IJBPAS, February, 2016, 5(2): 437-455

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

STUDY OF EFFECT OF HYDROALCOHOLIC EXTRACT OF WALNUT LEAF IN REDUCTION OF OXIDATIVE STRESS DUE TO PARACETAMOL IN LIVER OF RAT

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ABSTRACT

Paracetamol is a centrally-acting nonsteroidal anti-inflammatory drug overdose in the production of Reactive Oxygen Species by severe liver oxidases and the mechanisms causing severe damage to some tissues, especially by the liver. Walnut shell has a different collection of antioxidants that can neutralize released radicals and oxidative stresses due to their agents the manufacturer. Therefore, due to the antioxidant properties of Juglans regia, this study to evaluate the antioxidant effect of ethanol extracts of leaves of walnut (JRLE) against paracetamol induced by oxidative stress in rats was conducted.

In this experimental study, 60 male SD rats weighing 210±20 g, aged 8-7 weeks were randomly divided into four groups. Group 1 was selected as a control. In groups 2 and 4, JRLE 200 (mg/kg) were treated for 14 consecutive days. Groups 3 and 4 single dose of paracetamol 835 (mg/kg) on the eighth day trial received intraperitoneal administration of walnut leaves in groups 2 and 4 continued to 6 days. Finally, tissue levels of Malondialdehyde, glutathione and activities

of superoxide dismutase, Catalase, glutathione peroxides and glutathione Reductase in liver homogenates were measured. Finally, these findings were compared to histopathologic findings.
Results: In group 4, JRLE significantly decreased the levels of MDA ($p<0.05$) and increased levels of antioxidants in the liver. Enzymatic histopathologic findings were also consistent with the pathological changes.
Conclusion: JRLE to its antioxidant properties, protect to rat liver against oxidative stress induced by paracetamol.

Keywords: Juglans regia, paracetamol, oxidative stress, antioxidant, liver, rat

INTRODUCTION

The liver is the largest organ in the body that constitutes about 3 to 5% of body mass. The liver is divided into two main lobe and left lobe and two annexes (square, tail) as well. Also, one of the most significant acts of the liver is metabolism of various substances, detoxification of environmental pollutants and chemical agents (1). In most cases in function detoxification, metabolic activation by liver microsomal cytochrome P450 enzymes and activated causing toxic metabolites that can cause damage to various organs such as the liver (2). Thioacetamide, carbon tetrachloride, ethanol and acetaminophen including substances that enter the body after metabolized by the enzyme cytochrome P450 detoxification system (3). Released radicals are atoms or molecules that are active in the recent of their atomic layer intense affinity to their surrounding molecules And if they can preventing the combined activity leads to tissue damage and cardiovascular problems including heart disease and cancer.

Oxidative stress due to an imbalance between the production of released radicals inside the body and antioxidant defensive mechanisms can be achieved in organisms lipid peroxidation in the wall of the living cells is one of the most important goals of released radicals. This condition not only affects the structure and function of the wall but also some of the products resulting from the oxidation of biomolecules such as MDA can be demonstrated to cytotoxic and genotoxic effects. The presence of free radicals, peroxides, particularly key role in the pathogenesis of a number of diseases such as diabetes, heart disease - cardiovascular, cancer, aging and other diseases (6).

Although the synthetic antioxidants widely used, especially in the food industry, Many of these compounds have adverse effects to human health (7, 8). For example, there is

much evidence that confirm the toxicity and adverse effects of dietary antioxidants such as butyl hydroxy Anisole artificially added to food, butyl hydroxy toluene and tert beta hydroxy kinon. In addition, the risk of liver damage and cancer in laboratory animals is of the disadvantages of using artificial antioxidants (8, 9). Due to this fact, natural antioxidants, mainly medical plants, fruits and vegetables has become more popular among consumers and looks at prevention are important for a number of diseases. Since plants are an important source of antioxidants, research in this area is increasing. Plants enriched antioxidant compounds that can protect cells from damage or be Oxidative (10). Natural antioxidants increase the plasma antioxidants and reduce the risk of some diseases such as cancer, heart disease and stroke (11).

