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Comparative Evaluation of Hepatoprotective Activities of Single and Combined Administration of Silymarin, Ficus Leaves and pomegranate Peel Extracts on CCl_4 Indced Liver Injury: Preliminary Study

Rania, A. H. Abd El-Aal, Fatma, M. Sabry, Amira, M. El-Khawaga and Nahed, M. A. Hassanein

Developmental Pharmacology Department, National Organization for Drug Control and Research (NODCAR), Cairo Egypt

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ABSTRACT

Background: Natural products from plants have received considerable attention in recent years due to their diverse pharmacological properties, including antioxidants and hepatoprotective activities. The individual hepatoprotective effects of herbs such as *Silybum marianum* (Sliymarin) (Sily), *Ficus carica* Linn leaves (FLE) and *Punica granatum* Linn (Pomegranate peel extract) (PPE) extracts have been extensively in hepatotoxic damage. However, few studies on the hepatoprotective role of these herbs when administered in combination against liver damage were conducted.

Aim: To investigate the hepatoprotective effect of FLE and PPE either alone or in combination with silymarin (as reference drug) on carbon tetrachloride CCl_4 induced liver injury in male albino rats.

Methods: Adult male Sprague Dawely rats were randomly divided into seven groups of ten rats each; including control and CCl_4 treated groups. The remaining five groups were divided according to the received treatment into Sily (100 mg/kg.b.wt./day, P.O), FLE (500 mg/kg.b.wt./day, P.O), PPE (500 mg/kg.b.wt./day, P.O), Sily+FLE and Sily + PPE treated groups. The oral administration of different extracts was performed two weeks prior CCl_4 administration and continued till the end of the experiment (10 weeks). Liver injury was performed with an oral administration of 2.0 ml/kg.b.wt. of 20% CCl_4 in olive oil, twice a week for 8 weeks.

Serum analysis was performed to assay the levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglycerides and total cholesterol. Moreover, glutathione (GSH) and malondialdehyde (MDA) were estimated in rats' livers. Biochemical observations were also supported with histopathological examination.

Results: Serum ALT activity of CCl_4 treated rats exhibited slight increase in serum ALT, however after combined treatments rats exhibited pronounced increase in their serum ALT activity. Each of single Sily and PPE induced significant decrease in serum ALP activity as compared to CCl_4 group. Administration of CCl_4 for 8 weeks increased hepatic MDA and total NO metabolite content. Histopathological examinations revealed severe liver damage.

Single Sily and PPE could restore cholesterol to basal values at 6 weeks. Single FLE increased serum triglycerides level, while combined FLE could restore it to the normal value at 10 weeks of treatment. Although combined treatments potentiate the increase in serum cholesterol levels after 6 weeks of treatment nevertheless at the end of 10 weeks, all the utilized treatment could restore serum cholesterol level to the normal value.Administration of single and combined PPE potentiated CCl_4 induced increase in serum triglycerides level. While single Sily had no influence on CCl_4 induced increase in hepatic NO metabolites and MDA contents, single PPE and both combined treatments could restore hepatic MDA and NO metabolites content to their basal values. Both combined treatments, single Sily and PPE induced increase in the hepatic GSH content.

Histopathological examination showed significant regression in the hepatic damage and decreased punctate necrosis as thin strands of fibroblasts proliferated around the hepatocytes in both single FLE and PPE groups were detected. Combined treatments showed moderate fibrosis compared to CCl_4 group.

Conclusion: The data indicated that single FLE and PPE ameliorate CCl_4 induced liver injury compared to combined treatments.

Corresponding Author: Rania, A. H. Abd El-Aal, Developmental Pharmacology Department, National Organization for Drug Control and Research (NODCAR), Cairo Egypt.
E-mail: raniaawad350@yahoo.com

Key words: *Silybum marianum*, *Ficus carica* Linn , *Punica granatum* Linn, CCl_4, hepatoprotective, rat.

Introduction

The liver is a vital organ of the human body that is responsible for numerous fundamental and important roles, including digestive and excretory functions, in addition to nutrient storage and metabolic functions, synthesis of new molecules, and purification of toxic chemicals (Guerra *et al*., 2016). Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects. This is one of the reasons for many people in the world over including those in developed countries turning complementary and alternative medicine. Many traditional remedies employ herbal drugs for the treatment of liver ailments (Mitra *et al*., 2000). A number of plants have been shown to possess hepatoprotective property (Jamshidzadeh, *et al*., 2006)

Milk thistle (*Silybum marianum*) is the most ancient and broadly used therapeutic plant for its useful effects on liver and other organs (Negi *et al*., 2008). This plant is native to the Mediterranean and grows throughout Europe and North America. Silymarin, a polyphenolic flavonoid, is isolated from the milk thistle (Pepping, 1999). Silymarin is a combination of some bioflavonoids found in the fruit, seeds and leaves of this plant (Karimi *et al*., 2011). Besides the antioxidant effect (Karimi *et al*., 2006), Sily indicates effective anti-inflammatory (Nazemian, 2010), antifibrotic (Karimi *et al*., 2011), antineoplastic (Ramakrishnan *et al*., 2009), immunomodulating (Taghiabadi *et al*., 2012) and membrane stabilizing properties in different animal and human studies (Muthumani and Milton, 2014). It is established that Sily has been utilized medicinally to cure liver diseases including viral hepatitis, cirrhosis and alcoholic liver disorders (Saller *et al*., 2007).

Ficus carica Linn (family: Moraceae) is commonly referred to as "fig". Fig is one of the only five plants mentioned in the holy Quran along with the olives, grapes, pomegranate, and dates. Its fruit, root, and leaves are used in the native system of medicine in different diseases (Vikas *et al.*, 2010). The tree is one of the oldest plants cultivated in the Mediterranean (Trad *et al*., 2012) and has been traditionally used for metabolic, cardiovascular, respiratory, antispasmodic, and anti-inflammatory disorders (Vikas *et al.,* 2010). It is rich in minerals, vitamins, dietary fibers, and phenolic contents which play an important role in its antioxidant capacity (Veberic *et al.,* 2008), and exert positive effects to the human health (Çalişkan and Aytekin, 2011).

The bark, leaves, and fruits of Fig are used in traditional medicine for treatment of different disorders such as gastrointestinal, diabetes, skin diseases, ulcers, dysentery, hemorrhoids (Patil et al., 2010 , Joseph and Raj, 2011) and used in metabolic, cardiovascular, respiratory, antispasmodic, and anti-inflammatory remedies (Duke *et al*., 2002). The anti-inflammatory activity could be related to the antiradical activity of the extracts and by extension, to their chemical composition (Ali *et al*., 2012). Leaves were the only material displaying a protective effect (Patil and Patil, 2011a&b).

Some recent works have reported that fig antioxidants can protect lipoproteins in plasma from oxidation and produce a significant increase in plasma antioxidant capacity for 4 h after consumption (Vinson *et al*., 2005). The antioxidant potential of various parts of fig leaves, pulp and peel were evaluated; all these parts had an inhibition activity on DPPH (2,2-Diphenyl-1-picrylhydrazyl) and nitric oxide radicals depending on the concentration. Leaves were the most effective part (Oliveira *et al* 2009, Pande and Akoh, 2010). Its leaves are commonly used to cure hemorrhoid and clear away heart ache (English Chinese Medical Dictionary, 1986). Furthermore, it has also been shown to possess anti-hypertension and anti-cancer effects (Yin *et al*. 1997 & 1998). Psoralen and bergapten are two of major active components of *Ficus carica L.* leaves. Psoralen has anti-tumor, anti-bacterial and anti-viral activities while bergapten is used to cure vitiligo, psoriasis, and alopecia areata (English Chinese Medical Dictionary, 1986).

The species *Punica granatum* L. belongs to the Punicaceae family. It is one of the natural delicious fruit consumed worldwide. In Ayurvedic medicine, it is described under its Sanskrit name 'dalima' (fruit) as a blood purifier (Jurenka, 2008). *Punica granatum* Linn is a shrub or small tree and considered to be a native of Iran and Afghanistan. It is also found growing wild in the warm valleys and outer hills of the Himalayas.

