Biochemical Studies On The Hepatoprotective Effects Of Pomegranate And Guava Ethanol Extracts

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ABSTRACT: The hepatoprotective effect of pomegranate and guava against carbon tetrachloride-induced hepatotoxic in rats liver was evaluated. The hepatoprotective period, rats treated with pomegranate peel and guava leaves ethanolic extracts or silymarin for 21 consecutive days could significantly decrease in the liver weight when compared with CCl_4-treated group. In the curative period, rats treated with pomegranate peel and guava leaves ethanolic extracts or silymarin completely restored the increase of liver weight and no significant difference when compared with normal group. In the hepatoprotective period, rats treated with pomegranate peel ethanolic extracts or silymarin during CCl_4 administration significantly increased in the serum total protein content and albumin when compared with CCl_4-treated group. No significant effects were observed on the serum globulin contents compared with normal control. In the curative groups, the highest increase in serum total protein content and globulin were noticed in the rats treated with pomegranate peel ethanolic extracts. In the hepatoprotective and curative periods, rats treated with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly decreased in the activities of ALT, AST, GGT, lysosomal enzymes (ACP, -GAL and -NAG) and lipid peroxidation when compared with CCl_4-treated group. Significantly increases were found in the activities of SOD and CAT enzymes when compared with CCl_4-treated group. The content of reduced glutathione and GST in all treatments generally decreased as compared with normal group except treatment rats with guava leaves ethanolic extracts. In the hepatoprotective and curative period, the highest damages in liver tissue were found in the order carbon tetrachloride > guava leaf ethanolic extract > silymarin > pomegranate peel ethanolic extract > normal control. This clearly explained the reason for the antioxidant activity of guava leaves, pomegranate peel and silymarin.

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INTRODUCTION

Human are continuously exposed to different kinds of chemicals such as food additives, industrial chemicals, pesticides and other undesirable contaminants in the air, food and soil (Stavric, 1994). Most of these chemicals induce a free radical-mediated lipid peroxidation leading to disruption of biomembranes and dysfunction of cell and tissues (Cho *et al*., 2003). Antioxidants play a significant role in protecting living organism from the toxic effect of various chemicals by preventing free radical formation (Sheweita *et al*., 2001).

There are approximately 5000 known plant phenolics and model studies have demonstrated that many of them have antioxidant activity (Robards *et al*., 1999). The antioxidative effects of natural phenolic compounds in pure forms or in their extracts from different plant sources such as vegetables, fruits and medicinal plants were studied *in vitro* using different model systems of oxidation (Pietta *et al.*, 1998 and Yen and Hsieh, 1998) led to speculation about the potential benefits of ingestion of plant phenolics. Their antioxidant activity is generally based on the number and location of hydroxyl groups present as well as the presence of a 2-3 double bond and 4-oxofunction (Rice-Evans and Miller, 1998). The growing interest in the antioxidant properties of the phenolic compounds in vegetables and fruits derives from their strong activity and low toxicity compared with those of synthetic phenolic antioxidants, such as BHT (butylated hydroxytoluene) (Marinova and Yanishlieva, 1997).

Different extracts (alcoholic and aqueous) of both dried and fresh leaves of Guava (*Psidium Guajava* L) were screened for analgesic, anti-inflammatory and antiulcer activity in albino rats (Kulkarni et al., 1999). All the extracts for qualitative analysis to identify the nature of chemical substance extracted, this is further supported by I.R. spectral data. The activities were found to be dependent on the type of the solvent used for extraction and method adopted for extraction. However, significant analgesic and anti-inflammatory activity were found with fresh leaves extract, antiulcer activity showed by aqueous extract.

All the activities were carried out in different experimental models to explain the possible mechanism responsible for the activities. Phytochemical studies have identified more than 20 compounds in guava extracts. The major constituents of its leaves were identified to be tannins, -sitosterol, maslinic acid, essential oils, triterpenoids and flavonoids (Begum *et al*., 2002).

Punica granatum L. (Punicaceae), commonly called pomegranate, was a large deciduous shrub or small tree used medicinally in Europe, Indo-China, the Philippine Islands and South Africa. The plant was used in folklore medicine for the treatment of various diseases, such as ulcer, hepatic damage, snakebite, etc. The unripe fruit was a good appetizer and tonic, useful in vomiting, but causes biliousness. The ripe fruit was a tonic, astringent to the bowels, aphrodisiac, cures biliousness, fever, heart diseases, sore throat, stomatitis, etc. The rind of the fruit is antihelminthic, useful in diarrhea, dysentery and ulcer (Kirthikar and Basu, 2000). Pomegranate juices showed an antioxidant activity three times higher than red wine and a green tea infusion (Gil *et al*., 2000). Pomegranate juices also displayed potent antiatherogenic action in atherosclerotic mice and humans (Aviram *et al.,* 2000).

Antioxidant-rich fractions were extracted from pomegranate (*Punica granatum)* peels and seeds using ethyl acetate, methanol, and water. The extracts were screened for their potential as antioxidants using various *in vitro* models, such as -carotene-linoleate and 1,1-diphenyl-2-picryl hydrazyl (DPPH$^{\bullet}$) model systems. The methanol extract of peels showed 83 and 81% antioxidant activity at 50 ppm using the -carotene-linoleate and DPPH$^{\bullet}$ model systems, respectively. Similarly, the methanol extract of seeds showed 22.6 and 23.2% antioxidant activity at 100 ppm using the -carotene-linoleate and DPPH$^{\bullet}$ model systems, respectively. As the methanol extract of pomegranate peel showed the highest antioxidant activity among all of the extracts (Chidambara *et al.,* 2002)

Silymarin was a flavonoid obtained from *Silybum marianum* or milk thistle and was composed of three isomers: silybinin, silydianin and silychristin (Wagner, 1986), silybinin being quantitatively the most important (Bosisio *et al*., 1992). Wills and Asha (2006 a) showed standard drug silymarin a remarkable protection of serum AST, ALT and LDH levels towards CCl_4 induced hepatotoxicity. Silymarin-treated rats completely prevented the lowering of hepatic GSH. Silymarin also protected the liver from elevating MDA levels and was comparable with normal values. Rats treated with silymarin after the establishment of toxic injury showed recovery of liver tissue from the absence of centrilobular or bridging necrosis or mild hepatitis.

