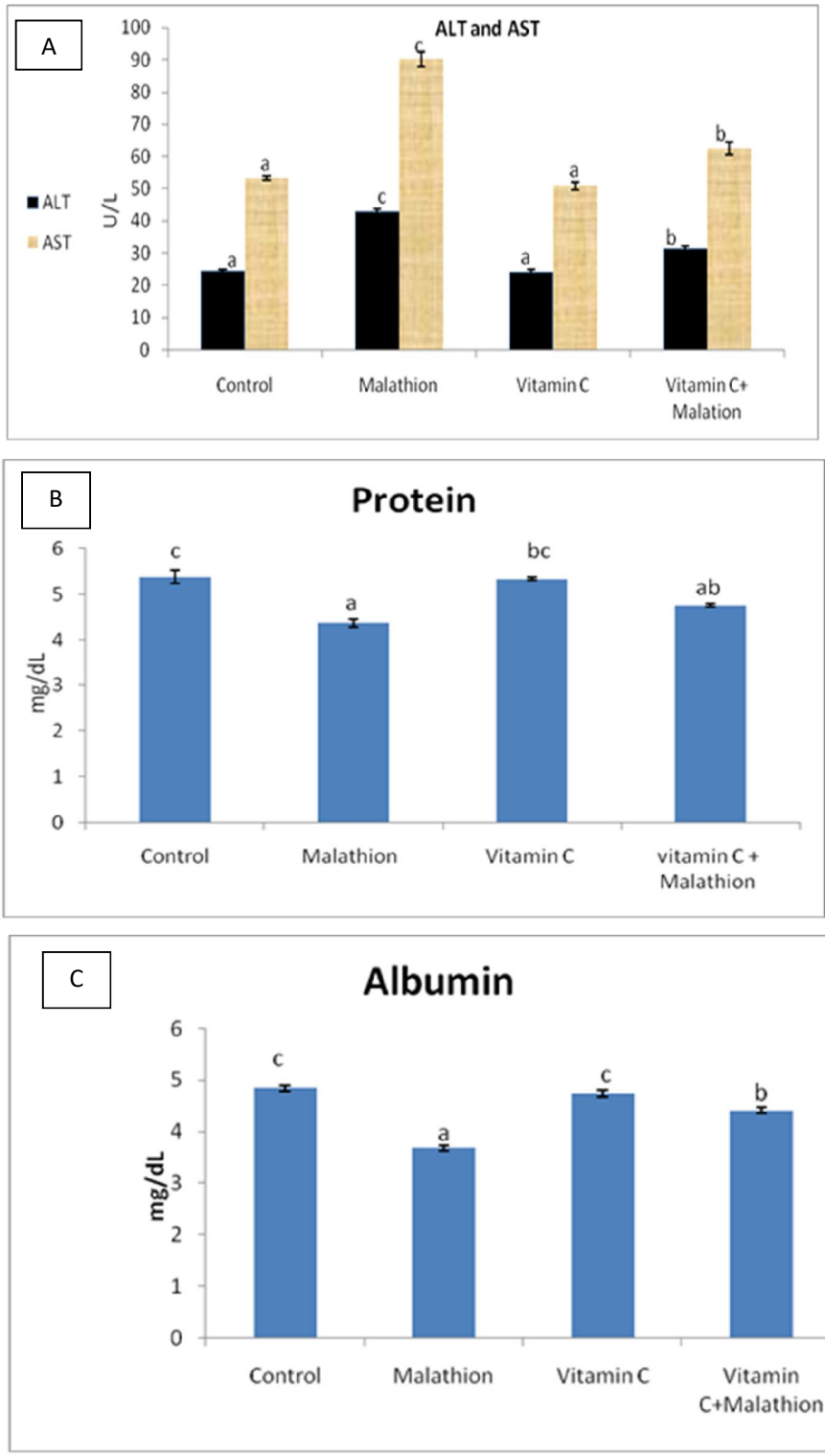

Fig. 2: Effect of malaththion and vitamin C on biochemical parameter (ALT, AST, Protien and Albumin) of all studied groups

Each bar represents a mean value ± SE (n=6). The presence of different letters on each bar means significant differences between groups. ANOVA test followed by multiple comparisons between groups at $p < 0.05$.