The pomegranate fruit consists of the peel, seeds, and the arils. The peel makes up about 50% of the fruit, whereas the arils and seeds make up 40% and 10%, respectively. The peel is rich in many compounds such as phenolics, flavonoids, ellagitannins and proanthocyanidin compounds, complex polysaccharides, and many minerals including potassium, nitrogen, calcium, magnesium, phosphorus, and sodium (Viuda-Martos *et al*. 2010).

Several recent studies have demonstrated that *Punica granatum L.* extract mediates a wide spectrum of biological activities including antibacterial, antiviral, antifungal, cytotoxic, antioxidant and immuno-potentiating activities (Wang *et al*., 2013). Pomegranate has been widely used by traditional medicine for the treatment of different types of diseases, including cancer, dyslipidemia of obesity, cardiovascular disorders, diabetes, male infertility, Alzheimer's disease and aging (Sadeghipour *et al*., 2014 , Sreekumar *et al*., 2014).

Pomegranate peel extract (PPE) attenuates liver damage in high lipid diet fed rats (Parmar and Kar, 2008), decreased lipid peroxidation in hepatic, cardiac, and renal tissues and at the same time it had a facilitatory effect on the scavenging capability of superoxide anion and hydrogen peroxide (Sadeghipour *et al*., 2014). Formerly, it was shown that pomegranate peel extract supplementation alleviated oxidative damage of the liver and enhanced the hepatic structure and function in rats exposed to bile duct ligation as well as prevents liver fibrosis ((Toklu *et al*., 2007).

The individual hepatoprotective effects of herbs such as Sliy, FLE and PPE have been investigated in hepatotoxic damage. However, few studies on the hepatoprotective role of these herbs when administered in combination against liver damage, particularly against chronic liver injury were conducted. In the present study, the hepatoprotective effect of FLE and PPE either alone or their combined treatments with Sily (as reference drug) were examined on CCl_4 induced liver injury.

Materials and Methods

Plant Materials:

I) One kilogram of *Ficus carica* Linn leaves was collected from El-monofia region, Egypt.
II) One Kilogram of *Punica granatum* L. pericarp was collected from El-Fayoum region, Egypt.

The samples of *Ficus carica* Linn leaves *and Punica granatum* peels were authenticated, based on its microscopic and macroscopic characteristics, by Applied Research Center of Medicinal Plants (ARCMP), at National Organization for Drug Control and Research (NODCR).

Preparation of Crude Extract of the Ficus carica L. Extract:

The air dried *Ficus carica* Linn leaves were ground to powder. The powder (550 grams) was extracted with 70% aqueous ethanol several times till exhaustion. The collective ethanol extract was filtered, and the filtrate was concentrated to dryness under reduced pressure (at temperature 60ºC). The solvents evaporated using a rotary evaporator. The dry extract was stored in a refrigerator at 4°C for the subsequent use.

Preparation of the Punica granatum L. Pericarp (Pomegranate Peel) Extract:

Pomegranate peel extract was prepared according to the method described by Abdel Moneim, 2012. Air-dried powder (850g) of pomegranate peels was extracted with 70% aqueous ethanol several times till exhaustion and kept at 4◦C for 24 h. The obtained extract was concentrated under reduced pressure (at a bath temperature of 60◦C) and dried in a vacuum evaporator. The residue was dissolved in distilled water and used in this experiment.

Measurement of extract total phenolic content:

Total phenolic content of the extract was assessed by the Folin-Ciocalteu method. Briefly, 20 µl extract 85% plus 1.58 ml deionized water and 100 µl Folin- Ciocalteu reagents were mixed. After 30 Sec., 30 µl Na_2CO_3 was added to the mixture. Then, the mixture was incubated at 20°C for 2 h. Finally, the absorbance was read in 765 nm.

Experimental Animals:

Male Sprague Dawley rats (300~350 g) were obtained from the animal house of NODCAR. The animals were acclimated for 1 week prior to the experiments, and housed in an air-conditioned animal room with a 12/12h light/dark cycle at a temperature of 22±1◦C. The animals were provided with a laboratory diet and water *ad libitum*. All experimental protocols involving the use of animals were conducted in accordance with National Institutes of Health (NIH) guidelines and approved by the Committee on NODCAR.

The long history of ethno medicinal application, with no reports of any serious side effect, suggests that *Ficus carica* could be considered as safe. In acute toxicity studies carried out on albino mice, the ethanol, chloroform, and petroleum ether extract of *Ficus carica* were found to be safe at a dose of 5000 mg/kg of body weight (Patil and Patil, 2011b).

The oral LD_{50} of the pomegranate fruit extract in rats and mice was found to be greater than 5000 mg/kg body weight. In the subchronic study, Wistar strain rats were administered via gavage 600 mg/kg body weight/day of the extract for 90 days, the no observed-adverse-effect level (NOAEL) for pomegranate fruit extract was determined as 600 mg/kg body weight/day (Patel *et al*., 2008)

Experimental Design

For CCl_4 subchronic toxicity studies, 8 week experiment was designed with some modifications (Shyu *et al*., 2008). Adult male rats of Sprague-Dawley strain were randomly divided into seven groups with ten animals in each. The first group was served as control group received only vehicles; olive oil (0.5 ml/kg b.wt.) and fed with a normal diet. The second group, the toxicant (CCl_4) group received oral administration of 2 ml CCl_4/kg.b.wt. (20% CCl_4/olive oil) twice a week for 8 weeks. The remaining five groups were divided according to the received treatment into single treatment groups and combined groups. The single groups were Sily group (100 mg/kg.b.wt.), FLE group (500 mg/kg.b.wt., Mohan *et al*., 2007) and PPE group (500 mg/kg.b.wt.). For combined treatments, the groups were Sily + FLE group and Sily + PPE. All treatments were given orally through a feeding tube daily for 10 weeks. These single and combined treated groups were also received oral administration of 2 ml CCl_4/kg.b.wt.(20% CCl_4/olive oil) twice a week for 8 weeks. After 24 hr of last dosing, rats were euthanized under ether anesthesia, blood was withdrawn from the orbital sinus of each animal and serum was obtained by blood centrifugation at1500 × g for 10 min, at 4°C.The liver was dissected out and used for biochemical estimation and histopathological examination.

The biochemical assays on blood samples were conducted at 6 and 10 weeks to verify the hepatoprotective effects of Sily, FLE and PPE singly or in combined treatments.

Determination of Serum Hepatic Function Parameters:

Serum ALT Determination:

Serum ALT was determined colorimetrically according to Reitman and Frankel (1957).

Serum Alkaline Phosphatase Determination:

Alkaline phosphatase was determined colorimetrically according to Babson *et al*., (1966) using commercial kit (Quimica Clinica Aplicada S. A, Spain).

Serum Cholesterol Determination

Serum total cholesterol was determined by quantitative-enzymatic colorimetric determination kit (Stanbio Laboratory, USA). The method is adopted by Allain *et al*., (1974).

Serum Triglycerides Determination

According to the manufacturer, triglyceride level was measured by a triglyceride assay kit (Bio-diagnostic, Egypt) according to Fossati and Prencipe method (1982).

Collection of Livers for Hepatic Tissue Oxidative Stress Markers

After blood collection, the livers were identified and carefully dissected out from each rat. The right lobe of the liver was rinsed in ice cold 1.15% KCl solution in order to preserve the oxidative enzyme activities of the liver and to prevent the breakdown of the hepatic antioxidant biomarkers before being stored.

Estimation of Lipid Peroxidation Assay

Malondialdehyde in liver homogenate was determined by reaction with thiobarbituric acid reactive substances (TBARS) according to Mihara and Uchiyama (1978). The results were expressed as nmol/gm tissue.

Determination of Liver Tissue Reduced Glutathione Activity (GSH)

The reduced glutathione (GSH) content in the liver tissue was estimated according to the method described by Ellman (1959) with some modification as described by (Nurrochmad *et al*., 2010). It was expressed as µmol GSH/gm tissue.