Carbon tetrachloride (CCl_4), a well-known model compound for producing chemical hepatic injury, requires biotransformation by hepatic microsomal cytochrome P-450 to produce toxic metabolites, namely trichloromethyl free radicals ($CCl_3^{\bullet}$) and subsequent derivative $Cl_3COO^{\bullet}$ (Brautbar and Williams, 2002). A number of investigators have previously demonstrated that antioxidants prevent CCl_4 toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation (Teselkin et al., 2000), suppressing alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (Lin and Huang, 2000), and increasing antioxidant enzyme activity (Kumaravelu et al., 1995).

The aim of this study was to evaluate in vivo hepatoprotective and curative effects of pomegranate and guava on carbon tetrachloride (CCl4)-induced hepatotoxic in rats liver in order to find new potential sources of natural antioxidants.

MATERIALS AND METHODS

MATERIALS

-Source of samples

Pomegranate and guava samples used in this experiment were purchased from local markets in Cairo, Egypt.

-Source of animals

The male albino rats (initial weight about 200±5g) were obtained from Faculty of Veterinary Medicine, Cairo University.

-Chemicals

All chemicals were of analytical grade. Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Solvents were purchased from Merck (Darmstadt, Germany). Assay kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Bio-diagnostic, France and assay kit for - glutamyl transferase (GGT) was obtained from Raichem, Division of Hemagen Diagnostics, Inc. (Martinsried, Germany). Total protein and albumin assay kits were purchased from Biosystem S.A. Costa Brava 30, Barcelona (Spain).

METHODS

-Preparation of sample

Samples were flushed by tap water then washed in distilled water for three times and cut into small pieces before being dried in a hot air-blowing oven at 50°C. They were ground to a fine powder in a mechanical blender and kept in refrigerator prior to extraction.

-Preparation of ethanol extract

The samples were extracted according to the method of Panovska et al. (2005). Ten g of each dry powder were extracted with 100 ml of 70% ethanol in a

screw-capped flask and shaken at room temperature for 24 h. The extracts were centrifuged at 5000g for 10 min while the residue was re-extracted under the same conditions twice and filtered through a Büchner funnel with filter paper (Whatman No.1). The 70% ethanol extracts were concentrated under reduced pressure, lyophilized to obtain powders, and stored at 4 °C until assay.

-Experimental design

Male albino rats (initial weight about 200±5g) were obtained from Faculty of Veterinary Medicine, Cairo University. The rats were raised in the animal's house of Regional Center for Food and Feed, Agriculture Research Center. The rats were kept in separated cage under normal laboratory conditions (temperature remain 25±2°C). All rats were adapted for two-weeks before the initiation of experiment. During this period, rats were allowed free access of water and American Institute of Nutrition standard reference diet (AIN-76A) was used as the basal diets. AIN-76A consists of 20% casein, 0.3% DL-methionine, 55% corn starch, 10% sucrose, 5% cellulose, 5% corn oil, 3.5% AIN-76A mineral mixture, 1.0% AIN-76A vitamin mixture, and 0.2% choline bitartrate (American Institute of Nutrition, 1977). The body weight of each rat was determined before start of experiment and every three weeks till the end of experiment (six weeks).

-Hepatoprotective period of the two extracts

Fifty rats were divided into 5 groups/10 rats each. Hepatotoxicity by carbon tetrachloride administration was induced for a period of 3 weeks, except normal control and it was treated as follows:

Group I (Normal control): Rats were given corn oil (1ml/200 g B.W, oral) twice a week; (Mon. and Thurs) and daily doses 1% gum acacia (1ml/200 g B.W, oral).

Group II (CCl_4): Rats were given 3.3% CCl_4 in corn oil (1ml/200 g B.W, oral) twice a week; (Mon. and Thurs) and daily doses of 1% gum acacia (1ml/200 g B.W, oral) (Hoefler *et al*., 1987).

Group III (Pomegranate peel extracts): Rats were given 3.3% CCl_4 in corn oil (1ml/200 g B.W, oral) twice a week; (Mon. and Thurs) and daily doses of (50 mg pomegranate peel extracts/ kg B.W, suspended in 1% gum acacia, oral) basis of the LD_{50} value has been reported by (Chidambara *et al*., 2002b).

Group IV (Guava leaf extracts): Rats were given 3.3% CCl_4 in corn oil (1ml/200 g B.W, oral) twice a week; (Mon. and Thurs) and daily doses of (500 mg guava leaf extracts/kg B.W, suspended in 1% gum acacia, oral) basis of the LD_{50} value has been reported (Jaiarj *et al*., 1999).

Groups V (Silymarin)**:** Rats were given 3.3% CCl_4 in corn oil (1ml/200 g B.W, oral) twice a week; (Mon. and Thurs) and daily doses of (50 mg silymarin/ kg B.W, suspended in 1% gum acacia, oral) basis of the LD_{50} value has been reported (Wills and Asha, 2006 b).

