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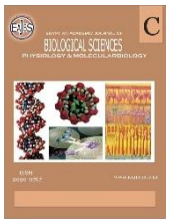

Role of Some Food by Product Extracts Compared to Silymarin as Protective Agents against Acute Liver Injury in Paracetamol Overdose-Exposed Rats

Wafaa A. Gaballah, Asmaa A. Faramawy and Samah A. El-Hashash*
Nutrition and Food Science Department, Faculty of Home Economics, Al-Azhar University, Nawag, Tanta City, P.O. Box 31732, Egypt.
***E-mail:** Wafaaahm709@gmail.com ; drasmaafaramawy555@gmail.com ; samahel-hashash@azhar.edu.eg

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ABSTRACT

In paracetamol (PCM) overdose-exposed rats, the hepato-protective properties of ethanolic extracts from grape seeds and peanut skins (GSEE and PSEE, respectively) were compared to silymarin in this study. The total amount of phenolic and flavonoids as well as the ability to scavenge radical DPPH of GSEE and PSEE were measured. Twenty adult male albino rats were split evenly into five groups for the in vivo evaluation; including the control group (GI), while PCM treated group (GII) got paracetamol once orally for 14 days at a dosage of 640 mg/kg/day, with no protective treatment. Silymarin (50 mg /kg /day), and 200 mg /kg /day of either GSEE or PSEE were given concurrently with PCM to GIII, GIV and GV, respectively. PCM overdose led to a significant elevation in the activities of transaminases and alkaline phosphatase (84.00±10.64, 396.00±49.44, and 579.67±72.00 U/L *versus* 44.00±5.72, 178.00±22.09, and 322.0±40.58 U/L in control group), along with an obvious decline in serum proteins. In the liver, the lipid profile was disrupted associated with a marked elevation in oxidative and inflammation markers, which was further confirmed by histological examination. These disorders were alleviated by the administration of food waste extracts. Even while PSEE showed a little increase in total phenolic, flavonoids and DPPH radical scavenging ability (604.45, 542.05, and 618.59 *versus* 583.70 mg gallic acid equivalent, 503.80 mg catechin equivalent, and 550.44 mg Trolox equivalent, respectively/g GSEE), GSEE was the nearest treatment when compared to silymarin. This study suggests GSEE and PSEE as alternative therapies for paracetamol-induced hepatotoxicity.

INTRODUCTION

Around the world, acute liver failure (ALF) is a serious illness with a high morbidity and short-term death rate (Anand *et al*., 2020). ALF refers to a clinical manifestation of encephalopathy that occurs a few weeks following the development of a coagulopathy-related liver disease. It may be linked to rapidly progressive multiple-organ failures, which can have disastrous consequences. The best course of treatment for ALF at the moment is liver transplantation because the prognosis is currently quite bad (Manka *et al*., 2016; Goldberg *et al*., 2023).

The underlying etiology of ALF differs depending on the area and stage of development. In developed countries, the large majority of cases were found to arise secondary to drug-induced liver injury, frequently from paracetamol (PCM), while across the developing countries, viral hepatitis was recognized as the main leading cause (Manka *et al*., 2016; Devarbhavi *et al*., 2021; Goldberg *et al*., 2023).

However, PCM–induced ALF has elevated in the developing world over the past few years. In Egypt, hospital-based prospective studies, from 2015 via the COVID-19 pandemic and till now, reported high percentages of PCM-intoxicated cases (Hegazy and Elfiky, 2016; Abdelhamid, 2021; Mostafa *et al*., 2022).

Paracetamol/acetaminophen, N-acetyl-p-aminophenol, is among the most popular and efficient analgesics and antipyretics in the world. It is available as over-the-counter (OTC) and prescription formulations, as well as single- or multi-component preparations (Saccomano, 2019). Notwithstanding the many therapeutic advantages, the unmonitored use of PCM is a concerning indication that has to be addressed in order to prevent the high incidence of unintentional toxicity. Hepatocytes are said to be protected against PCM toxicity by inhibiting oxidative stress and reducing the inflammatory response (El-Boshy *et al*., 2019; Wan *et al*., 2020).