Many studies have been conducted on the antioxidant properties of plants and their active components have been studied. Many plant compounds have antioxidant properties such as polyphenols (5). Polyphenolic compounds especially flavonoids have protective effect against liver injury induced by toxins and released radicals (12, 13).

Walnut leaves are widely used in traditional medicine for the treatment of chronic diseases. Also they have anticancer, antioxidant and blood purifying properties. Walnut leaves are rich in antioxidants such as phenolic compounds. Phenolic acids and flavonoids are the main groups of phenolic compounds in leaves of walnut. The most important phenolic acids in walnut leaves, are caffeic acid, chlorogenic acid and coumaric acid (14, 15). On the other hand, the important flavonoids in leaves of walnut are gallic acid, quercetin derivatives, Pantosid, quercetin arabinoside, ferulic acid and ferulic acid, kaempferol, quercetin derivatives coumaric acid (14, 16). Antioxidant activity in walnut leaves are swept the free radicals with unsaturated Nafto kinon of-ß has been shown (17). Walnut leaves on Flavonoids Quercetin is one of the chemically induced damage in human lymphocytes protects and increased plasma antioxidant capacity and leads to genomic stability in rats with liver cirrhosis. The walnut leaf flavonoid protects the cells were collected by released radicals and prevents DNA damage and mutations (16). Polyphenolic compounds and flavonoids, can also kill cells against glutathione depletion, increasing the capacity of the antioxidant enzymes protect glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase (18, 19).

Many studies have shown that overproduction of ROS can increase Oxidative stress and thus attachment mechanism of injury that can lead to disease progression cycle, such as heart disease, diabetes, liver damage, cancer and aging (20, 21, 22, 23). So maintaining a balance between ROS and antioxidant enzymes superoxide dismutase and catalase and Glutathione peroxidase particularly is important and can be major mechanism to prevent damage by Oxidative stress. This balance seems to act as an important role in drug toxicity, such as acetaminophen (24). Paracetamol is a drug from NSAID (non steroidal anti inflammatory drug) group which produce excess oxygen species by serious liver oxidases (25). So this process to intensify the development of oxidative stress and liver damage produced by acetaminophen has an important role (26).

The question is whether walnut leaves induced the protective effect of paracetamol hepatotoxicity or not? According to long history of medicinal plants in different parts of the flora and vegetation of native walnut climate in the present study on the antioxidant effects of oxygen free radicals (ROS) Such as superoxide anion, hydrogen peroxide, hydroxyl radicals and oxidative stress, which have direct proportion to the effects of hydroalcoholic extract of Cuscuta on levels of antioxidant enzymes in rat liver histopathology and paracetamol-induced hepatotoxicity.

MATERIAL AND METHODS

The walnut hulls, and then the powder were dried in shade. One hundred grams of this powder in 50% alcohol for 9 hours at 50$^{\circ C}$ was placed in a Soxhlet extractor machine and the extract obtained after passing through the filter, dry and waxy substance produced was maintained at 4$^{\circ C}$. The present study is experimental. The population of the study includes 60 male SD rats weighing 210±20 g, aged 7-8 weeks. 1 week before the beginning of the study, animals were treated for compatibility with the environment. Polyethylene cages for animals at 23±2 degrees and 60% humidity and 12 hours light and 12 hours dark light conditions with free access to standard pellet food and water were stored desired amounts. After 7 days of adaptation to environmental conditions, the rats were randomly divided into 4 groups of 15 each, including:

1) Normal Control; NC: receiver 10 ml/kg sodium chloride 0.9 % orally for 14 days.

2) Juglans Regia Leaf Extract; JRLE: for 14 days at a dose of 200 mg/kg/day orally.