Blood samples were collected from the retro-orbital vein of each animal at zero time by using a glass capillary tube. After 3 weeks intoxication of CCl_4, twenty five rats were fasted for 12h, blood samples were collected from the retro-orbital vein of each animal using a glass capillary tube and then sacrificed by cervical decapitation.

-Curative period of the two extracts

The experiment was extended for another 3 weeks with twenty five rats divided into (5 groups/6 rats) where the CCl_4 dosing was stopped for all groups as follows:

Group I (Normal control) and **Group II** (CCl_4): Rats were given daily doses 1% gum acacia (1ml/200 g B.W, oral).

Group III (Pomegranate peel extracts): Rats were given daily doses of (50 mg pomegranate peel extracts/kg B.W, suspended in 1% gum acacia, oral).

Group IV (Guava leaf extracts): Rats were given daily doses of (500 mg guava leaf extracts/kg B.W, suspended in 1% gum acacia, oral).

Groups V (Silymarin): Rats were given daily doses of (50 mg silymarin/kg B.W, suspended in 1% gum acacia, oral).

After other 3 weeks rats were fasted for 12h, blood samples were collected from the retro-orbital vein of each animal using a glass capillary tube and then sacrificed by cervical decapitation.

-Preparation of serum

The blood samples were allowed to coagulate and then serum was separated by centrifuged at 3000 rpm for 20 min, collected into sterilized tubes and stored at -20°C. Aspartate aminotransferase (AST) (Reitman and Frankel, 1957), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), **-** glutamyl transferase (GGT) (Orlowski and Meister, 1963), total protein (Gornall *et al.,* 1949) and albumin (Doumas *et al*., 1971) were determined.

-**Preparation of liver samples**

Animals were killed by cervical decapitation and their livers were rapidly removed, weighed and divided into three part.

Preparation of liver Homogenate

Liver homogenate was prepared according to the method described by El-Demerdash *et al.* (2005). Dissected livers were excised, washed with ice-cold 0.9% NaCl (w/v) to remove the blood, cut into small pieces by fine scissors, and then homogenized (10%w/v) separately in ice-cold 1.15% KCl-0.01M sodium phosphate buffer, pH 7.4 with a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 10,000 xg 20 min at 4 °c. Supernatant

of the liver homogenate was collected into sterilized tubes and stored at -20°C until analysis of reduced glutathione (Ahmed *et al.,* 1991), glutathione-S-transferase (Habig *et al.,* 1974), catalase (Sinha, 1972), superoxide dismutase (Marklund and Marklund 1974) activity and lipid peroxidation (Uchiyama and Mihara, 1978).

Preparation of lysosomal fraction

Lysosomal fraction was prepared according to the method of (Tanaka and Iizuka, 1968).

Determination of lysosomal marker enzyme activities

The activity of four lysosomal acid hydrolases, acid phosphatase (ACP), N-acetyl-β- glucosaminidase (β-NAG) and β-galactosidase (β-GAL) has been measured according to the method described by Van Hoof and Hers (1968) with a slight modification by Younan and Rosleff (1974).

Histological examination of liver

Autopsy samples were taken from liver of sacrificed rats in both control and experimental groups and fixed in 10% formalin saline solution for ten hours at least then washed in tap water for 12 hours. Serial alcohol (methyl, ethyl and absolute) were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3 micron thickness by sledge microtome. The obtained tissue sections were collected on the glass slides and stained by hematoxylin and eosin stain (Banchroft *et al.*, 1996) for histopathological examination by the light microscope.

Statistical analysis of the data

Statistical analysis for each of the collected data was done in the following procedure outlined by

The treatment means were compared using the least significant difference test (LSD) at the 5% level of probability as outlined by Waller and Duncan (1969). Using the in the Duncan test institute program used a computer in the statistical analysis.

RESULTS AND DISCSSION

Interest in the search for new natural antioxidant has grown dramatically over the past years because reactive oxygen species production and oxidative stress has been shown to be linked to aging related illnesses (Finkel and Holbrook, 2000) and a large number of other illnesses. So, the aim of this study was to evaluate in *vivo* antioxidant activities of pomegranate and guava in order to find new potential sources of natural antioxidants.

***In vivo* antioxidant activities models systems**

As the results of the *in vitro* study demonstrated that the ethanolic extracts of guava leaves**,** cinnamon bark and pomegranate peel have excellent antioxidants activities out of 15 plant samples (data not shown). In these experiments, it was interesting to investigate the *in vivo* antioxidant potential as hepatoprotective and curative effects of guava leaves and pomegranate peel ethanolic extracts or silymarin on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rat's liver.

Effect of plant ethanolic extracts or silymarin treatments on body weight, liver weight and ratio of hepatotoxic rats

The results which reported in Table (1) showed that the liver weights of rats in each group. In the hepatoprotective period, rats administered with CCl_4 alone were significantly ($P < 0.05$) increased in their liver weights when compared with the normal control group. Treatment with pomegranate peel and guava leaves ethanolic extracts or silymarin for 21 consecutive days could significantly decrease the liver weight when compared with CCl_4-treated group. In the curative period, rats treated with pomegranate peel and guava leaves ethanolic extracts or silymarin completely restored the increase of liver weight when compared with CCl_4-treated group which showed also some recovery in this respect. These results are in agreement with the findings of Hung *et al*. (2006) showed that CCl_4-treated rats increased the relative organ weights of liver.