Over the last few decades, there has been considerable emphasis on finding new therapeutic strategies, particularly of natural origin, for modulating oxidative stress and inflammation-induced health disturbances (Cabello-Verrugio *et al*., 2017). This is because allopathic medicines, rather than their high cost, usually exert side effects and affect the quality of life. Hence, extracts of herbs and food byproducts as well as their bioactive compounds encompassing hepato-protective potential may serve as suitable and safer alternatives (Izzo *et al*., 2016).

Silymarin, also known as *Silybum marianum* (Silybi mariani fructus extractum), is obtained from the dried seeds and fruits of the milk thistle plant. Because of its chemical components (polyphenols, flavonolignans, and flavonoids), it has been used extensively as a biological and health-promoting agent. Its antioxidant, anti-fibrotic, and anti-inflammatory properties have been documented (Gillessen and Schmidt, 2020; Hashem *et al*., 2021). It was also reviewed for its preventive capacity for chronic liver diseases (Aghemo *et al*., 2022).

Grape (*Vitis vinifera* L.) seeds are one of the most important food industry byproducts. They have a high medicinal value and are nutrient-rich. It has been reported that 15 phenolic compounds, including (+)-catechin, (−)-epicatechin, proanthocyanidin, gallate, flavonols, and others, are primarily present in grape seed extract (GSE). Numerous researches on GSE and its constituent parts have revealed that it possesses pharmacological properties like neuro-protection, lowering cholesterol, bacteriostatic, anti-inflammatory, anticancer, and reduced blood pressure (Rodríguez-Pérez *et al*., 2019; Chen *et al*., 2020). Moreover, its hepato-protective effects were cited in many animal studies (Osuntokun *et al*., 2020; Abu Hafsa and Hassan, 2022).

It is well known that skins, a low-value byproduct of the peanut (*Arachis hypogea* L.) industries, have high polyphenol content (Makau *et al*., 2018). The color of peanut skin varies from light brown to deep crimson. The redness and hue angle of the peanut skin not only demonstrated strong correlations with total polyphenol content but also with antioxidant capacity, suggesting a strong correlation between total polyphenols and antioxidant capacity (Chukwumah *et al*., 2009).

In general, there is a bad and continuous need for natural dietary interventions which have the ability to face the mechanisms of toxic actions of PCM. At the same time, little data is available on the efficiency of food byproduct extracts against paracetamol overdose–induced hepatotoxicity. Hence, in rats given an overdose of paracetamol, the hepato-protective effects of ethanolic extracts from grape seeds and peanut skins were compared to silymarin in this study.

MATERIALS AND METHODS

1. Plant Materials and Preparation:

Grape (*Vitis vinifera* L., variety Roumy Ahmer) fruits and peanut *(Arachis*

hypogea L.) pods were purchased from the local market, Tanta City, Gharbia Governorate, Egypt. After authentication by a senior botanist at the Agriculture Faculty, Kafrelsheikh University, grape fruits were cleaned and free from evidence of insect infestation and objectionable materials. Seeds were separated from the pulp and dried in the shade. Regarding peanut pods, they were mildly roasted and manually shelled, and then skins were removed. After that, both materials were ground using a hammer mill (Thomas Willey mills, model Ed-5, Germany), and kept frozen at –20 ˚C till extraction. To prepare ethanolic extracts, 2 kg of each powder were blended with 4 L ethanol (95%) at ambient temperature. This was done for three days with the aid of an overhead stirrer (Betatek Inc. for Pharmaceutical Manufacturing, Toronto, Ontario) in total darkness. The blends were filtered and placed in a rotary evaporator (WB 2001; Heidolph, Schwabach, Germany) to evaporate the solvent, at 40°±5°C.

2. Drugs, Chemicals and Reagents:

Paracetamol (PCM), tween 80, and saline (0.9%) were purchased from PHARMATRADE Company for trading medical and pharmaceutical products, Mansoura, Dakahlia Governorate, Egypt. Silymarin was obtained from SEDICO Pharmaceutical Company for trading pharmaceutical & biotechnology products in the form of sachets; each contains 140 mg. Al-Gomhoria Company for trading drugs, chemicals and medical instruments, Cairo, Egypt, supplied the chemicals; formalin, ethanol (95%), and others. Sigma (St. Louis, MO, USA) also provided the diagnostic kits.