3) Toxicant Control; Atm: for 14 days sodium chloride 0.9 % orally. In day 8 single

dose of acetaminophen 835 mg/kg (27) injected intraperitoneally.

4) JRLE+Atm: 14 days of the alcoholic extract of walnut leaves orally/day 8 intraperitoneal injection of acetaminophen.

Acetaminophen injection was solved in polyethylene glycerol 40%. Histopathologic studies 24 hours after acetaminophen administration, all animals with dislocation of the cervical vertebrae Comfortable killed (euthanasia). Rat liver samples were immediately removed and left part of the hepatic lobe diaphragmatic stabilization to in 10% buffered formalin (PH=7.4) placed for 24 hours and then dehydrated in ethanol solution was incubated in paraffin.

Then the tissue sections using conventional methods of pathology, 5m serial sections prepared by cutting a section and a total of 10 sections stained with hematoxylin-eosin 3 of each sample were prepared. Inflammation in the portal, hepatic cell necrosis and inflammatory cell infiltration semi-quantitative (semi quantitative scale) were evaluated according to the method proposed by Frei et al in 1984. Severity of injuries rated from zero to 4 (zero: no harm, least damage: 1, mild injury: 2, moderate damage, severe injury 3 and 4). All ratings were used to a magnification of 100 × and 5 microscopic fields of each section into a model of Nikon optical microscope (ECLIPSE E 200, made in Japan).

The activity of the total antioxidant:

Along with liver biopsy for histopathological studies, another part of the liver of rats was prepared in cold saline wash and approximately 10% of the 1.15 percent (w/v) KCl. Homogenates was used at a speed of 700 rpm for 10 min at 4°C by centrifugation and flotation solution to measure lipid peroxidation by measuring the amount of Malondialdehyde: MDA And also for antioxidant enzyme superoxide dismutase The activity of (SOD), catalase, Glutathione peroxidase (GPx) and Glutathione reductase (GR) .

- MDA, as a measure of lipid peroxidation, was measured in the form of (Thiobarbituric acid reacting substances) TBARS and Esterbauer and Cheesman using the TBARS value were reported as nanomol mg protein (29).
- SOD activity was measured by Nishikimi (30) and adjusted by the method of Kakkar (31). Each unit of SOD activity was determined as in enzyme concentration required for 50% inhibition of the production of color in 1 min under the study.
- CAT activity was examined by Claiborne (32), based on the decomposition of hydrogen peroxide.

- The activity of glutathione peroxidase was examined using Rotruck et al (33), based on the reaction: $H_2O_2 + 2GSH \rightarrow 2\ H_2O +$ GSSG and expressed as mol min/mg protein.

- The activity of glutathione reductase was measured using Mohandas et all according to the reaction: $NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH$

Statistical analysis:

Data were analyzed by SPSS-13 software package. Quantitative data obtained were examined as the mean ± standard deviation (Mean ± SD) presented significant differences between groups by analysis of variance (ANOVA) and post hoc Tukey test at $\alpha = 0.05$ Differences in the $p < 0.05$ was considered significant.

RESULTS

The results of antioxidant factors:

As shown in Figures 1, 2, 3, 4, 5 and 6 and also can be seen in Table 1, Intracellular amounts of antioxidant enzymes and glutathione and Malondialdehyde amount in group NC (normal saline) and JRLE (recipient of the alcoholic extract of Juglans regia) but the group did not show significant differences Atm (recipient acetaminophen) prescribing the drug significantly reduced amounts superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glutathione compared with the control group ($p < 0.001$)for catalase and glutathione reductase and $p<0.01$for superoxide dismutase, glutathione peroxidase and glutathione reductase). The level of MDA show increased significantly compared with the control group ($p<0.05$). In group 4 (JRLE+Atm), simultaneous use of with acetaminophen prevented ethanol extracts of the leaves of walnut and the amount of reduced glutathione, superoxide dismutase catalase, glutathione reductase and glutathione peroxidase induced by acetaminophen. On the other hand walnut leaf extract that inhibits the increase of Malondialdehyde, so there wasn't any significant difference between the control group (NC) and this group.