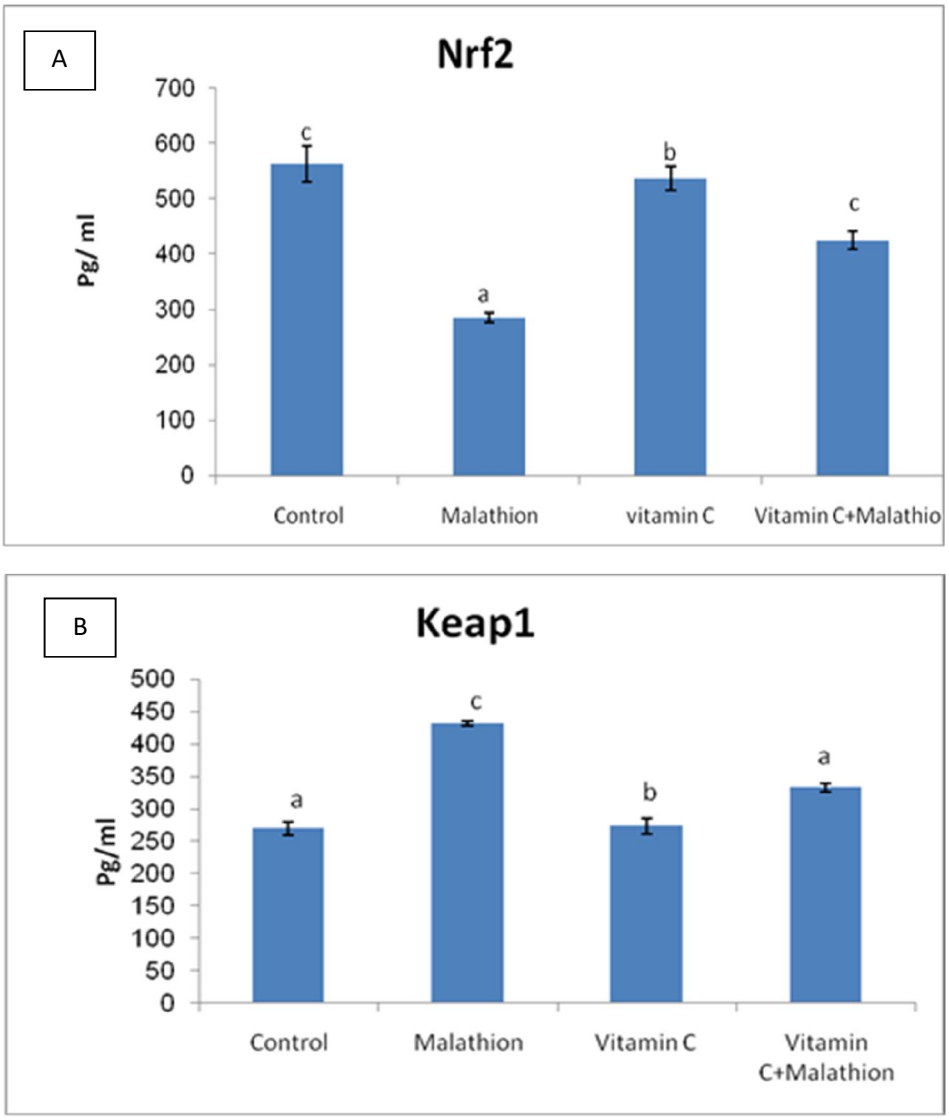

Fig. 3: Effect of Malathion and Vitamin C on Nrf2 and Keap1of all studied groups in mitocondria

Each bar represents a mean value ± SE (n=6). The presence of different letters on each bar means significant differences between groups. ANOVA test followed by multiple comparisons between groups at $p < 0.05$.