3. Phytochemical and Free Radicals Scavenging Activity:

With some modifications, the Folin-Ciocalteu procedure (Hagerman *et al*., 2000) was used to determine the total phenolic content. 250 µL of Folin-Ciocalteau reagent was added to the test tube containing the extract (100 µL), and the volume was adjusted to 3.5 mL using distilled water. After five minutes, 1.25 mL of a 20% aqueous sodium carbonate solution was added to the mixture to neutralize it. The absorbance was measured at 725 nm in relation to the solvent blank after 40 minutes. Using a calibration curve made with gallic acid, the total phenolic content was calculated and expressed as milligrams of gallic acid equivalent (mg GAE) per gram of sample.

The aluminum chloride ($AlCl_3$) colorimetric assay was used to determine the total flavonoid content in accordance with Zhishen *et al*. (1999). 100 µL of extract was combined with 300 µL of 5% sodium nitrite ($NaNO_2$). After 6 minutes, 300 µL of a 10% $AlCl_3$ solution was added, and distilled water was used to adjust the volume to 2.5 mL. A centrifuge at 5000 g for 10 minutes was used after adding 1.5 mL of 1 M NaOH after 7 minutes. At 510 nm, the supernatant's absorbance was measured in relation to the solvent blank. Using a calibration curve made with catechin, the total flavonoid content was calculated and expressed as milligrams of catechin equivalent (mg CE) per gram of sample. If the measured absorbance value fell outside of the standard curve's linear range, more dilution was applied.

The stable DPPH* was used to measure the extracts' ability to scavenge free radicals (Hwang and Thi, 2014). DPPH* was finally concentrated to 200 µM, and the reaction volume was 3.0 mL. After 60 minutes of dark incubation, the absorbance was measured at 517 nm against a blank of pure methanol. The following formula was used to determine the percentage inhibition of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical:

100 × [(A control – A sample)/ A control] = the inhibition (%)

Where A control is the absorbance of the reaction under control, which is made up of all the reagents save the test compound, the absorbance with the test compound serves as A sample. Trolox was used to create the standard curve. Trolox equivalent (TE) per milligram (mg) of sample was used to express the results. If the measured DPPH value was higher than the standard's linear range, more dilution was required.

4. Animals and Ethics Permission:

Twenty adult male Sprague-Dawley albinos, weighing between 120 and 140 g, were acquired from the Helwan Farm, VI Organization, in Cairo, Egypt. They were housed in polypropylene cages with a 12-hour light/dark cycle, relative humidity of 50 ±20%, and room temperature of 22±1 ºC. There was an endless supply of water. Before starting the experiment, they had a week to get used to the lab environment. Generally speaking, the Animal Ethics Committee (KFS-IACUC/133/2023) of Kafrelsheikh University's Faculty of Veterinary Medicine reviewed and approved all animal procedures based on the National Committee for Research's code of practice for the care and use of animals for scientific purposes.

5. Experimental Diet:

The Agricultural Development Company, 6-October City, Giza Governorate, Egypt, was the supplier of the balanced diet pellets used for animal feeding purposes. Sunflower oil (15%), yellow corn (49%), soybean meal (44%), wheat bran (10%), molasses (3%), common salt (0.5%), ground limestone (0.2%), dicalcium phosphate (0.1%), lysine (0.2%), dl-methionine (0.7%), and mineral-vitamin premix were already included in the diet (Atta *et al*., 2020; El-Hashash *et al*., 2023).

6. Study Design:

Rats were weighed at the conclusion of the adaptation period and then randomly assigned to five groups consisting of four rats each. The first group was given a normal diet along with 0.05% tween 80 dissolved in 0.9% NaCl solution (0.5 mL/rat). Every day, paracetamol (PCM, 640 mg/kg body weight p.o.) was given to group II (positive control) to induce toxicity according to Islam *et al*. (2021). Groups III, IV and V received silymarin (50 mg/kg p.o.), 200 mg/kg of grape seed ethanolic extract (GSEE), and 200 mg/kg of peanut skin ethanolic extract (PSEE), respectively, along with 640 mg PCM/kg body weight p.o. daily. Freshly made distilled water was used to dissolve PCM, GSEE, and PSEE, and 0.5% sodium carboxymethyl cellulose (CMC-Na) distilled water solution was used to suspend silymarin. Effective nontoxic doses of GSEE, PSEE and silymarin were selected according to previous studies on animals (Giribabu *et al*., 2018; Fuadiyah and Kurniawan, 2021; Islam *et al*., 2021). The feeding trial lasted for 14 days. In the meantime, animals had unlimited access to food and water, and their body weight was measured once a week. By altering each rat's body weight, dosages of PCM, silymarin and plant extracts were also changed.