Determination of Liver Nitrate/Nitrite Content

Total nitrate/nitrite accumulation in liver was performed according to Miranda *et al*., (2001). It was expressed as µmol/gm tissue.

Histopathological Examination

For qualitative analysis of liver histology, the rats' livers samples were fixed for 48 h in 10% formalin-saline and dehydrated successfully in different mixtures of ethyl alcohol-water, cleaned in xylene, and embedded in paraffin. Paraffin liver sections of 4-5µm thickness were hematoxylin-eosin stained. These stained sections were examined under a photomicroscope by a single observer in a blinded-manner for histopathological examination.

Statistical Analysis

Data were analyzed with a one-way analysis of variance (ANOVA) followed by a *post hoc* least significant difference (LSD) test in order to measure statistical significance of the differences observed (SPSS). All data are presented as the mean ± standard error of the mean (SEM) and *P* values of 0.05 or less were considered to be statistically significant.

Results

The preliminary phytochemical screening of FLE and PPE showed the presence of carbohydrates and/or glycosides, flavonoids, phenols and tannins and sterols and/or triterpenes and resins. Both FLE and PPE extracts were free from volatile oils and anthraquinones (table, 1). FLE contains resins and PPE contains saponine and alkaloids (Egyptian Pharmacopeia 1984, Harborne 1998 & British Pharmacopeia 2014).

Table 1: The preliminary phytochemical screening of *Ficus Carica* and *Punica granatum L.* pericarp (Pomegranate Peel) Extract:

Components / Extract	Carbohydrates and /or glycosides	Flavonids	Polyphenols and /or tannins	Sterols and/or triterpenes	Saponins	Resins	Alkaloids
Ficus carica	++	++	+	+	-	+	-
Punica granatum	++	+	++	++	+	-	+

Effect of Extracts on Liver Function:

Serum ALT and ALP Activities

Serum ALT and ALP activities were measured to assess the effects of the treatments on liver functions. The obtained results revealed that at week 6, serum ALT activities of CCl_4 and single treated groups (Sily, FLE and PPE) exhibited significant increases by 41% *(P*<0.01), 41% *(P*<0.01), 61% and 67% (*P*<0.001), respectively as compared to control group (Fig.1).

At week10, CCl_4 group still had increased serum ALT (*P*<0.05) and ALP (*P*<0.001) activities compared with the control group (Figs 2&4). While serum ALT activities still has increased among CCl_4, single FLE and PPE groups at week 10, serum ALT activity of Sily group exhibited normal activity (Fig., 2).

While single Sily pre-treatment significantly decreased serum ALP activity by 61% (P<0.001) at week 6 and restore it to the normal value at week 10, single FLE pre-treatment had no influence on CCl_4 induced elevation on serum ALP activity (Fig. 4). On the other hand, single PPE pretreatment for 10 weeks induced significant decrease in serum ALP activity by 20% (P<0.01) as compared to CCl_4 group (Fig. 4) ,

Combined FLE and PPE pre-treatments exhibited pronounced increase in serum ALT activity by 73% and 104% (both *P*<0.001) and ALP activity by 86% (*P*<0.01)and 58% (*P*<0.001), respectively, as compared to CCl_4 group at 6 week (Figs. 1&3).

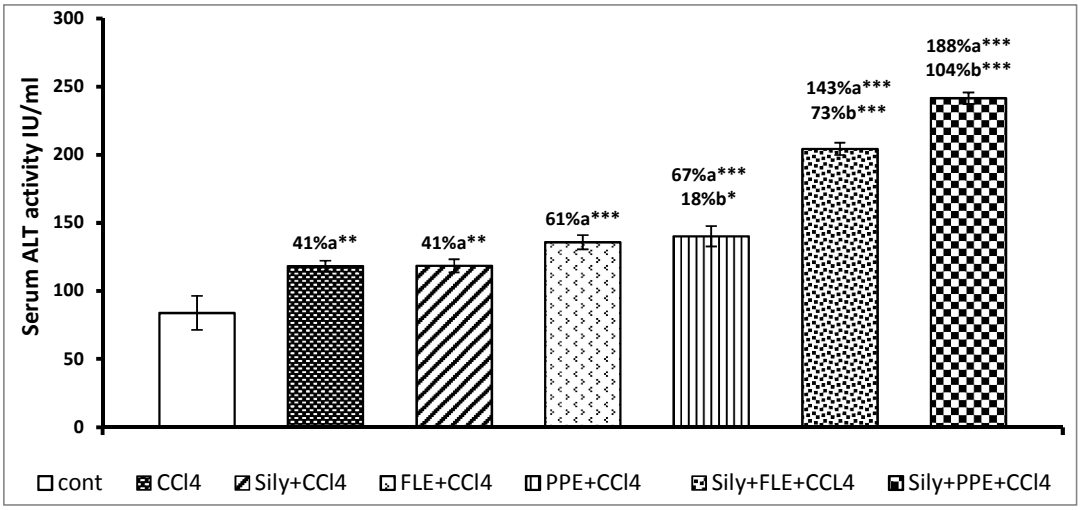

Fig.1: Effect of Silymarin, Ficus leaves and Pomegranate precarbe extracts on ALT activity after 6weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD) test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate

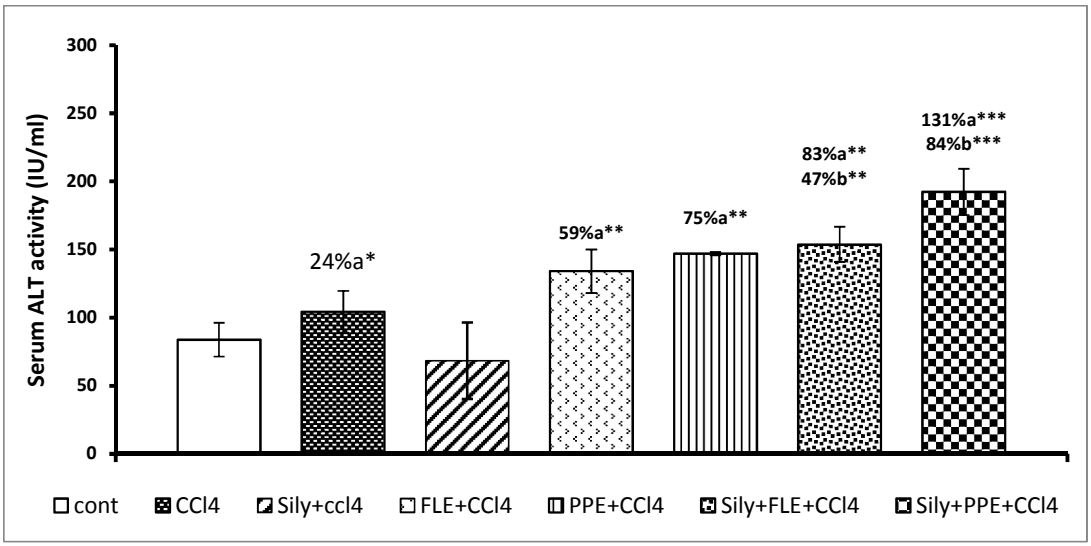

Fig. 2: Effect of Silymarin, Ficus leaves and Pmegranate precarb extracts on serum ALT activity after 10 weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