Effect of plant ethanolic extracts or silymarin treatments on serum protein contents of hepatotoxic rats

The effect of pomegranate peel and guava leaves ethanolic extracts or silymarin on serum proteins (total protein, albumin and globulin) were determined and results are given in Tables (2 and 3). In the hepatoprotective period, the CCl_4-treated group showed a significant reduction in the serum total protein content. Treatment with pomegranate peel ethanolic extracts or silymarin during CCl_4 administration significantly increased the serum total protein content but it was no significant effect in treated rats with guava leaves ethanolic extracts when compared with CCl_4-treated group. In the curative period, the highest increase in serum total protein content was noticed in the rats treated with pomegranate peel ethanolic extracts and no significant difference was found when compared with normal group.

From the obtained data in both hepatoprotective and curative periods, treated rats with carbon tetrachloride showed a significant decrease in the serum albumin contents. Treatment rats with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly increase the serum albumin contents when compared with CCl_4-treated group.

No significant effects were observed on the serum globulin contents between all groups and normal control in the hepatoprotective period. In the curative period, a relative high increase in serum globulin

content was noticed in the rats treated with pomegranate peel ethanolic extracts. These results are in agreement with those obtained by Zafar and Ali (1998), who observed decreased level of albumin and protein in rats after treatment with carbon tetrachloride.

Effect of plant ethanolic extracts or silymarin treatments on serum aminotransferases ALT and AST activities of hepatotoxic rats

Results reported in Tables (4) revealed the effect of pomegranate peel and guava leaves ethanolic extracts as well as, standard control drug "silymarin" treatments on serum amino transferases ALT and AST activities of hepatotoxic rats.

As indicated from the results in hepatoprotective and curative periods of CCl_4 treated rats showed a significant increase on the activities of ALT and AST enzymes. Treatment of the hepatotoxic rats with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly decreased the activities of ALT and AST enzymes when compared with CCl_4-treated group in the hepatoprotective and curative groups. In the curative period, the inhibitory effects of pomegranate peel ethanolic extracts or silymarin have a highly potent effect on the activity of AST enzyme compared with CCl_4-treated group and no significant difference when compared with normal group.

These results are in agreement with Hung *et al.* (2006), who stated that CCl_4-treated rats causes liver damage and significantly ($p < 0.05$) increased the AST and ALT levels in serum as compared with the control group. These results were confirmed by the data of Wills and Asha (2006 b) who showed that the standard drug silymarin have a remarkable protection of serum AST and ALT levels towards CCl_4 induced hepatotoxicity.

Effect of plant ethanolic extracts or silymarin treatments on serum -glutamyl transferase (GGT) activity of hepatotoxic rats

The results reported in Tables (5) showed that treatmented rats with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly decrease the activity of GGT enzyme when compared with CCl_4-treated group in the hepatoprotective period. In the curative period, the highest relative decrease on serum GGT enzyme activity was noticed in the rats treated with pomegranate peel ethanolic extracts and there was no significant difference when compared with normal group. These results are in agreement with Li *et al.* (2006), who concluded that pomegranate peel extract appeared to have more potential as a health, supplement rich in natural antioxidants.

Effect of plant ethanolic extracts or silymarin treatments on liver homogenate reduced glutathione (GSH) content of hepatotoxic rats.

Glutathione (GSH) constitutes the first line of defense against free radicals and other oxidant species. The liver homogenate reduced glutathione (GSH) content is presented in Table (6). The results in both hepatoprotective and curative periods, showed that the content of liver homogenate reduced glutathione in all treatments generally decreased as compared with normal group except with guava leaves ethanolic extracts treatment which cause significant increase in the reduced glutathione content when compared with other treatments during hepatoprotective and curative periods with no significant difference when compared with normal control group. This potential antioxidant activity may be due to the high content of phenolic compound in guava leaves ethanol extract that played a significant role on the antioxidant activity as stated by Tachakittirungrod *et al.* (2007). On the other hand these results are in agreement with Hung *et al*., (2006), who showed that CCl_4-treated rats significantly ($P < 0.05$) decreased the GSH content in liver as compared with the control group. Other reason for the high potential antioxidant activity of guava leaf extracts could be due to the presence of some of natural products with high antioxidant activity.

Effect of plant ethanolic extracts or silymarin treatments on liver homogenate lipid peroxidation contents on hepatotoxic rats

MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade, was widely used as marker of lipid peroxidation.

The liver homogenate lipid peroxidation contents are given in Table (7). In the hepatoprotective period, a significant increase in liver homogenate MDA level was observed in CCl_4 alone treated rats when compared with the normal control group. However, CCl_4 induced elevation of the liver homogenate MDA levels were lowered significantly by the treatment of the rats with the pomegranate peel, guava leaves ethanolic extracts or silymarin when compared with CCl_4-treated group. The remarkably reducion in the homogenate MDA levels were noticed in the rats treated with pomegranate peel ethanolic extracts or silymarin and these were no significant difference when compared with normal control group.

In the curative period, the same trend was observed in treatment animals with pomegranate peel, guava leaves ethanolic extracts or silymarin significantly which reversed the elevation of hepatic MDA formation when compared with CCl_4-treated group**.** Also no significant effects were observed between the rats treated with pomegranate peel, guava leaves

ethanolic extracts or silymarin and normal control.
These results are in agreement with Teselkin *et al*. (2000), who demonstrated that antioxidants prevent CCl_4 toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation. These results were confirmed by the data of Chidambara *et al.* (2002 b) and Ajaikumar *et al*. (2005) who showed the tissue lipid peroxidation level decrease in the *Punica granatum* extract treated groups of animals as compared to the control group. Also Wills and Asha (2006 b) found that standard drug silymarin have a remarkable protection of the liver from elevating MDA levels towards CCl_4 induced hepatotoxicity.