At the end, the animals were weighed, and allowed to fast for the entire night, and then blood samples were taken from the rats' retro-orbital venous plexus and put into sterile dry centrifuge tubes. Serum samples were separated carefully at room temperature using centrifugation (3000 rpm) for 10 min. After that, they were put into Eppendorf tubes and frozen at -20°C until testing in the lab.

Upon blood collection, rats were anesthetized by intraperitoneal injection with 70 mg/kg pentobarbital sodium, then painlessly sacrificed and livers were carefully removed, dried with filter paper, weighed, and rinsed in 0.9 g/100 mL of ice-cold saline. Following that, a specimen from each liver was stored at -80°C for homogenation *as a prelude* to molecular and other biochemical investigations. For histopathological analysis, the other specimen was submerged in a 10% buffered neutral formalin solution (Abou El-Naga *et al*., 2022).

7. Preparation of Liver Tissue Homogenates:

Liver tissue was homogenized with a Potter-Elvenhjem tissue homogenizer (20-30 up and down strokes), then divided into 3 parts. The 1st part was used to estimate lipids based on the procedure outlined by Folch *et al*. (1957). The tissue specimen (0.2 g) was blended with 1.8 mL of two mixed solvents 2:1 (v/v) of chloroform-methanol. The resulting homogenate was then put directly into 2 mL Eppendorf tubes. After standing at -20 °C for 10 minutes, the homogenate was centrifuged for 10 minutes at 2400 rpm. The solution has two phases, an upper aqueous layer (25%) of methanol (polar part) and a

lower layer (75%) of chloroform (nonpolar part). The chloroform layer was aspirated into a clean tube, and it was then thoroughly rinsed with 0.2 mL of 0.05 M potassium chloride before being centrifuged for 10 min at 2400 rpm. The two layers were separated into the clean Eppendorf tubes and then were kept frozen (- 20 °C) until needed for further studies.

For studies on oxidative stress and lipid peroxidation, exactly 0.2 *g* of liver tissue (the 2nd part) were individually homogenized in 1.8 mL homogenizing cold (0°C) buffer (pH 7.2) using a Teflon pestle homogenizer. After being placed into a 2 mL Eppendorf microtube, the entire contents were centrifuged for 10 minutes at 3000 rpm. Until needed for further use, the supernatant was kept at - 20 °C.

A manually sterilized mortar was used to homogenize 25–30 mg of liver tissue (the third part) for molecular analysis. Next, 2-mercaptoethanol and 0.6 ml of lysis buffer were added. After vortexing the mixture for at least 40 seconds, each volume of cell homogenate received one volume of 70% ethanol, and the mixture was again vortexed to thoroughly mix and discard any visible precipitates. As instructed by the manufacturer, the process was finished.

8. Biochemical Analysis:

8.1. Liver Enzymes and Serum Proteins:

The activities of transaminases (ALT and AST) and alkaline phosphatase (ALP) were measured in serum utilizing the techniques of Reitman and Frankel (1957) and Kind and King (1954), correspondingly. Total protein (TP) and albumin were additionally ascertained in serum according to referenced methods (Gornall *et al*., 1949; Doumas *et al*., 1997).

8.2. Lipid Profile in Liver Tissue Homogenates:

The levels of triglycerides (TG) and cholesterol (Cho.) were determined in liver tissue homogenates using the method of Folch *et al*. (1957). For TG quantification, using an Ecotherm (heating/cooling dry bath), 50 microliters of the lipid extract were transferred into an Eppendorf tube and evaporated to dryness at 60 °C. Next, 200 µL of ethyl alcohol was added and vortexed. Following that, 1 mL of the reconstituted triacylglycerol reagent was added to the tubes, which then swirled, and incubated for 5 minutes at 37 °C. The absorbance at 546 nm was measured 60 minutes later in comparison to the reagent blank.