3.4. Histopathological examination of liver:

Microscopically, liver of rats from control normal group (G1) revealed the normal histological structure of hepatic lobules (Figs. 4). Moreover, liver of rats treated with Malathion (50 mg/Kg/day) (G2) intraperritoneally, showed focal hepatic necrosis associated with inflammatory cells infiltration, hemorrhage and portal fibrosis (Fig. 5). Inflammatory cells infiltration and degeneration of some hepatocytes /day) orally (G3), showed no histopathological alterations (Figs.7). On the other hand, liver of rats treated with vitamin C with Malathion (G4) revealed slight portal infiltration (Fig. 8). Whereas, cytoplasmic vaculation, congestion of central vein and dilatation of hepatic sinusoids were noticed in liver section of rats treated with vitamin C with Malathion (G4) (Fig.9).

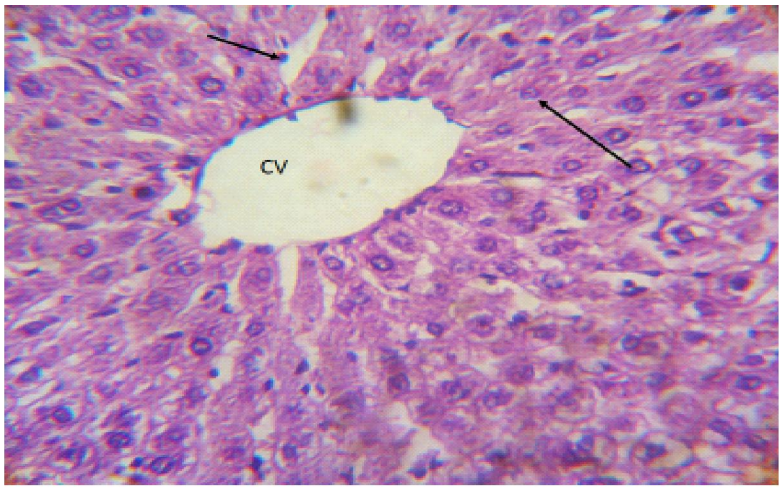

Fig. 4: Photomicrograph of liver section of rats from control normal group revealed the normal histological structure of hepatic lobules (H&E, 400).

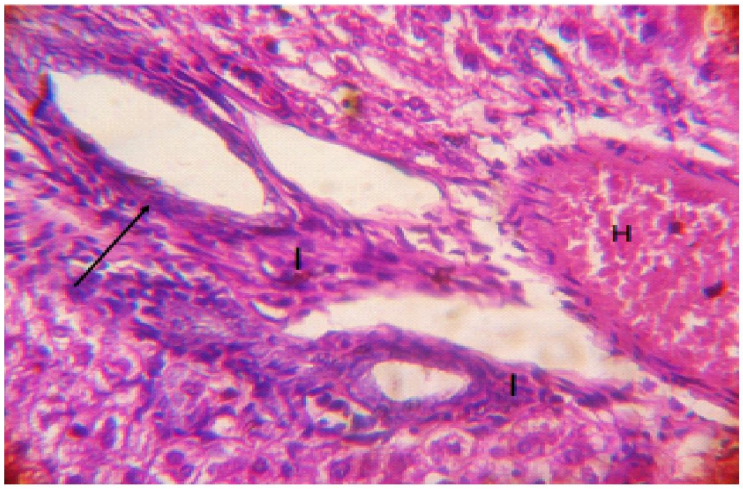

Fig. 5: Photomicrograph of liver section of rats treated with Malathion (50 mg/Kg/day) intraperritoneally, showed inflammatory cells infiltration, hemorrhage and portal fibrosis (H&E, 400).