Effect of plant ethanolic extracts or silymarin treatments on liver homogenate glutathione-S-transferase (GST) and superoxide dismutase (SOD) activity of hepatotoxic rats

From results in Table (7) it is clear that the effect of pomegranate peel and guava leaves ethanolic extracts as well as, standard drug "silymarin" on liver homogenate glutathione-S-transferase (GST) activity of CCl_4-induced hepatotoxicity rats. As indicated from the results in hepatoprotective period, treated rats with carbon tetrachloride showed a significant decrease in the GST activity. Treatment rats with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly increased the GST activity when compared with CCl4-treated group. Guava leaves ethanolic extract exerted a highly inhibitory effect on the decrease of GST activity than other treatment groups.

In the curative period as can be seen, the highest increase on homogenate glutathione-S-transferase activity was noticed in the rats treated with guava leaves ethanolic extracts with no significant difference when compared with normal group. These results are in agreement with Qian and Nihorimbere (2004), who found that remarkably high total phenolic content for ethanol guava leaf extracts**.** This study revealed that guava leaf extracts are could have an effective other potential compounds of natural antioxidant activity.

Superoxide dismutase is the first enzyme involved in the antioxidant defenses against reactive oxygen species (ROS) by dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). The liver homogenate superoxide dismutase activities are presented in Tables (7).

As indicated from the results in hepatoprotective and curative periods, the treated rats with carbon tetrachloride showed a significant decrease in the activity of SOD enzyme. Treatment rats with pomegranate peel and guava leaves ethanolic extracts or silymarin significantly increase the activity of SOD enzyme when compared with CCl_4-treated group during the hepatoprotective and curative periods. These results indicated that no significant effects in the activity of superoxide dismutase enzyme between rats treated with pomegranate peel and guava leaves ethanolic extracts or silymarin in both hepatoprotective and curative stages.

These results are in agreement with Shih *et al.* (2005), who confirmed that the hepatic activity of superoxide dismutase was markedly decreased by CCl_4 treatment. Similar trend of the treatment rats with pomegranate peel extract at 50 mg/ kg body weight was observed by Chidambara *et al.* (2002 b), who found that treatment rats with CCl_4 decreased the level of SOD. Pretreatment rats with pomegranate peel methanolic extract at 50 mg/ kg body weight for 14 days followed by CCl_4 treatment causes preservation of SOD enzyme activity. Also Ajaikumar *et al.* (2005) showed in the *Punica granatum* extract treated groups of animals; the *in vivo* antioxidant level of superoxide dismutase was increased near to the normal values.

Effect of plant ethanolic extracts or silymarin treatments on liver homogenate catalase (CAT) activity of hepatotoxic rats.

Catalase is the second enzyme involved in the antioxidant defenses against reactive oxygen species (ROS) by conversion of H_2O_2 to H_2O and O_2. The liver homogenate catalase activities are illustrated in Table (8). In the hepatoprotective period, rats administered with CCl_4 alone were found significantly decreased in liver homogenate catalase activity when compared with the normal control group. Treatment with pomegranate peel and guava leaves ethanolic extracts or silymarin for 21 consecutive days could significant increase the liver homogenate catalase activity when compared with CCl_4-treated group. Also non significant effects were observed between groups of rats administrated with pomegranate peel and guava leaves ethanolic extracts or silymarin in homogenate catalase activity.

In the curative period, no significant differences were observed in the liver homogenate catalase activity between groups of rats treated with pomegranate peel, guava leaves ethanolic extracts or silymarin and normal control. These results are in the line with the findings of Chidambara *et al.* (2002 b), who found that rats treated with CCl_4 decreased the level of catalase activity. Pretreatment rats with pomegranate peel methanolic extract at 50 mg/ kg body weight for 14 days followed by CCl_4 treatment causes preservation of catalase enzyme activity. On the other hand, these results are in agreement with Qian and Nihorimbere (2004) who revealed that guava leaf extracts are an effective potential source of natural antioxidants.

Effect of plant ethanolic extracts or silymarin treatments on liver homogenate lysosomal

enzymatic release activities of hepatotoxic rats.

As illustrated from data in Table (9), that the lysosomal enzymatic release activities of three marker enzymes of rat liver (ACP, -GAL and -NAG) were enhanced in (CCl_4)-induced hepatotoxicity rats.

In the hepatoprotective period, the CCl_4-treated group showed a significant increase in the activities of three marker lysosomal enzymatic activities (ACP, -GAL and -NAG). Treatment with pomegranate peel and guava leaves ethanolic extracts or silymarin during CCl_4 administration significantly decreased the activity of ACP lysosomal enzyme when compared with CCl_4-treated group and no significant differences were observed between these groups of rats. Also no significant changes were observed in the activity of -GAL lysosomal enzyme between pomegranate peel ethanolic extracts or silymarin groups and normal group. The administration of pomegranate peel and guava leaves ethanolic extracts or silymarin to rats significantly decreased the activity of -NAG lysosomal enzyme when compared with CCl_4-treated group and no significant effects were observed between groups of rats treated with pomegranate peel ethanolic extracts or silymarin.

In the curative period, it is clear that the general trend of the relative activity of ACP lysosomal enzyme was similar in pomegranate peel and guava leaves ethanolic extracts or silymarin treatments to the hepatoprotective period. Administration with pomegranate peel ethanolic extracts or silymarin caused the highest decrese in the activity of -GAL lysosomal enzyme compared with normal group. Pomegranate peel ethanolic extracts and silymarin have a highly potent effect on the activity of - NAG lysosomal enzymatic compared with CCl_4-treated group and no significant difference when compared with normal group.