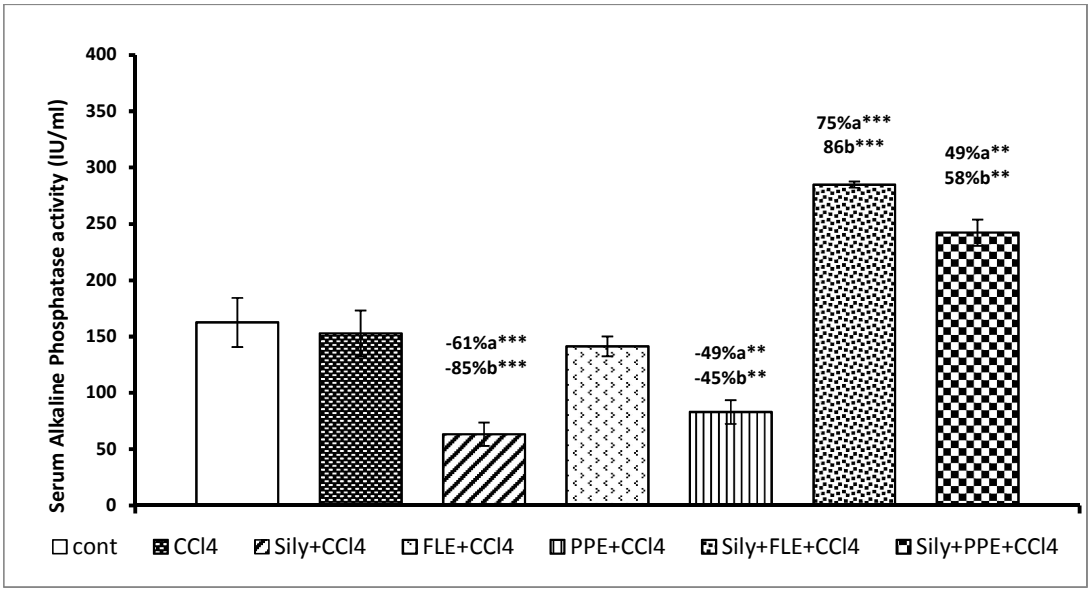

Fig. 3: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on serum alkaline phosphatase activity after 6 weeks of CCl_4 adminstration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

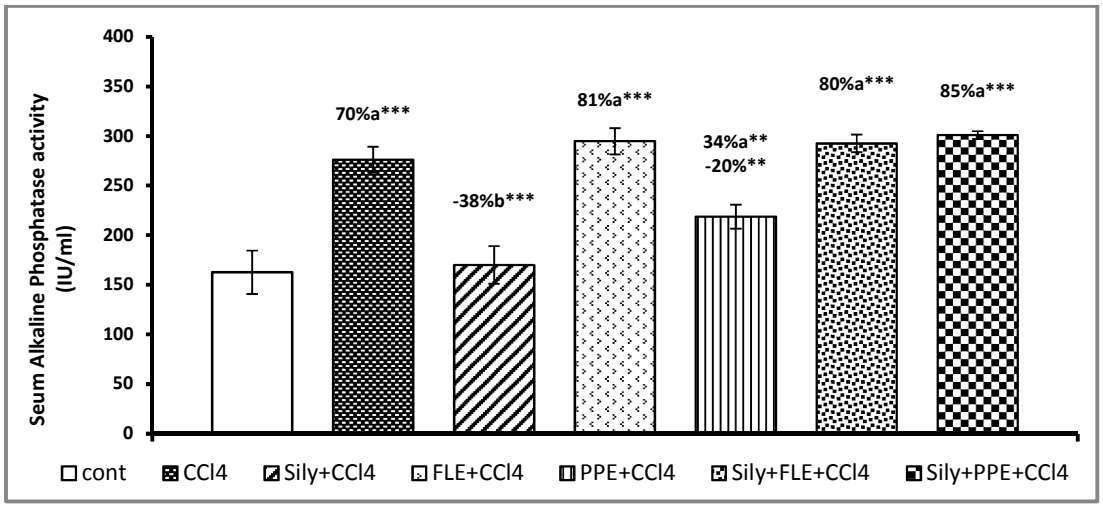

Fig. 4: Effect of Silymarin, Ficus and Pomegranate Precarb extracts on serum alkaline phosphatase activity after 10 weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

Serum Triglyceride and Total Cholesterol Concentrations

Serum lipid concentrations were determined to evaluate the effects of the treatments on lipid profiles. After the induction of liver injury, CCl_4 induced elevation in serum triglycerides (41.832 ± 4.149 vs 31.47 ± 2.655) and total cholesterol concentrations (67.8 ± 6.256 vs 55.47 ± 5.22) at 10 week (Figs 6&8). Sily had no influence on serum triglycerides level after 6 and 10 weeks, while PPE increased serum triglycerides concentrations by 104% (P<0.001) after 6 weeks and 130% (P<0.001) after 10 weeks as compared to CCl_4 group (Figs 5&6).

Each of FLE, PPE single treatment and combined FLE treatment could restore serum total cholesterol level to the normal value at week 10 (Fig. 8). While combined PPE-pretreatment induced increments in serum triglycerides by 217% (P<0.001) and cholesterol levels by 45.5%, (P<0.01) at 6 week as compared to CCl_4 group (Figs 5&7), such increases were attenuated at week 10 to reach 53% (P<0.05) for triglycerides and to the normal value for cholesterol (Figs.6&8).

Effects of Extracts on Hepatic MDA and GSH Contents

The end product of lipid peroxidation, hepatic MDA level, exhibited significant increase in CCl_4 group by 148%than control (P< 0.001, Fig.9). While Sily failed to induce any alteration on CCl_4 induced increase in hepatic MDA, single PPE and combined FLE pretreatments could restore the hepatic MDA content to the normal value (Fig.9) On the contrary, FLE extract potentiated the increase in the hepatic MDA content by 53% (p<0.001) as compared to CCl_4 group (Fig 9).

The data represented in figure (10) reveled that, meanwhile, CCl_4 administration decreased the hepatic GSH content than control group, single Sily and PPE treatment efficiently increased hepatic GSH content by 244% and 111% (P<0.001 and P<0.01, Fig. 10), respectively as compared to CCl_4 group. On the other hand, single FLE extract had no influence on hepatic GSH content. Combined FLE and PPE pre-treatments also efficiently increased hepatic GSH content by 255% and 131% (both P<0.001), respectively as compared to CCl_4 group (Fig. 10).

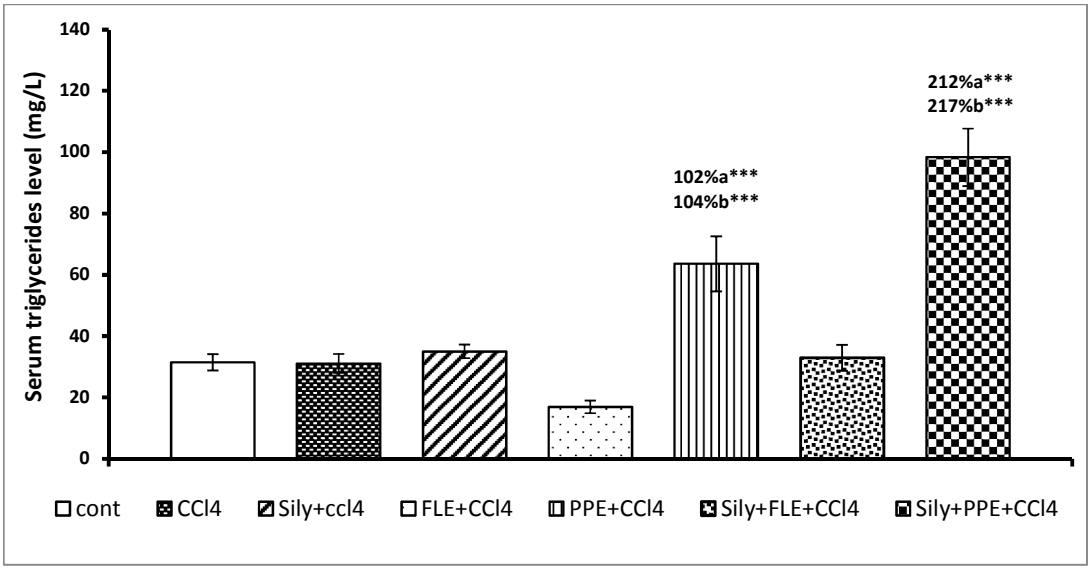

Fig. 5: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on serum Triglycerids level after 6 weeks of CCl_4 administration in Rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