As for cholesterol (Cho.), using an Ecotherm (heating/cooling dry bath), 50 microliters of the lipid extract were transferred into an Eppendorf tube and evaporated to dryness at 60 °C. Then, vortexing of 20 µL of a 1:1 v/v chloroform and triton-X100 mixture was applied. After that, the tubes were filled with 1 mL of cholesterol reagent (from Randox assay kit), stirred, and kept at 37 °C for five minutes. The absorbance at 546 nm was measured 60 minutes later in comparison to the reagent blank.

8.3. Oxidative and Anti-Oxidative Markers in Liver Tissue Homogenates:

Ohkawa's method (Ohkawa *et al*., 1979) was used to determine malondialdehyde (MDA), the lipid peroxidation biomarker, in liver tissue homogenate. Additionally, using established procedures, the levels of reduced glutathione (GSH) and superoxide dismutase (SOD) activity were determined according to Ellman (1959) and Misra and Fridovich (1972), respectively.

9. Quantitative Expression of Liver Genes:

Using a Bio-Rad spectrophotometer, ribonucleic acid (RNA) from liver tissues was isolated and its purity was assessed at an OD of 260/280 nm. A Quanti-Tect kit was used to reverse transcribe 1 µg of RNA. Real-time polymerase chain reaction (RT-PCR) was used to create the newly synthesized single-strand complementary DNA (cDNA) in two steps. The Bio-Rad thermal cycler was utilized to amplify and quantify the cDNA using the SYBR Green Master Mix kit. Table 1 lists the primer sequences for oxidative stress, inflammation, and apoptosis. The $2^{-\Delta\Delta CT}$ method was used to quantify the genes under examination, which included tumor necrosis factor-alpha (TNF-α),

interleukin 10 (IL-10), and heme oxygenase-1 (HO-1). To standardize the investigated genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal standard) was utilized as a housekeeping gene (Somade *et al*., 2020).

Table 1. The primer sequences utilized for the qRT-PCR analysis of rats' livers.

Gene	Primer	Sequences (5'→3')	Accession No
TNF-α	Forward	5′- AAA TGG GCT CCC TCT CAT CAG TTC -3′	(NM_012675.3)
	Reverse	5′- TCT GCT TGG TGG TTT GCT ACG AC-3′	
IL-10	Forward	5′- GCTCAGCACTGCTATGTTGC-3′	NM_012854
	Reverse	5′- TTGTCACCCCGGATGGAATG-3′	
HO-1	Forward	5′-GAG ACG GCT TCA AGC TGG TGA TG-3′	(NC_005118.4)
	Reverse	5′-GTT GAG CAG GAA CGC AGT CTT GG-3′	
GAPD	Forward	5′-ATG GGA GTT GCT GTT GAA GTC A-3′	(NM_017008.4)
	Reverse	5′-CCG AGG GCC CAC TAA AGG-3′	

10. Liver Histopathology:

After being submerged for 24 hours in 10% phosphate-buffered neutral formalin, liver specimens were cleaned, dehydrated in escalating serial ethanol, and cleared with xylene. Subsequently, the slices underwent paraffin processing and were removed at a thickness of 5 _m. Hematoxylin and eosin (H&E) were used in standard histology procedures to stain the sections (Bancroft and Layton, 2013).

11. Statistics:

The mean of at least three independent values was used to express the chemical results. Gene expression results were presented as mean ± standard error of the mean (SEM), whereas biochemical results were expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's and Duncan's post hoc descriptive tests (SPSS version 22 for Windows; SPSS, IBM, Chicago, IL, USA). At least three separate experiments' worth of data was represented in the data. For gene expression results, significance was presented as ***, **, *****, and ******** ($P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively), while for other results, it was set at $P \leq 0.05$.