These results indicated that the active ingredient of these extracts have an inhibitory effects on membrane permeability of lysosomes. It may be due to the effect of antioxidant compounds present on the ethanolic extracts on the constituents of phospholipids bilayers of the membrane of rat liver lysosomes. This inhibitory effect appeared to have stabilizing effect on the membrane fluidity.

Table 1. Effect of plant ethanolic extracts or silymarin treatments on body weight, liver weight and ratio of hepatotoxic rats.

Tratments	**Hepatoprotective period (after three weeks)**				**Curative period** (after six weeks)**			
	Body weight (g)	**Liver weight (g)**	**Ratio (%)**	**Relative (%)**	**Body weight (g)**	**Liver weight (g)**	**Ratio (%)**	**Relative (%)**
Normal control	*217.33 ± 19.7^{a}	6.40 ± 0.17^{c}	2.94	100	254.00 ± 2.64^{c}	7.93 ± 0.23^{b}	3.12	100
(3.3% CCl_4)	174.66 ± 4.72^{b}	9.63 ± 0.80^{a}	5.51	187.28	204.68 ± 7.45^{d}	9.26 ± 0.15^{a}	4.36	145.43
Pomegranate peel	230.00 ± 14.7^{a}	6.86 ± 0.25^{bc}	2.98	101.38	268.00 ± 2.0^{ab}	8.36 ± 0.23^{b}	3.12	99.95
Guava leaf	221.00 ± 17.3^{a}	7.60 ± 0.95^{b}	3.43	116.77	262.33 ± 7.23^{bc}	8.43 ± 0.66^{b}	3.21	102.92
Silymarin	228.66 ± 14.3^{a}	6.96 ± 0.25^{bc}	3.04	103.45	276.00 ± 7.93^{a}	8.53 ± 0.35^{b}	3.09	98.98
	LSD 0.05= 27.44	**LSD 0.05= 1.06**			**LSD 0.05= 11.01**	**LSD 0.05= 0.67**		

Each value in the table was obtained by calculating the average of six experiments ± standard deviation, The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05,* These are the same values of zero time for all treatments,**CCl_4 dose was stopped for all groups.

Table 2. Effect of plant ethanolic extracts or silymarin treatments on serum total protein content of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)		Curative period** (after six weeks)	
	Total protein (g/l)	Relative (%)	Total protein (g/l)	Relative (%)
Normal control	*66.68±1.36^{a}	100	67.08±1.02^{a}	100
(3.3% CCl_4)	45.88±2.61^{c}	68.81	48.20±2.47^{d}	71.86
Pomegranate peel	54.91±3.58^{b}	82.34	64.37±1.01^{ab}	95.96
Guava leaf	49.67±3.68^{c}	74.50	58.46±1.15^{c}	87.15
Silymarin	54.78±0.95^{b}	82.16	63.70±1.59^{b}	94.96
LSD 0.05	4.87		2.93	

Each value in the table was obtained by calculating the average of three experiments ± SD. The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 3. Effect of plant ethanolic extracts or silymarin treatments on serum albumin and globulin contents of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)				Curative period** (after six weeks)			
	Albumin (g/l)	Relative (%)	Globulin (g/l)	Relative (%)	Albumin (g/l)	Relative (%)	Globulin (g/l)	Relative (%)
Normal control	*43.25±0.94^{a}	100	23.42±1.21^{a}	100	44.20±2.0^{a}	100	22.87±2.0^{a} ^{bc}	100
(3.3% CCl_4)	24.83±2.84^{c}	57.42	21.04±3.18^{a}	89.85	28.83±2.7^{c}	65.23	19.36±2.17^{c}	84.66
Pomegr. peel	32.74±1.48^{b}	75.70	22.16±2.87^{a}	94.60	38.09±1.42^{b}	86.19	26.27±0.76^{a}	114.84
Guava leaf	30.28±1.11^{b}	70.00	19.39±4.41^{a}	82.81	37.88±2.8^{b}	85.71	20.57±4.53^{bc}	89.93
Silymarin	32.25±1.24^{b}	74.56	22.53±0.77^{a}	96.18	38.83±3.0^{b}	87.85	24.86±3.34^{ab}	108.69
	LSD 0.05= 3.04		**LSD 0.05= 5.14**		**LSD 0.05= 4.53**		**LSD 0.05= 5.11**	

Each value in the table was obtained by calculating the average of three experiments ± standard deviation, The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 4. Effect of plant ethanolic extracts or silymarin treatments on serum ALT and AST activity of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)				Curative period** (after six weeks)			
	ALT (U/ml)	Relative (%)	AST (U/ml)	Relative (%)	ALT (U/ml)	Relative (%)	AST (U/ml)	Relative (%)
Normal control	*44.68±4.15[c]	100	*64.21±6.76[c]	100	44.53±4.41[b]	100	63.91±6.60[c]	100
(3.3% CCl_4)	103.18±16.11[a]	230.91	215.55±8.26[a]	335.68	94.25±9.05[a]	211.63	198.56±3.79[a]	310.69
Pomegr. peel	61.45±6.37[b]	137.51	93.34±2.69[b]	145.36	51.24±5.02[b]	115.06	71.73±3.92[bc]	112.24
Guava leaf	65.93±5.48[b]	147.53	102.92±29.27[b]	160.29	55.05±6.03[b]	123.61	82.25±9.38[b]	128.71
Silymarin	61.97±5.17[b]	138.69	95.26±15.10[b]	148.35	52.48±5.26[b]	117.84	74.86±4.97[bc]	117.13
LSD 0.05	15.74		28.25		11.24		1.09	