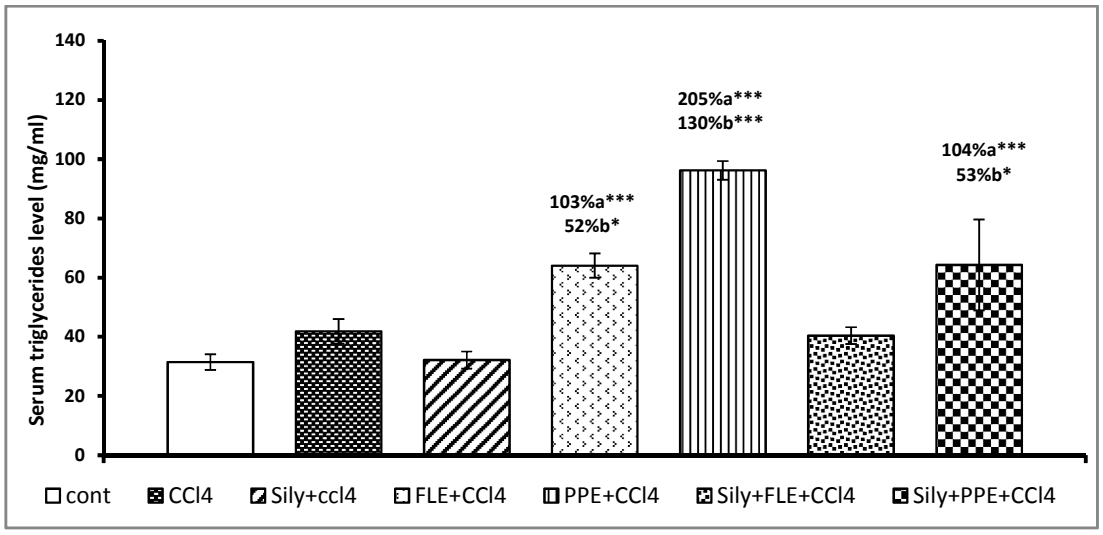

Fig. 6 : Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on serum Triglycerids level after 10 weeks of CCl_4 administration in Rats.

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus. PPE: Pomegranate

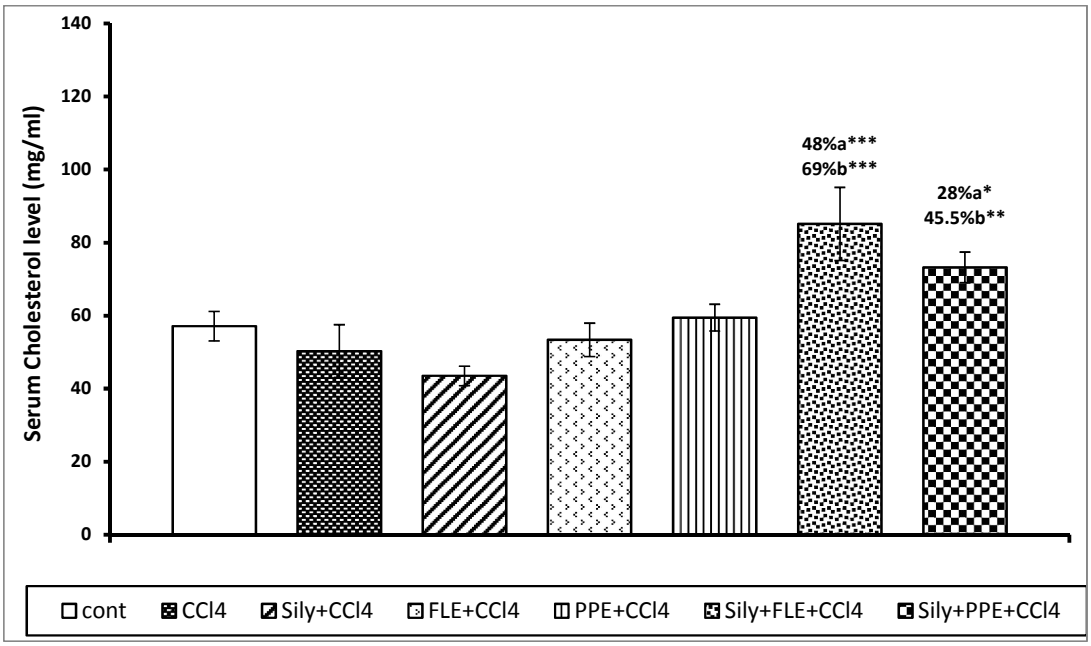

Fig. 7: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on serum cholesterol level after 6 weeks of CCl_4 administration in rats

- Data are represented asMean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

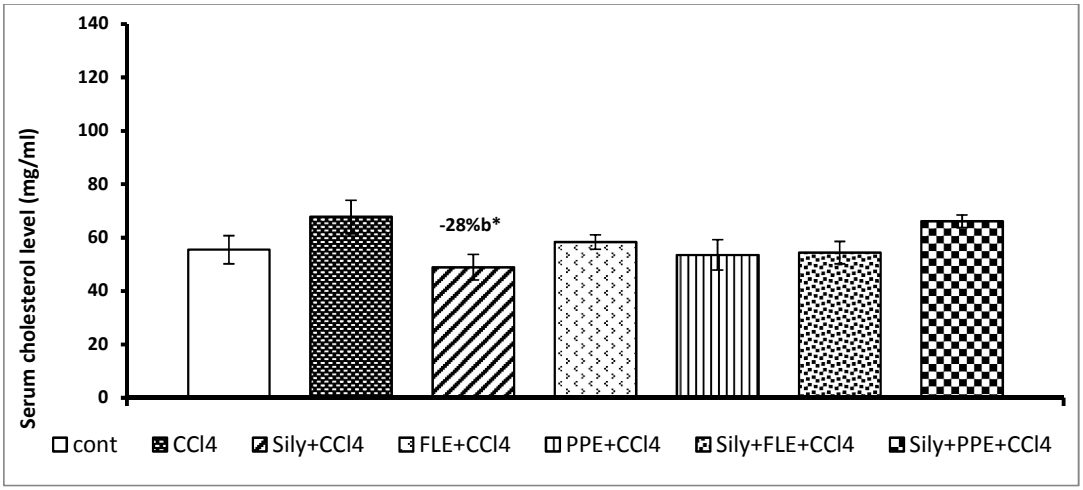

Fig. 8: Effect of Silymarin, Ficus leaves and Pomegranate precarb extracts on serum cholesterol level after 10 weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: pomegranate.

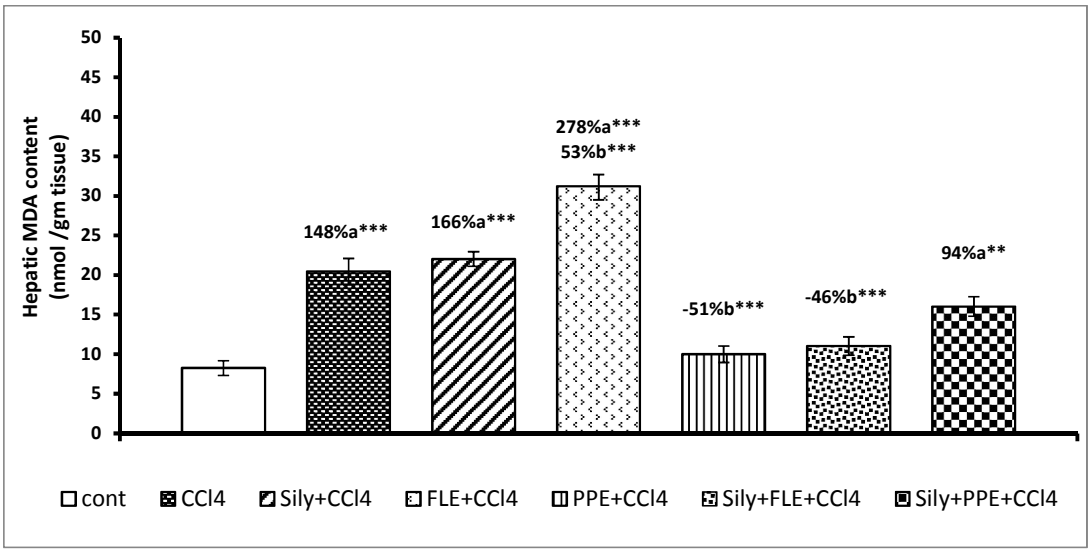

Fig. 9: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on hepatic MDA levels after 10 weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus. PPE: Pomegranate.