RESULTS

1. Total Phenolic and Flavonoids as Well as the Ability to Scavenge DPPH Radical:

Results presented in Table 2 indicated that total phenolic and flavonoids as well as the ability of PSEE to scavenge DPPH radical were slightly higher than those of GSEE (604.45 mg GAE, 542.05 mg CE, and 618.59 mg TE/g, respectively *versus* 583.70 mg GAE, 503.80 mg CE, and 550.44 mg TE/g, respectively).

Table 2. Total phenolic and flavonoids as well as the ability of both GSEE and PSEE to scavenge DPPH radical.

Test sample	Total phenolic (mg GAE/g)	Total flavonoids (mg CE/g)	DPPH (mg TE/g)
GSEE	583.70	503.80	550.44
PSEE	604.45	542.05	618.59

DPPH= 2, 2-Diphenyl-1-picrylhydrazyl, GSEE= Grape seed ethanolic extract, PSEE= Peanut skin ethanolic extract, GAE= Gallic acid equivalent, CE= Catechin equivalent, TE = Trolox equivalent.

2. Liver Enzymes:

Rats given PCM had a clear disruption in their liver functions. Table 3 demonstrated a substantial rise ($P \leq 0.05$) in transaminases (ALT and AST) and ALP activities in comparison to the control group. Silymarin and both GSEE and PSEE administration improved these markers significantly ($P \leq 0.05$) in PCM–received rats. Silymarin was so efficient that could

normalize serum activity levels of ALT and ALP. No significant differences were noticed between plant extracts-received groups in all studied liver enzymes, however, and compared to silymarin, GSEE induced the same effects, while PSEE induced the same effects on only AST and ALP activities.

Table 3. Effect of both grape seeds and peanut skins ethanolic extracts *versus* silymarin on the activities of liver enzymes in serum of paracetamol -overdosed rats.

Groups / Parameters	Control	PCM	Silymarin	GSEE	PSEE	*P* value
ALT (U/L)	44.00±5.72 [a]	84.00±10.64 [d]	52.67±6.60 [ab]	62.00±7.65 [bc]	66.33±8.49 [c]	0.000
AST (U/L)	178.00±22.09 [a]	396.00±49.44 [c]	236.67±29.17 [b]	267.00±33.66 [b]	292.00±36.74 [b]	0.000
ALP (U/L)	322.0±40.58 [a]	579.67±72.00 [c]	403.33±50.95 [ab]	432.83±54.70 [b]	474.00±59.24 [b]	0.000

Results are expressed as mean ± SD, n= 4. Values within the same row with completely distinct alphabets are significantly ($P \leq 0.05$) different from each other. The small letter "a" refers to the smallest value. PCM= Paracetamol, GSEE= Grape seed ethanolic extract, PSEE= Peanut skin ethanolic extract, ALT= Alanine aminotransferase, AST= Aspartate aminotransferase, ALP= Alkaline phosphatase.

3. Serum Proteins and Lipid Profile in Liver Tissue Homogenates:

Another consequence of liver function disturbance was noticed. Serum levels of total protein (TP) and albumin (alb.), as illustrated in Table 4, were decreased in PCM-administered rats compared to the control group, however, only albumin decrease was significant ($P \leq 0.05$). Silymarin, GSEE, and PSEE groups exhibited improved levels of TP and albumin compared to the PCM group. Their albumin levels showed no significant differences between them and the control group or among each other. Statistically, silymarin, however, was the best treatment. Table 4 also showed the effect of both grape seeds and peanut skin ethanolic extracts *versus* silymarin on liver tissue homogenates' lipid profile of PCM-exposed rats. Similar to serum proteins, liver levels of TG and cholesterol in PCM-administered rats rose significantly ($P \leq 0.05$) compared to the control group. Concurrent administration of silymarin, GSEE, or PSEE with PCM improved these markers significantly ($P \leq 0.05$). Silymarin was so efficient that could return these lipids to their normal levels recorded by the control group. No significant differences were noticed between the two extracts (GSEE and PSEE) -received groups in both lipids, however, and compared to silymarin, GSEE exerted the same effects, while PSEE had the same effects on only liver cholesterol content.

Table 4. Effect of both grape seeds and peanut skins ethanolic extracts *versus* silymarin on serum proteins and lipid profile in liver tissue homogenates of paracetamol -overdosed rats.