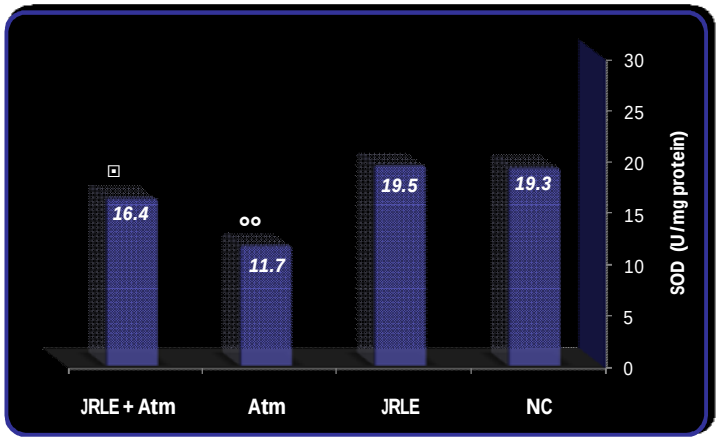

Figure 1: Comparison of superoxide dismutase amounts in rat liver of treatment groups
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control
OO: p<0.01 in comparison with the control group. □: p< 0.05 compared with acetaminophen group.

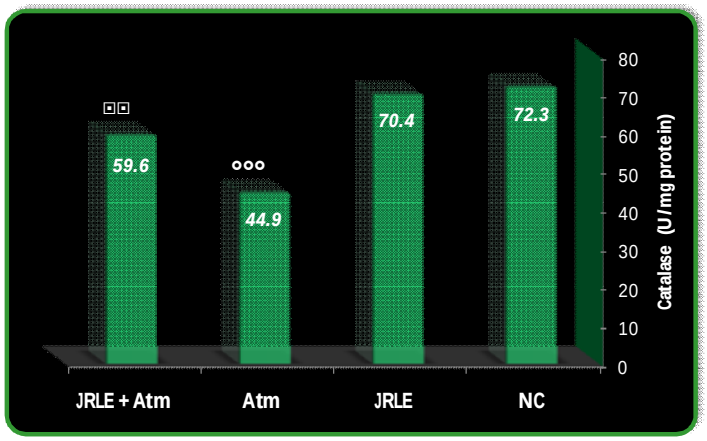

Figure 2: Comparison of amounts of the rat liver catalase in treatment groups
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control
OOO: P<0.001 in comparison with the control group. □□: p <0.01 compared with acetaminophen group.

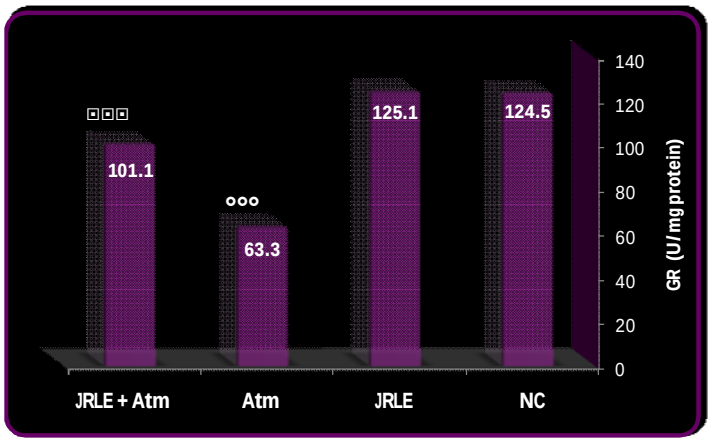

Figure 3: Comparison of glutathione reductase in the liver of rats treatment groups
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control
OOO: P<0.001 in comparison with the control group. □□□: p <0.01 compared with acetaminophen group.