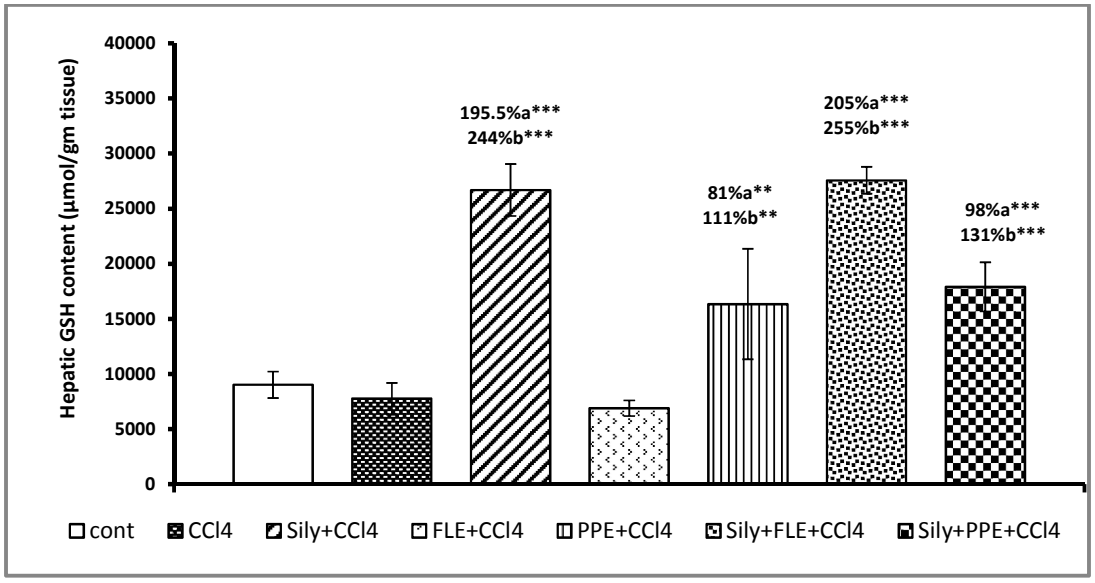

Fig. 10: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on hepatic GSH content after 10 weeks of CCl_4 administration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at P<0.05.
- (b) Significantly different from CCl_4 group at P<0.05.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate.

Effect on Hepatic Nitrate/Nitrite Content

Hepatic NO metabolite content of CCl_4 rats exhibited significant increase by 42% ($p<0.05$) as compared to control group (Fig.11). Single Sily pretreatment failed to induce any alteration on CCl_4 induced increase in the hepatic NO metabolite content, while single FLE and PPE and combined FLE showed an attenuation in hepatic NO metabolite content, nevertheless, combined PPE pretreatment could restore it to the basal value (Fig. 11).

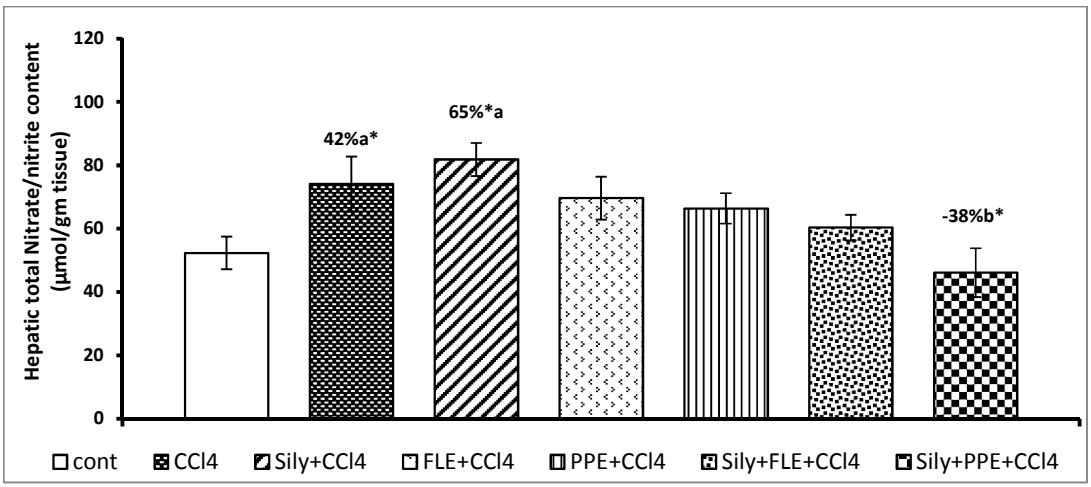

Fig. 11: Effect of Silymarin, Ficus leaves and pomegranate precarb extracts on hepatic Nitrate/Nitrite content after 10 weeks of CCl_4 adminstration in rats

- Data are represented as Mean±SEM (n=10)
- Statistical analysis was carried out using one way ANOVA, followed by post hoc least significant difference (LSD)test
- (a) Significantly different from control group at $P<0.05$.
- (b) Significantly different from CCl_4 group at $P<0.05$.
- Sily: Silymarin FLE: Ficus PPE: Pomegranate

Histopathological Findings:

As shown in Figure (12), typical photomicrographs of the control group depicted normal histoarchitecture pattern of hepatic lobules consists of central vein and radiating array of regular hepatocytes of well-preserved cytoplasmic and nuclear appearance (Fig. 12). Liver histoarchitecture pattern of CCl_4 animals showed the presence of multiple fibrotic nodules and extensive fibrosis predominantly in the periportal areas with briding of fibroblasts around lobules of hepatocytes (Fig.13). CCl_4 toxicity involved remarkable distortions of liver architecture which included considerable degree of apoptosis diffused throughout hepatic parenchyma with prominent peri-portal inflammatory and kupffer cells infiltration, congestion of blood vessels and fatty globules accumulation (Fig. 13&14).

Livers of rats treated with Sily showed thin strands of fibroblasts around lobules of hepatocytes and steatosis of hepatocytes (Fig.15).

Liver sections of rats pre-treated with PPE and FLE prior CCl_4 exposure showed significant reductions in liver tissue damage and decreased punctate and focal necrosis as thin strands of fibroblasts proliferated around the hepatocytes were detected (Figs.16&17). These results indicated that hepatic damage was diminished in extent compared to CCl_4. These improved signs of alleviated hepatocellular injuries, reduced fibrosis and regression of the hepatic damage indicating the hepatoprotective effect of the single FLE and PPE pretreatment.

Regarding the combined pre-treatments, livers of these animals showed moderate fibrosis, steatosis and apoptosis of hepatocytes (Figs. 18&19).

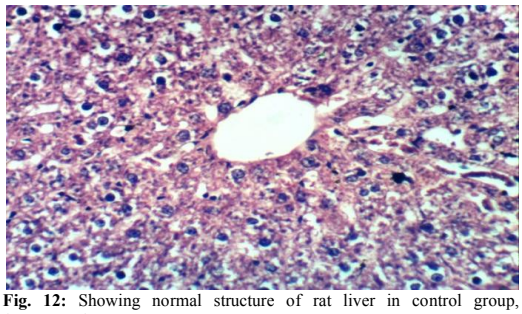

Fig. 12: Showing normal structure of rat liver in control group, (H&EX100).

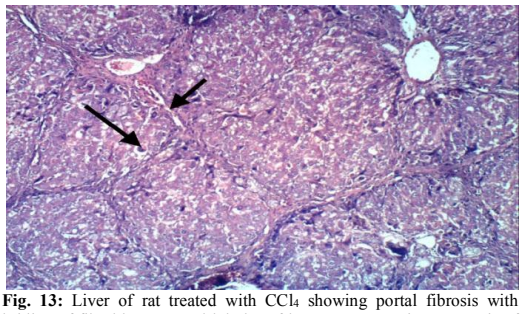

Fig. 13: Liver of rat treated with CCl_4 showing portal fibrosis with briding of fibroblasts around lobules of hepatocytes. Notice apoptosis of hepatocytes (H & E X 100).