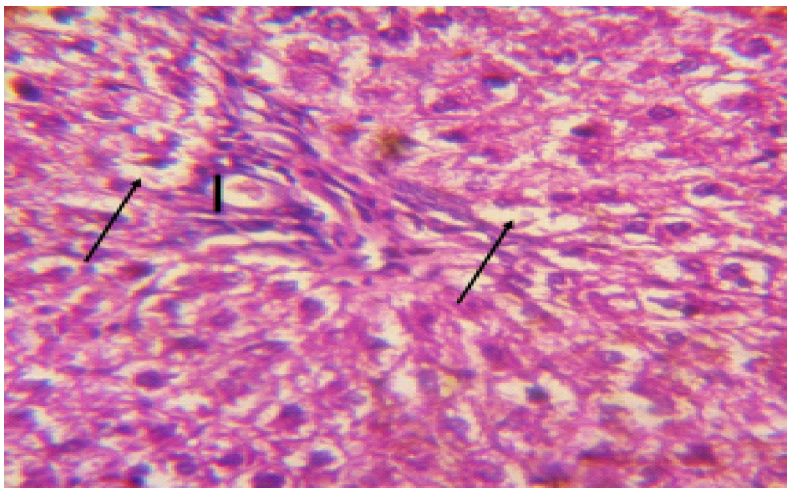

Fig. 6: Photomicrograph of liver section of rats treated with Malathion showed inflammatory cells infiltration and degeneration of some hepatocytes (H&E, 400).

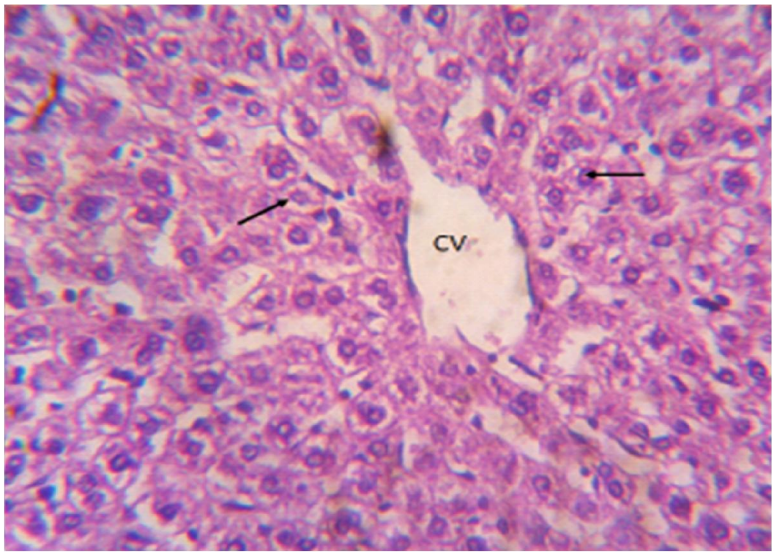

Fig.7: Photomicrograph of liver section rats treated with vitamin C (100 mg/Kg /day) orally, showed no histopathological alterations (H&E, 400)**.**

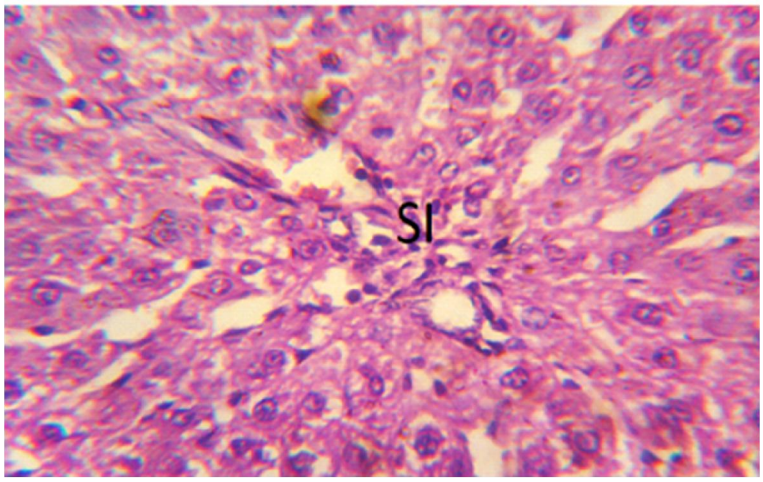

Fig. 8: Photomicrograph of liver section of rats treated with vitamin C combined with Malathion revealed slight portal infiltration and dilatation of hepatic sinusoids (H&E, 400).