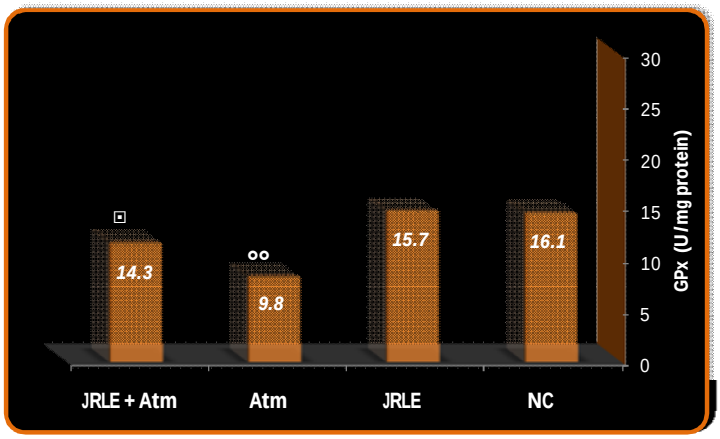

Figure 4: Comparison of the rat liver glutathione peroxidase in treatment groups.
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control.
OO: P<0.01 in comparison with the control group. □: p <0.05 compared with acetaminophen group.

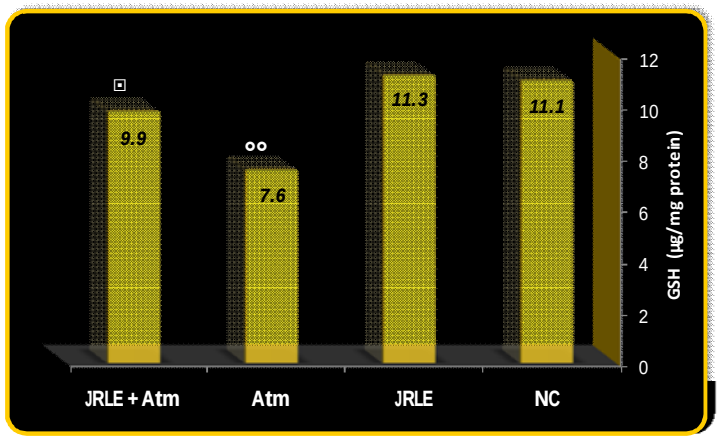

Figure 5: Comparison of rat liver reduced glutathione amounts in treatment groups
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control.
OO: P<0.01 in comparison with the control group. □: p <0.05 compared with acetaminophen group.

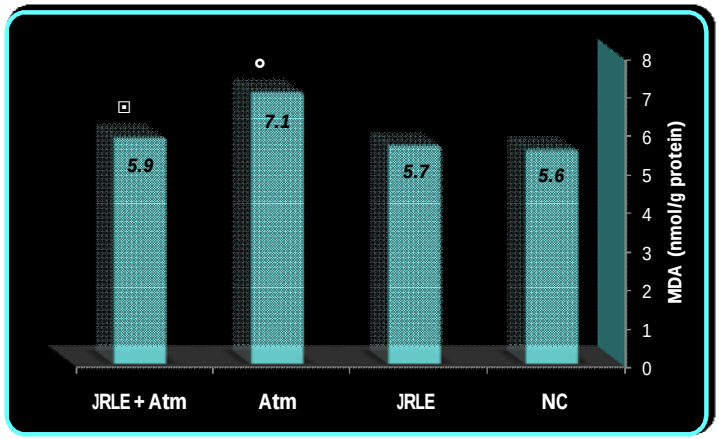

Figure 6: Comparison of rat liver MDA amounts of in treatment groups
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control.
O: P<0.05 in comparison with the control group. □: p <0.05 compared with acetaminophen group.