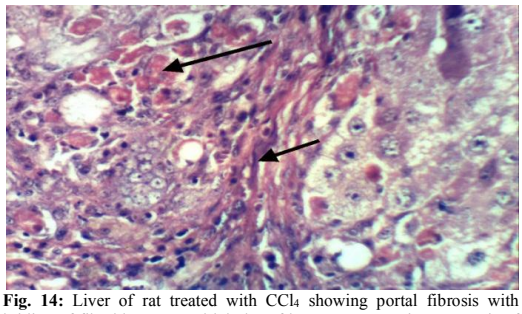

Fig. 14: Liver of rat treated with CCl_4 showing portal fibrosis with briding of fibroblasts around lobules of hepatocytes. Notice apoptosis of hepatocytes (H & E X 400)

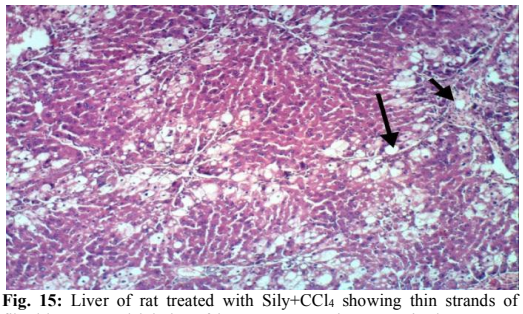

Fig. 15: Liver of rat treated with Sily+CCl_4 showing thin strands of fibroblasts around lobules of hepatocytes. Notice steatosis hepatocytes (H & E X 100

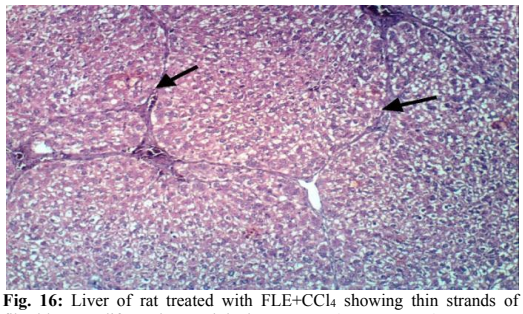

Fig. 16: Liver of rat treated with FLE+CCl_4 showing thin strands of fibroblasts proliferated around the hepatocytes (H & E X 100).

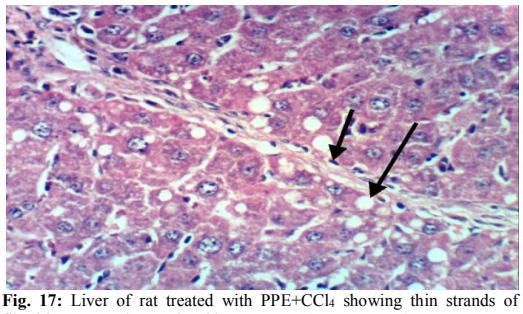

Fig. 17: Liver of rat treated with PPE+CCl_4 showing thin strands of fibroblasts around vacuolated hepatocytes. (H & E X 400).

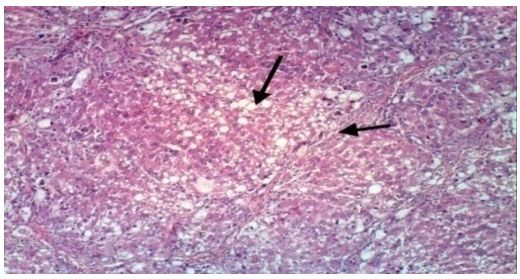

Fig. 18: Liver of rat treated with Sily+FLE+CCl_4 showing few strands of fibroblasts around lobules of vacuolated hepatocytes. (H & E X 100).

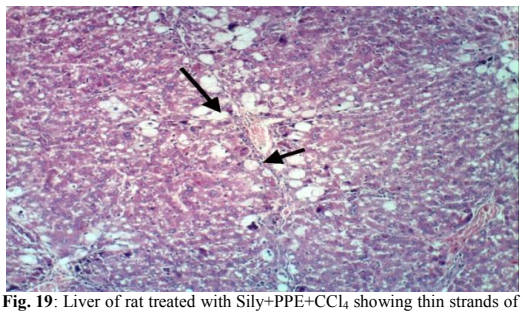

Fig. 19: Liver of rat treated with Sily+PPE+CCl_4 showing thin strands of fibroblasts around lobules of hepatocytes. Notice steatosis of hepatocytes (H & E X 100).

- Sily: Silymarin FLE: Ficus PPE Pomegranate.

Discussion

Liver is a major site for metabolism of exogenous chemicals (pesticides, drugs, metals), resulting in the formation of metabolites which may be more or less toxic than the parent compound (Aghel *et al*. 2011). Induction of liver injury by CCl_4 is the best characterized experimental model of xenobiotic induced hepatotoxicity and is commonly used for screening of hepatoprotective agents. The mechanism of CCl_4 induced hepatic injury involves the biotransformation of CCl_4 by cytochrome

P450 to trichloromethyl radicals (CCl:3) which generates highly reactive free radicals such as trichloromethylperoxy (CCl3OO.). These free radicals react readily with polyunsaturated fatty acids leading to free radical chain reaction as well as membrane lipid peroxidation that disrupts the membrane integrity (Manibusan *et al*., 2007). Disruption of cell membrane integrity leads to cell injury,cellular necrosis and chronic liver injury with the result of liver fibrosis (Wasser and Tan 1999).

Aminotransferases are normally cytoplasmic but released into the circulation after cellular damage (Amacher 1998). The leakage of large quantities of enzymes into the blood stream is associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. The increase in the transaminases and ALP is a clear indication of cellular leakage and loss of functional integrity of the membrane resulting from liver damage (Saraswat *et al*., 1993). As previous results revealed that during 2 weeks of CCl_4 intoxication, serum ALT and AST exhibited pronounced increases reaching more than100 folds (Hou *et al*., 2014). Nevertheless, these increases decline to reach 3 times during two or three months (Muriel *et al*., 2005, Hou *et al*., 2014) and continued to be similar or lower to those of the control after 4 months (Muriel *et al*., 2005).

Disruption of the hepatic structural integrity by subchronic CCl_4 administration, in the present study, is reflected by slight increases in the serum activities of ALT and ALP indicating that, livers of these rats treated with subchronic CCl_4 administration were so damaged (Muriel *et al*., 2005). The results of histopathological examination provided further endorsement for the serum markers of hepatotoxicity (ALT and ALP), CCl_4 toxicity involved remarkable distortions of liver architecture. Livers of CCl_4 animals showed the presence of multiple fibrotic nodules and extensive fibrosis predominantly in the periportal areas, prominent periportal inflammatory kupffer cells infiltration, congestion of blood vessels and considerable degree of diffused apoptosis throughout hepatic parenchyma. It is well established that continuous exposure to CCl_4 leads to the development of severe hepatic fibrosis (Wasser and Tan 1999).

In the present study, CCl_4 significantly increased MDA and NO metabolite contents. It is well known that, increased NO production attributes to NF-κB-induced iNOS expression following CCl_4 challenge (Chamulitrat *et al*., 1995). The produced NO reacts with superoxide anion O2- to produce peroxynitrite (ONOO-) (Hon *et al*., 2002). Peroxynitrite increases lipid peroxidation leading to oxidative stress, hampering mitochondria and realase of Cytochrome c from the mitochondria and consequently apoptosis **(**Crompton 2000).

The found elevation in the level of end products of lipid peroxidation (MDA) in livers of rats treated with CCl_4, an observation similar to earlier reports, is attributed to enhanced lipid peroxidation (Drewa *et al.,* 2002), leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Shenoy *et al*., 2001; Wang *et al*., 2004).