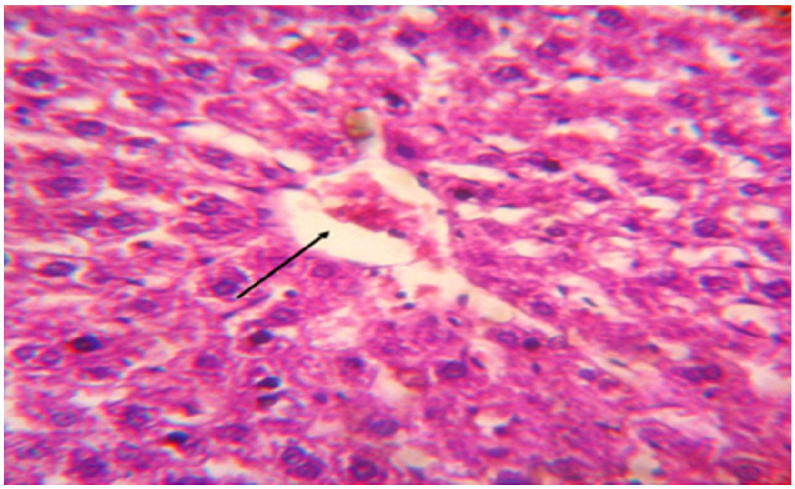

Fig. 9: Cytoplasmic vaculation and congestion of central vein were noticed in liver section of rats treated with with vitamin C combined with Malathion (H&E, 400).

4. Discussion

In the present study Malathion increased malondialdehyde level, whereas it decreased glutathione level these results in agreement with Akbel *et al*. (2018); Abdel-Razik and Hamed (2021). It is hypothesized that free radicals generated during metabolism of malathion by cytochrome P450-oxidase induced lipid peroxidation (Abdulaziz *et al*., 2011.). Treatment with vitamin C resulted in a significant decrease in the liver and mitochondrial content of MDA when compared with Malathion treated group.

The main result of the present study was that Malathion induced oxidative damages and mitochondrial dysfunction in rat liver. These results clearly indicate that Malathion increased liver oxidative damage in rat by prompting of LPO, GSH and SOD activities, and these results were conscious with those of previous investigators (Mohamed *et al*., 2015 and Abdulaziz *et al*., 2011) and in parallel with Ranjbar *et al*. (2014). Vitamin C was able to attenuate Malathion-induced changes in all of the tested parameters. In addition, vitamin C decreased the mitochondrial toxicity in liver. The key function of mitochondria is producing energy, by oxidative phosphorylation in the form of ATP, for cellular processes (Balaban *et al*., 2005). These functions are linked to neurodevelopment, connections, tissue differentiation, and plasticity (Bergman and Ben-Shachar, 2016). Mitochondrial dysfunction is a fundamental pathogenic mechanism that leads to several significant toxicities in mammals, especially those associated with the liver and spleen (Zanoli *et al*., 2012). Numerous studies have revealed that mitochondrial dysfunction is a significant mechanism of drug-induced toxicity, as well as the primary mechanism in several disorders and a prominent toxicological target (Bergman and Ben-Shachar, 2016). This study reported the direct indications of damage in the liver mitochondria induced by Malathion insecticide. It seems that the LPO byproducts induced a state of oxidative stress in mitochondria.

Mitochondrial GSH plays a key role in order to avoid the excessive accumulation of hydrogen peroxide and subsequent oxidative stress (Marí *et al.,* 2013). It is the primary non-protein thiol in cells, with the redoxactive thiol of its cysteine moiety serving as a cofactor for a variety of antioxidant and detoxifying enzymes. GSH is synthesized exclusively in the cytosol from its constituent amino acids, yet it is dispersed throughout the body, including mitochondria, where its matrix concentration equals that of the cytosol. This property and its negative charge at physiological pH, suggests the existence of specific carriers that transport GSH from the cytosol to the mitochondrial matrix, where it is involved in the detoxification of lipid hydroperoxides and electrophiles as well as the defense against respiration-induced ROS (Abdel-Razik and Hamed 2021).