Table 1: Effects of walnut leaf extract on antioxidant activity of rat's liver damage due to acetaminophen. Values are presented as Mean ± SD.

MDA (nmol/g protein)	GSH (µg/mg protein)	GPx (U/mg protein)	GR (U/mg protein)	Catalase (U/mg protein)	SOD (U/mg protein)	group
5.6±0.5	11.1±2.4	14.7±1.5	124.5±4.2	72.3±1.3	19.3±0.2	NC
5.7±0.4	11.3±1.8	14.9±0.9	125.1±3.7	70.4±2.6	19.5±0.5	JRLE
7.1±0.9	7.6±0.9	8.5±1.1	63.3±2.1	44.9±2.9	11.7±0.7	Atm
5.9±0.3	9.9±1.7	11.8±.8	101.1±2.6	59.6±2.4	16.4±.3	JRLE + Atm

SOD: superoxide dismutase, GR: Glutathione reductase, GPx: glutathione peroxidase, MDA:malon dialdehide
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control.

The results of Histopathology:

Rats in group 1 (NC) and lobular structure of the liver cell, it seemed natural and healthy. But in the livers of rats intoxicated with paracetamol (Atm), bleeding in the interstitial space and vacuolization of the cytoplasm of hepatocytes and liver foci scattered in different Lobules parts significant necrosis of hepatocytes and inflammatory cells infiltrated with inflammatory cells in medium Hyperemia with congestion around the central Small specific portal vein.

The groups that had received paracetamol poisoning (JRLE+Atm), treated with extract of walnut leaves, significantly was prevented the occurrence pathological changes in liver tissue of rats, only brief degeneration damaged, especially around the central small vein. But the general view seemed like controls. Microscopic findings separately in liver quantitatively are listed in Tables 2 treatment groups.

Table 2: Effect of walnut leaf extract on the amount of congestion and inflammation in the portal, and hepatic necrosis rate interstitial infiltration of inflammatory cells in the liver tissue of rats induced by paracetamol intoxication

treatment Groups				Intensity Score	
JRLE+Atm	Atm	JRLE	NC		
No.	No.	No.	No.		
5	0	15	15	Degree 0	Congestion and inflammation in the portal vein
4	0	0	0	Degree 1	
3	3	0	0	Degree 2	
1	6	0	0	Degree 3	
2	6	0	0	Degree 4	
6	0	15	15	Degree 0	Hepatic necrosis
1	0	0	0	Degree 1	
3	2	0	0	Degree 2	
3	6	0	0	Degree 3	
2	7	0	0	Degree 4	
6	0	14	14	Degree 0	Interstitial infiltration of inflammatory cells
2	0	1	1	Degree 1	
4	1	0	0	Degree 2	
2	7	0	0	Degree 3	
1	7	0	0	Degree 4	

0: no damage, 1: minimum damage, 2: mild damage, 3: moderate damage, 4: severe damage.
Atm: acetaminophen, JRLE: walnut leaves extract, NC: normal control, N: number

DISCUSSION

In addition to metabolism of carbohydrates, the basic function of liver is proteins and fats, detoxification, bile secretion and storage of vitamins. Therefore, maintaining a healthy liver is very important factor for the overall health of the body. But constantly it is exposed to environmental toxins in various ways and forbidden psychoactive factors, various clinical drugs, alcohol, etc., which eventually can lead to various diseases such as hepatitis and cirrhosis of the liver (35, 36). So nowadays it is a fatal disease of the liver and serious challenge for public health in the world As a result, investigation to find out appropriate alternative herbal medicines is necessary. Many plants have been used in the treatment of poisoning and liver diseases in traditional medicine; many of them contain the Flavonoids and Polyphenolic compounds (37, 38). Chu et al have shown that the polyphenol compounds and flavonoids, can kill protect against glutathione depletion by increasing capacity of the antioxidant enzymes glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase (19).