In order to ascertain whether FLE and PPE as a single or combined pre-treatment, would reduce and ameliorate liver damage, rats were pre-treated with 100 mg/kg Sily (standard reference drug), 500 mg /kg b. wt. FLE and 500 mg/kg PPE for 2 weeks before challenging them with 20% CCl_4 (2ml/kg.b.wt./twice a week/ 8 weeks) and continued for another 8 weeks.

The primary mechanism of hepatotoxicity of CCl_4 and its reactive metabolites involves the generation of propagating chain of reactive oxygen species and disruption of the antioxidant defense system leading to oxidative stress which plays a cardinal role in the pathogenesis of liver injury (Romero *et al.*, 1998). Inhibition of hepatotoxin binding to receptor sites on the hepatocyte membrane; increase in the level of reduced glutathione in the liver, stimulatory effect on ribosomal RNA polymerase and finally protein synthesis leading to increased hepatocyte regeneration, are considered as Sily hepatoprotective mechanisms of action (Dixit *et al*., 2007). In addition, previous studies showed that Sily has been to exert (1) antioxidant activity [Sily is an ROS scavenger and also reduces the loss of endogenous antioxidant enzymes such as glutathione reductase, peroxidase, catalase and superoxide dismutase (Kwon *et al*., 2013)]. Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes.; (2) anti-inflammatory activity; Sily interferes with the NF-κB-induced inflammatory cascade release by Kupffer cells (Dehmlow et al., 1996); and (3) antifibrotic activity; Sily has been shown to reduce liver collagen deposition in vivo in different models of chronic toxic liver damage (Lieber *et al*., 2003, Kim *et al*., 2012, Li *et al*., 2012). In general, Sily has antioxidant, anti-fibrotic, anti-proliferative, immunomodulatory, and antiviral properties (Vargas *et al*., 2014). The protective effect of Sily could be attributed to the antioxidant

agent and membrane-stabilizing actions (Mourelle *et al*., 1989), it protect harmful increase in the membrane ratios of cholesterol: phospholipids and sphingoimyline: phosphatidylcholine, thus providing protection from CCl_4 induced chronic liver damage (Muriel *et al*., 2005).

The current study demonstrated that Sily treatment for 10 weeks could restore each of serum ALT, ALP, triglycerides and cholesterol to their basal values and increased hepatic GSH content. Moreover, histopathological examination of silymarin rats' livers showed thin strands of fibroblasts and steatosis of hepatocytes.

Previous reports revealed that the effect of Sily to prevent CCl_4 induced lipid peroxidation and hepatotoxicity (Muriel and Mourelle, 1990) is attributed to its ability to normalize the levels of the transaminases that are elevated in hepatotoxicity (Sharma *et al*., 1991). Nevertheless, the present study revealed that single Sily pre-treatment failed to induce any alteration on CCl_4 induced increase in hepatic MDA and total NO metabolite contents. In addition, histopathological examination of Sily rats' livers provided further evidence for disruption of lipid metabolism showing steatosis of hepatocytes.

As previous results revealed that during 2 weeks of CCl_4 intoxication, serum ALT and AST exhibited pronounced increases reaching more than100 folds (Hou *et al*., 2014). Nevertheless, these increases decline to reach 3 times during two or three months (Muriel *et al*., 2005, Hou *et al*., 2014) and continued to be similar or lower to those of the control after 4 months (Muriel *et al*., 2005). Surprisingly, sera of rats pretreated with the combined FLE, single and combined PPE treatments exhibited elevation in ALT and ALP activities. One can reasonably infer from these results that these extracts pretreatment regimens delayed the onset of hepatic fibrosis and the development of CCl_4 hepatotoxicity. It worth noting that deferoxamine delays the development of the hepatotoxicity of acetaminophen (Schnellmann *et al*., 1999).

Hepatic injury has been shown to be associated with increased level of nitric oxide (NO); a potent inflammatory mediator although its role might be protective in certain conditions (Li and Billiar, 1999). NO is involved in pathogenesis of acute hepatotoxicity mainly through depletion of hepatic GSH and inactivation of antioxidant enzymes as well as generation of the highly toxic derivative, peroxy nitrite, through reaction with superoxide (Clancy and Abramson, 1995). Moreover, NO is an important vasodilator in the vascular system.

The current study demonstrated that, while Sily (the reference drug) treatment had no influence on CCl_4 induced elevation in the hepatic NO metabolites production, single FLE, PPE and both combined treatments effectively minimized its level to the basal values. It is possible that trichloromethyl radical or lipid peroxides generated by CCl_4 treatment may be scavenged by the utilizing extracts resulting in depression of lipid peroxidation in the liver. The antioxidant and free radical scavenging activity of PPE and FLE could be due to their flavonoids and phenolic compounds constituents. It has been suggested that the protective effect of plant extracts against CCl_4 induced liver damage may be attributed to the presence of flavonoids, tannins, triterpenoids and alkaloids (Gupta et al., 2004). Flavonoids are known to be antioxidants, free radical scavengers and anti-lipoperoxidants which cause hepato-protection (Mankani *et al*., 2005).The present phytochemical screening showed PPE contain flavonoids (+), polyphenols and /or tannins (++), sterols and/or triterpenes (++) and alkolids (+), while FLE extract contain flavonoids (++) and polyphenols and /or tannins (+) and sterols and/or triterpenes (+). The observed decrement in the hepatic MDA content (TBARS) level, as a marker of lipid peroxidation, of single PPE and combined administered groups indicate the ability of single PPE and combined treatments to alleviate oxidative stress. The results of PPE reduced hepatic MDA is consistent with Chidambara *et al.*, (2002).

Mammalian cells have a complex network of antioxidant enzymes, including glutathione peroxidases, superoxide dismutase and catalase, and non-enzymatic antioxidants, such as GSH, vitamin C, vitamin E and β-carotene, to scavenge ROS (Bose *et al*., 2012). GSH acts to protect normal cell structure and function by maintaining the redox homeostasis, quenching free radicals and participating in detoxification reactions. As well as functioning as a direct scavenger of free radicals, GSH is a co-substrate for peroxide detoxification by glutathione peroxidases (Fawole *et al*., 2012).

In the present study, The observed elevation of hepatic GSH content among single PPE, combined PPE and combined FLE treated rats, may explain their partial protective effects in alleviating liver fibrosis and could be related to their flavonoids and polyphenolic contents (Chidambara *et al*., 2002, Xu et al., 2009, Wang *et al*., 2015). The histolpathological examination of

single FLE and PPE pre-treatment groups showed significant reductions in liver tissue damage and decreased punctate and focal necrosis as thin strands of fibroblasts proliferated around the hepatocytes and steatosis of hepatocytes. These results indicated that hepatic damage was reduced in extent compared to CCl_4. In addition, the improved signs of alleviated hepatocellular injuries, reduced fibrosis and regression of the hepatic damage indicate and provide further evidence for the protective effect of the single FLE and PPE pre-treatment.

The present study showed that both FLE and PPE had protective effects against CCl_4 toxicity in hepatic tissues. Moreover, PPE was found to be more effective than FLE in the CCl_4 induced changes in GSH level and MDA content. Previous studies showed the protective effect of FLE against CCl_4 induced liver injury (Singab *et al*., 2010), which was comparable to that of Sily, a known hepatoprotective (Mohan *et al*., 2007, Aghel *et al*., 2011, Mujeeb *et al*., 2011). The discrepancy between the current and previous study may refer to duration of the treatment where all previous studies were for short term.

Because synergism was not observed in the overall spectrum of biochemical and histological changes in the liver, we thought that Sily and its combination with each of FLE and PPE may not have synergistic effects against CCl_4 induced oxidative damage in hepatic tissue. Combined treatments did not have stronger effects than their separate effect against CCl_4 induced liver damage. Our result indicated that combined pretreated animals showed moderate fibrosis, steatosis and apoptosis of hepatocytes. It seems that it was related to the antioxidant dose, because high doses of some antioxidants may be do not have a protective effect, and can exacerbate tissue damage (Azarkish *et al*., 2013).

Acknowledgments

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