Each value in the table was obtained by calculating the average of three experiments ± SD.
The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * these are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 5. Effect of plant ethanolic extracts or silymarin treatments on serum GGT activity of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)		Curative period** (after six weeks)	
	GGT (U/ml)	Relative (%)	GGT (U/ml)	Relative (%)
Normal control	*11.78±0.63[d]	100	12.89±2.30[d]	100
(3.3% CCl_4)	75.14±4.81[a]	637.50	71.08±6.08[a]	551.42
Pomegranate peel	34.99±3.88[c]	296.87	18.78±1.10[cd]	145.71
Guava leaf	43.09±2.92[b]	365.62	23.20±3.31[bc]	180.00
Silymarin	46.04±3.88[b]	390.62	27.62±2.21[b]	214.28
LSD 0.05	6.41		6.27	

Each value in the table was obtained by calculating the average of three experiments ± SD. The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 6. Effect of plant ethanolic extracts or silymarin treatments on liver homogenate reduced glutathione (GSH) content of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)		Curative period** (after six weeks)		Hepatoprotective period (after three weeks)		Curative period** (after six weeks)	
	GSH (µg/mg protein)	Relative (%)	GSH (µg/mg protein)	Relative (%)	100	Relative (%)	LP (nmol/ mg protein)	Relative (%)
Normal control	*8.34±0.34[a]	100	8.41±0.43[a]	100	267.08	100	2.43±0.10[b]	100
(3.3% CCl_4)	5.75±0.54[c]	68.89	5.97±0.34[c]	71.05	101.66	328.57	6.51±0.18[a]	267.08
Pomegranate peel	6.23±0.50[bc]	74.70	6.97±0.29[bc]	82.87	118.80	103.83	2.47±0.13[b]	101.66
Guava leaf	7.52±0.95[ab]	90.10	8.01±1.05[ab]	95.29	107.68	159.96	2.89±0.43[b]	118.80
Silymarin	6.13±1.25[bc]	73.45	6.71±0.74[bc]	79.82	2.74±0.02[c]	110.99	2.62±0.16[b]	107.68
LSD 0.05	1.45		1.16		0.54		0.43	

Each value in the table was obtained by calculating the average of three experiments ± SD. The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 7. Effect of plant ethanolic extracts or silymarin treatments on liver homogenate glutathione-S-transferase (GST) and superoxide dismutase (SOD) activity of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)		Curative period** (after six weeks)		Hepatoprotective period (after three weeks)		Curative period** (after six weeks)	
	GST (mM/min/ mg protein)	Relative (%)	GST (mM/min/ mg protein)	Relative (%)	SOD (U/mg protein)	Relative (%)	SOD (U/mg protein)	Relative (%)
Normal control	*25.15±0.61[a]	100	25.08±0.83[a]	100	*44.55±1.71[a]	100	43.68±2.24[a]	100
(3.3% CCl_4)	11.66±0.48[e]	46.36	13.37±1.67[c]	53.30	13.95±1.82[c]	31.31	14.86±2.57[c]	34.01
Pomegranate peel	15.20±0.91[c]	60.42	20.41±3.45[b]	81.38	22.90±1.68[b]	51.40	38.98±0.78[b]	89.25
Guava leaf	17.78±0.68[b]	70.68	24.85±1.24[a]	99.07	21.97±3.56[b]	49.30	38.68±1.46[b]	88.55

Silymarin	13.59±1.35[d]	54.03	17.03±1.19[b]	67.90	20.69±3.49[b]	46.43	36.48±2.09[b]	83.51
LSD 0.05	1.57		3.48		4.74		3.53	

Each value in the table was obtained by calculating the average of three experiments ± SD. The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 8. Effect of plant ethanolic extracts or silymarin treatments on liver homogenate catalase (CAT) activity of hepatotoxic rats.

Treatments	Hepatoprotective period (after three weeks)		Curative period** (after six weeks)	
	CAT (µmol/ mg protein)	Relative (%)	CAT (µmol/ mg protein)	Relative (%)
Normal control	*15.64±0.43[a]	100	15.50±0.43[a]	100
(3.3% CCl_4)	4.63±0.36[c]	29.62	4.88±0.06[b]	31.52
Pomegranate peel	6.41±0.74[b]	40.99	15.50±0.19[a]	99.95
Guava leaf	6.12±0.27[b]	39.17	15.41±0.31[a]	99.41
Silymarin	6.04±1.07[b]	38.63	14.88±0.49[a]	95.94
LSD 0.05	1.18		0.61	

Each value in the table was obtained by calculating the average of three experiments ± SD.
The various superscript letters indicate statistically significant differences in the Duncan test, with *P* <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

Table 9. Effect of plant ethanolic extracts or silymarin treatments on liver homogenate lysosomal enzymatic release activities of hepatotoxic rats.