Cos and Anderson Studies showed in 2001 and 2002 that phenolic acids and flavonoids, two major phenolic antioxidants, are available in walnut leaves That the phenolic acids walnut leaves contain coumaric acid, and chlorogenic acid (14, 15). However Wilms et al showed in 2005 that walnut leaves contain flavonoids such as kaempferol pantosid, quercetin and quercetin derivatives, Galactosid quercetin, Pantosid quercetin, arabinoside quercetin, Gizylosid quercetin and are Ramnosid quercetin (16).

Alongside these flavonoids, Maguire et al showed in 2004 that the plant contains a specific combination of antioxidant to neutralize active forms of oxygen that this antioxidant name's is sequalene that can prevent lipid peroxidation (39).

In general, with regard to the items specified that Walnut shell antioxidant concentrations similar to or better than vitamin E and beta-carotene acts as antioxidants (40, 41, 42).

Paracetamol is used as an analgesic and sedative drugs (43). The drug used in 1950s as an analgesic and antipyretic global consumption, but the adverse effects was unknown until 1966 (44). Paracetamol poisoning is usually caused by illness, vomiting, diarrhea, and sometimes jaundice shock starts in a few days (at extra cost), Hepatic Dysfunction, and in some cases leads to damage the myocardium and kidney (44). Studies conducted by James and colleagues (2003) also showed that the toxic effects of

paracetamol on the use of laboratory animals is excessive (43). The results of the present study is the liver toxicity of the drug in doses 835 mg/kg and so on, with results from other researchers confirmed the potential hepatotoxic drug is consistent. Paracetamol use in normal pathways mainly metabolized in the liver to glucuronide or Sulfation.

After entering the body, paracetamol metabolite by cytochrome P450 Active N l-P Benzoquinon imin (NAPQI) that converts the chemical that it is highly electrophilic and generally using glutathione-conjugated and thereby discharge liver glutathione stores. NAPQI conjugate followed by the kidneys and intestines and analysis of cysteine conjugate acid Mercaptoric and excreted through administrative procedure (43, 44). NAPQI, the property of neucleophilic, acts as a strong oxidant and can cause lipid peroxidation, damage to macromolecules and impaired mitochondrial function and increased permeability of the membrane (45). In other words NAPQI can simultaneously causes the formation of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, hydrogen peroxide and other reactive nitrogen species (RNS) and Peroxy nitrite is like Nitric oxide. Higher levels ROS and RNS can be biological molecules such as DNA, proteins and phospholipids attacked and eventually leads to lipid peroxidation, nitration of tyrosine and depletion of antioxidant enzymes (CAT, SOD and GPX) and thus can lead then to the Oxidative stress (46, 47).

Also, NAPQI can fractures DNA strands and progression of apoptosis and necrosis in the acetaminophen-induced liver toxicity (48, 49). The previous studies have shown that oxidative stress is a major mechanism of paracetamol-induced hepatotoxicity (50, 51 and 52).

The results of a recent study showed that excessive doses of paracetamol may lead to decreased levels of antioxidant enzymes (CAT, SOD and GPX) and a certain degree of hepatotoxicity creates during treatment .

The walnut leaf extract reduced the levels of CAT, SOD and GPX in Oxidative stress caused by ROS and RNS paracetamol are increased. In addition, increased levels of MDA in the group that had received paracetamol were concrete.

However, pretreatment with walnut leaf extract, reduced MDA levels. These results indicate that the reduction in the formation of lipid peroxidation prevent another incident of paracetamol Oxidative toxicity caused by leaf extract.

Generally, levels of antioxidant enzymes activity could be an indirect way to assess the

oxidant-antioxidant appropriate tissues (53). It's a known fact that Reactive oxygen species (ROS) damages such as superoxide radicals with very high performance, hydrogen peroxide, hydroxyl radicals and lipid Peroxy genase (LPO) in the presence of heavy metal ions in various cellular components such as proteins, fats and acids membrane nucleic (54).