It has been revealed that malathion-induced oxidative stress modulates SOD activity in the liver (John *et al*., 2001). Malathion changed the antioxidant enzymes such as SOD following subchronic exposure in animals (Akhgari *et al*., 2003, Possamai *et al*., 2007). It has been confirmed that malathion-induced oxidative stress is due to the inactivation of mitochondrial respiratory complexes (Ranjbar *et al*., 2010, Kalender *et al*., 2010). Abdel-Razik and Hamed *et al*., (2021) reported that mitochondrial SOD activity had lowered by half, resulting in a functional decrease in oxidative phosphorylation, an increase in oxidative stress, and an increase in the rate of apoptosis. According to the findings, mitochondrial SOD (Mn SOD) plays a crucial role in the redox state of mitochondria in cells and tissues (Buettner, 2011).The current results showed that SOD activity of liver and mitochondria was significantly inhibited this result in consistent with Abdel-Razik and Hamed (2021). The decreased SOD activity in chlorfenapyr (CFp) intoxicated rat may be owed to the consumption of this enzyme in converted O2- to H2O2 Abdel-Razik and Hamed (2021) so we can attributed SOD decrease to the same reason.

The result of the present study showed a significant increase in serum ALT, AST in Malathion-treated animals when compared with control, this result consistent with those of Mohamed *et al*. (2015) and Atef (2010). A significant increase in serum activities of AST and ALT considered like markers enzymes of hepatocyte cytolysis (Mohamed *et al*., 2015). The recorded increase of AST and ALT levels may be due to increased LPO, there is a high positive correlation between serum rates of AST and ALT and hepatic MDA level (Mohamed *et al*., 2015). When the liver cell membrane is damaged, several enzymes located in the hepatocyte cytosol, including, ALT, AST and ALP, are secreted into the blood (Ncibi *et al*., 2008). It has been shown that OrganoPhosphours insecticides can elevate the enzymatic activities of ALP, ALT, and AST (Ncibi *et al*., 2008). Moreover, Malathion raises the ALT and AST levels in rats (Rezg *et al*., 2008). Also, we Total protein and albumin reduce

by a significant value with Malathion, this result agree with Elzoghby *et al*. (2014). Several studies have shown that albumin production by liver can be decreased under organophsphours (OP) exposure (Kalender *et al*., 2005; Yousef *et al*., 2006; Ogutcu *et al*., 2008). It is possible that OP like Malathion alter protein and free amino acid metabolism and their synthesis in the liver (Ncibi *et al*., 2008). These results may be due to disturbance in protein synthesis in the liver which resulted from hepatocytes dysfunction (Pahwa and Chtterje, 1990).

The major natural antioxidant, which are derived from the natural sources by dietary intake, are vitamins A, C, E and carotenoids (Heistad, 2006). Accordingly, the natural antioxidant prevent oxidative damage as a factor in the pathophysiology and histopathology of various health disorders (Shireen *et al*., 2008; Budin *et al*., 2011). Vitamin C is one of the most widely available and non-enzymatic antioxidant molecules that have been used to mitigate oxidative damage (Naidu, 2003). It readily scavenges physiological organophosphate pesticide-induced hematological and biochemical alterations in humans and animals (Ambali *et al*., 2007; Aly *et al*., 2010; Karmmon *et al*., 2011). This relatively non-toxic antioxidant possesses great benefit in the amelioration of toxic effects by most xenobiotics (Uchendu *et al*., 2012).

In the present study, administration of vitamin C plus malathion result in ameliorate GSH, MDA, SOD, ALT, AST, total protein and albumin this finding agree with Ismail (2013); Elzoghby *et al*. (2014). Improvement of liver functions may be due to inhibition of lipid peroxidation that cause damage of cell wall, cell lyses' and necrosis, the most important free radical scavenger in extracellular fluids and protecting cell membranes from per oxidative damage (Sulak *et al*., 2005)