Lysosomal enzymatic release			Treatments				
			Normal control	**(3.3% CCl_4)**	**Pomegranate peel**	**Guava leaf**	**Silymarin**
Hepatoprotective period (after three weeks)	**ACP**	**nmol/ml/hr**	*1674.00±42.02[c] **LSD 0.05=89.84**	2662.26±50.86[a]	2095.74±59.26[b]	2151.24±55.62[b]	2138.91±33.96[b]
		% Relative	100	159.03	125.19	128.50	127.77
	-GA	**nmol/ml/hr**	*344.34±12.90[c] **LSD 0.05=**	826.72±20.46[a]	367.11±13.42[c]	438.35±28.38[b]	359.31±17.44[c]

	L		**25.23**				
		% Relative	100	240.05	106.61	127.30	104.34
	-NAG	**nmol/ml/hr**	*225.74±8.84^{d} **LSD 0.05=16.60**	377.91±10.34^{a}	248.78±7.03^{c}	308.82±12.14^{b}	250.48±5.85^{c}
		% Relative	100	167.40	110.20	136.8	110.95
Curative period (after six weeks)**	**ACP**	**nmol/ml/hr**	1679.42±45.84^{c} **LSD 0.05=52.93**	2574.31±21.10^{a}	1765.75±16.25^{b}	1811.01±25.21^{b}	1779.53±28.05^{b}
		% Relative	100	153.28	105.14	107.83	105.96
	-GAL	**nmol/ml/hr**	345.66±4.16^{b} **LSD 0.05=8.93**	815.95±3.50^{a}	336.56±4.03^{bc}	343.31±5.00^{b}	333.03±7.04^{c}
		% Relative	100	236.05	97.36	99.32	96.34
	-NAG	**nmol/ml/hr**	223.20±4.85^{c} **LSD 0.05=9.31**	368.52±6.34^{a}	229.53±5.16^{c}	244.05±5.90^{b}	226.51±2.40^{c}
		% Relative	100	165.11	102.83	109.34	101.48

Each value in the table was obtained by calculating the average of three experiments ± standard deviation, The various superscript letters indicate statistically significant differences in the Duncan test, with P <0.05, * These are the same values of zero time for all treatments, **CCl_4 dose was stopped for all groups.

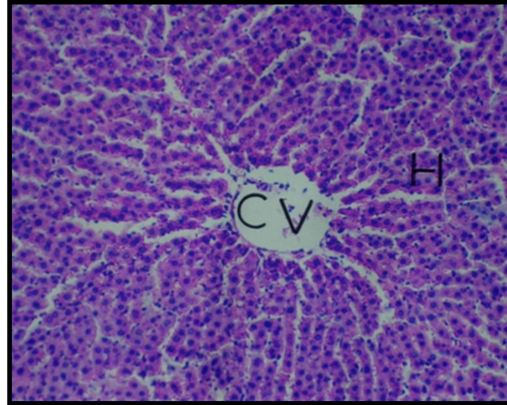

Fig. 1. Photomicrographs of liver sections of normal control rats. (C.V; central veins and H; hepatocytes).

M= mononuclear leucocyte

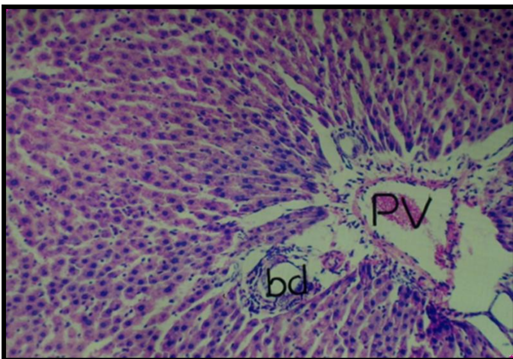

Fig. 2. Photomicrographs of liver sections of CCl_4-treated rats (C.V; central veins and inflammatory cells infiltration).

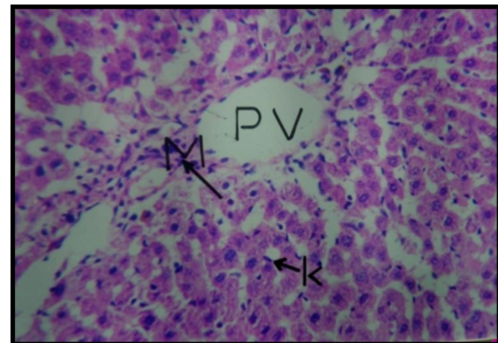

Fig. 3. Photomicrographs of liver sections of pomegranate peel ethanolic extract and CCl_4-treated rats. (C.V; central veins and K; kupffer cells).

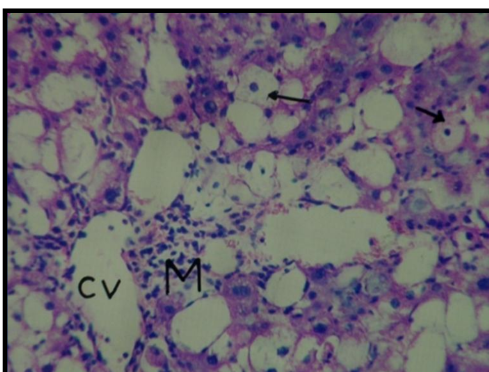

Fig. 4. Photomicrographs of liver sections of guava leaf ethanolic extract and CCl_4-treated rats. (P.V; portal veins and bd; bile ductules).

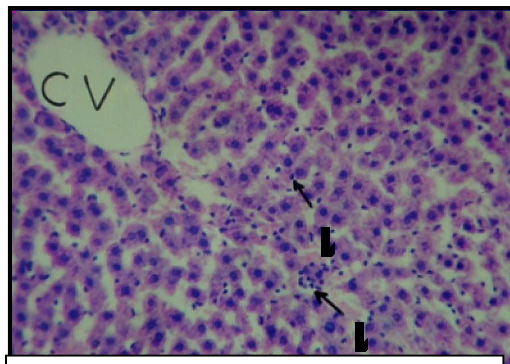

Fig. 5. Photomicrographs of liver sections of silymarin and CCl_4-treated rats. (P.V; portal veins, M; mononuclear leucocytes inflammatory cells