Thus, the intracellular and extra cellular environments, enzymatic and non-enzymatic antioxidants exist to detoxify ROS factors (55). Antioxidants act as released radical scavenger agents, hydrogen donors, electron donor, the parser peroxide, inhibitors of enzymes and chelator agents for heavy metal. As seen in this study, the antioxidant defense system specific functions such as superoxide dismutase (SOD), glutathione peroxidase, (GPX) and glutathione reductase (GR) had a significant reduction in poisoning with paracetamol group than the control group. these values were increased toxic group treated with walnut leaves. Superoxide dismutase has assigned to remove the catalytic conversion of the superoxide to H2O2 (56). Argano et al reported in 1997 decreased superoxide dismutase activity is likely to increase the production of H2O2 and O2, as well as reduced levels of protein (57).

Sindhu et al., 2004 also reported a decrease in enzyme activity may be due to decreased expression of genes producing them from free radical-producing agents (58). It is clear that glutathione peroxidase (GPX) in the process of dismantling or other peroxides H2O2 requires restoration form of glutathione (GSH) to be oxidized form (GSSG). However, glutathione reductase (GR), which is another part of the antioxidant defense system, the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) and the antioxidant defense mechanism thereby indirectly will support. As previously reported Quinlan in 1988, the foundations of the active part glutathione disulfide reductase may be a goal for the production of free radicals and inhibits its activity (59). GSH is a major endogenous antioxidant and as a cofactor for the removal of hydrogen peroxide and glutathione peroxidase Lypoperoxides used by families in which the restoration of glutathione (GSH) becomes to its oxidized form (GSSG) (60).

As seen in this study, a significant decrease in GSH levels in the groups receiving paracetamol compared with the control group were recorded.

While groups received walnut leaves, the values were comparable to the control group

and the results of walnut leaves is introduced as improving the oxidation process.

Finally oxidized glutathione by glutathione reductase again (GSR) to restoration (GSH) into the intracellular GSH level and thus maintain the proper level of GSH and it is an effective measure to maintain structural integrity, functional and physiological cell membrane. Saturated fatty acids on lipid peroxidation of cell membranes occurs and finally progresses to adverse reactions of free radicals.

Hydroxyl radicals started to process variations of active and reactive oxygen (ROS) and remove hydrogen atoms produces a radical lipid and therefore due to lipid peroxidation, a number of compounds, especially alkanes, Malone lipid (MDA) and isoprotane are formed. In the present study, MDA levels in the groups receiving paracetamol had a significant increase compared with the other groups, but its amount was very low in the groups receiving walnut leaves. Reactive oxygen species (ROS) reacts to lipid peroxidation, and leads to oxidative changes of lipids. It has been shown that lipid peroxidation is non-enzymatic antioxidants by free radicals producing drugs as a sign of decreased antioxidant defense system (61). The MDA, one of the final products of lipid peroxidation as a mutagenic and genotoxic agent that can lead to the development of various cancers (62). Hence factors that can changes the level of lipid peroxidation in organs with polyunsaturated fatty acids (PUFA) and can be inhibited antioxidant status and effectively be added to the diet. So what was said, increasing amounts of antioxidant enzymes and reducing lipid peroxides is recommended as the main mechanism of walnut leaf extract in preventing the progression of liver damage caused by paracetamol and the results of this study are added to the available data on antioxidant potential of Juglans regia.

A histopathologic finding of the present study is consistent with the results of oxidative stress induced by toxic doses of paracetamol and walnut leaf extract is an antioxidant function.

In this study we investigate the histopathologic changes were well be seen in the liver, changes such as necrosis of The liver lobule, fat changes, hepatic degeneration and interstitial infiltrate of lymphocytes and inflammatory cells infiltration and serious congestion in the portal on the toxicity of paracetamol.

However, treatment with walnut leaf extract could significantly inhibit the creation of histopathologic changes.

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