Nuclear factor-erythroid 2-related factor-2 (Nrf2), a redox sensitive transcription factor, is the most important regulator of the antioxidant and cytoprotective defense system in the body (Loboda *et al*., 2016). In its inactive state, Keap1, a protein that inhibits the translocation of Nrf2 into the nucleus, sequesters Nrf2. ROS produced in the cell are able to react with critical cysteine sulfhydryl groups present in Keap1, leading to its degradation by the ubiquitine proteasome system and activation of Nrf2. The active Nrf2 then translocates into the nucleus and transactivates the expression of a broad spectrum of antioxidant and cytoprotective genes containing an antioxidant response element in their promoters (Kim *et al*., 2010). Thus, Nrf2 protects the body against damage by oxidants and other endogenous or exogenous insults and exerts a preventive effect against various disorders including cancer, heart disease or diabetes (Hybertson and Gao. 2014). Notably, the beneficial effects of dietary secondary plant metabolites in the prevention of various diseases including coronary heart disease or diabetes are induced by an activation of Nrf2, which also proves important role of this transcription factor for human health (Qin and Hou 2014; Merry and Ristow, 2016).

In the present study, Nrf2 increased significantly in Malathion group. While it decreased in a significant manner by using vitamin C with Malathion this finding agree with Vadhana *et al*. (2013); Satta *et al*. (2017) who reported an increase in Nrf2 mRNA level when old age rats exposure to low doses of permethrin in long-term consequences leading to cardiac hypotrophy and with (Rodrigues *et al*., 2019). Oxidative stress can act directly on the Nrf2-Keap-1 complex or alternatively, *via* activation of protein kinases (P13 K, p38, ERK, PKC, JNK) causing phosphorylation and subsequent release of NRF2 from its inhibitory protein (Son *et al*., 2008).

Keap1 is an adapter of Cul3-based E3 ubiquitin ligase that can inhibit Nrf2 and Nrf2-dependent transcription levels, and anchor Nrf2 in the cytoplasm under normal conditions (Deng, 2014). In the present study keap1 increased significantly with malathion when compared with control group this finding in agreement with (Juan *et al*., 2017) who reported that mRNA expressions of IL-1α, IL-1β, IL-6, MAPK3, NF-κB, SIRT1, TNF-α, Keap1, GPX2, and Caspase-3 were significantly increased in the GLP- Glyphosate (N-phosphonomethyl-glycine, GLP) (herbicide) treated groups of rat liver compared to the control group. However, when cells undergo oxidative stress due to exogenous stimulus, Nrf2 will separate from Keap1 and enter into nucleus, leading to the accumulation of nuclear Nrf2. And nuclear Nrf2 can activate the transcription of a series of antioxidant and detoxification genes like glutathione S-transferase (GST) and UDP-Glucuronosyl Transferase (UGT) to prevent oxidative stress, by binding to the antioxidant response element (ARE) and the small Maf protein (musculoaponeurotic fibrosarcoma oncogene homolog) (Guo *et al*., 2015). While malathion + vitamin C ameliorate Nrf2 and Keap1 in the present study.

In the histological study, a marked congestion and hemorrhage were observed in tissues seemed to be a sort of inflammation induced by toxic agent such pesticides. Histamine liberated from

damaged cells is an important factor in the vascular response that follows injury, causing increased blood flow into the capillary bed and vessels, which induced vasodilation. The inflammation entails both residing and circulating inflammation cells movement and stimulation and cytokine production Triggered inflammatory cells and produced cytokines stimulate fibroblasts to divide, migrate, secrete and generate collagen (Nagao *et al*., 2003).

Hepatotoxic materials cause damage to hepatocytes those results in hepatic fibrosis characterized by the deposition of collagen, proteoglycans, and glycoproteins caused by dysregulation of physiological wound healing. Whereas, xenobiotic metabolites induce hepatocyte damage through inflammation-related cytokines and fibrogenic mediators induced by the release of reactive oxygen species (Kisseleva and Brenner 2006). Necrosis, which was observed in liver tissues of rats treated with Malathion, may be attributed to hypoxia (reduced oxygen in the blood). Hypoxia leads to both necrosis and apoptosis (Shimizu, *et. al*., 1996).

5. Conclusion

The findings of this study unveiled the hazardous effects of Malathion repeated exposure on the tested rats, raising concerns about this pesticide that possesses a potential hazard to human health. In addition, it is important to note that some adverse effects may be caused by impurities and metabolites. Hence, it is important to do more toxicological studies about these chemicals and their impurities. Vitamin C can ameliorate the hazardous effects of Malathion.

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