Groups / Parameters	Control	PCM	Silymarin	GSEE	PSEE	*P* value
TP (g/dL)	8.05±1.08	6.63±0.81	7.76±0.98	7.45±0.92	7.35±0.93	0.326
Alb. (g/dL)	4.62±0.57 [b]	3.37±0.42 [a]	4.29±0.54 [b]	4.06±0.51 [ab]	3.98±0.49 [ab]	0.040
Liver TG (mg/g)	3.31±0.41 [a]	6.97±0.87 [d]	3.83±0.48 [ab]	4.49±0.56 [bc]	4.91±0.61 [c]	0.000
Liver Cho. (mg/g)	2.20±0.29 [a]	3.90±0.49 [c]	2.65±0.33 [ab]	2.90±0.35 [b]	3.16±0.40 [b]	0.000

Results are expressed as mean ± SD, n= 4. Values within the same row with completely distinct alphabets are significantly ($P \leq 0.05$) different from each other. The small letter "a" refers to the smallest value. PCM= Paracetamol, GSEE= Grape seed ethanolic extract, PSEE= Peanut skin ethanolic extract, TP= Total protein, Alb.= Albumin, Liver TG= Liver triglycerides, Liver Cho.= Liver cholesterol.

4. Antioxidants (GSH and SOD) *versus* Oxidative Marker (MDA) in Liver Tissue Homogenates:

Liver tissue homogenates of rats from different groups were checked for their oxidants/antioxidants status. In Table 5, it could be noticed that the GSH level and the activity of the SOD enzyme were reduced significantly ($P \leq 0.05$) compared to the control group. Conversely, the lipid peroxidation marker, MDA, was increased significantly ($P \leq 0.05$). GSEE and PSEE administered with PCM side by side supported the antioxidant defense system in liver tissues, evidenced by the significant rise in the studied antioxidants (GSH and SOD), besides the significant reduction in MDA level ($P \leq 0.05$). The by-product extracts were comparable to the silymarin-treated group. No significant differences were found among silymarin, GSEE, and PSEE-treated groups in MDA and GSH levels. In general, silymarin was the most efficient, as it could normalize both MDA and GSH levels and resulted in the closest level of SOD activity to that of the control group.

Table 5. Effect of both grape seeds and peanut skins ethanolic extracts *versus* silymarin on oxidative/antioxidant profile in liver tissue homogenates of paracetamol -overdosed rats.

Groups / Parameters	Control	PCM	Silymarin	GSEE	PSEE	*P* value
MDA (nmol/ g)	8.20±1.03 ^{a}	22.38±2.79 ^{c}	10.67±1.32 ^{ab}	12.28±1.55 ^{b}	13.18±1.66 ^{b}	0.000
GSH (mmol/g)	3.03±0.37 ^{c}	1.25±0.16 ^{a}	2.69±0.35 ^{bc}	2.44±0.31 ^{b}	2.28±0.28 ^{b}	0.000
SOD (U/g)	238.27±29.85 ^{d}	81.80±10.23^{a}	200.9±25.12^{c}	165.03±20.09^{b}	144.33±18.03^{b}	0.000

Results are expressed as mean ± SD, n= 4. Values within the same row with completely distinct alphabets are significantly ($P \leq 0.05$) different from each other. The small letter "a" refers to the smallest value. PCM= Paracetamol, GSEE= Grape seed ethanolic extract, PSEE= Peanut skin ethanolic extract, MDA= Malondialdehyde, GSH= Reduced glutathione, SOD= Superoxide dismutase.

5. Hepatic Gene Expression:

According to the present findings, the PCM-treated rats' liver TNF-α and IL-10 mRNA expressions were significantly higher than those of the control group ($P \leq 0.0001$ and $P \leq 0.001$, respectively). In contrast, silymarin, GSEE and PSEE-treated groups showed significant downregulation compared to the PCM-treated one ($P \leq 0.0001$ for TNF-α, and $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.01$, respectively for IL-10). As for the mRNA expression of the hepatic HO-1, PCM-treated rats showed non-significant upregulation compared to the control group ($P > 0.05$). Silymarin, GSEE and PSEE administration along with PCM led to significant upregulation compared to both PCM ($P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.05$, respectively) and control ($P \leq 0.001$) groups (Fig. 1).

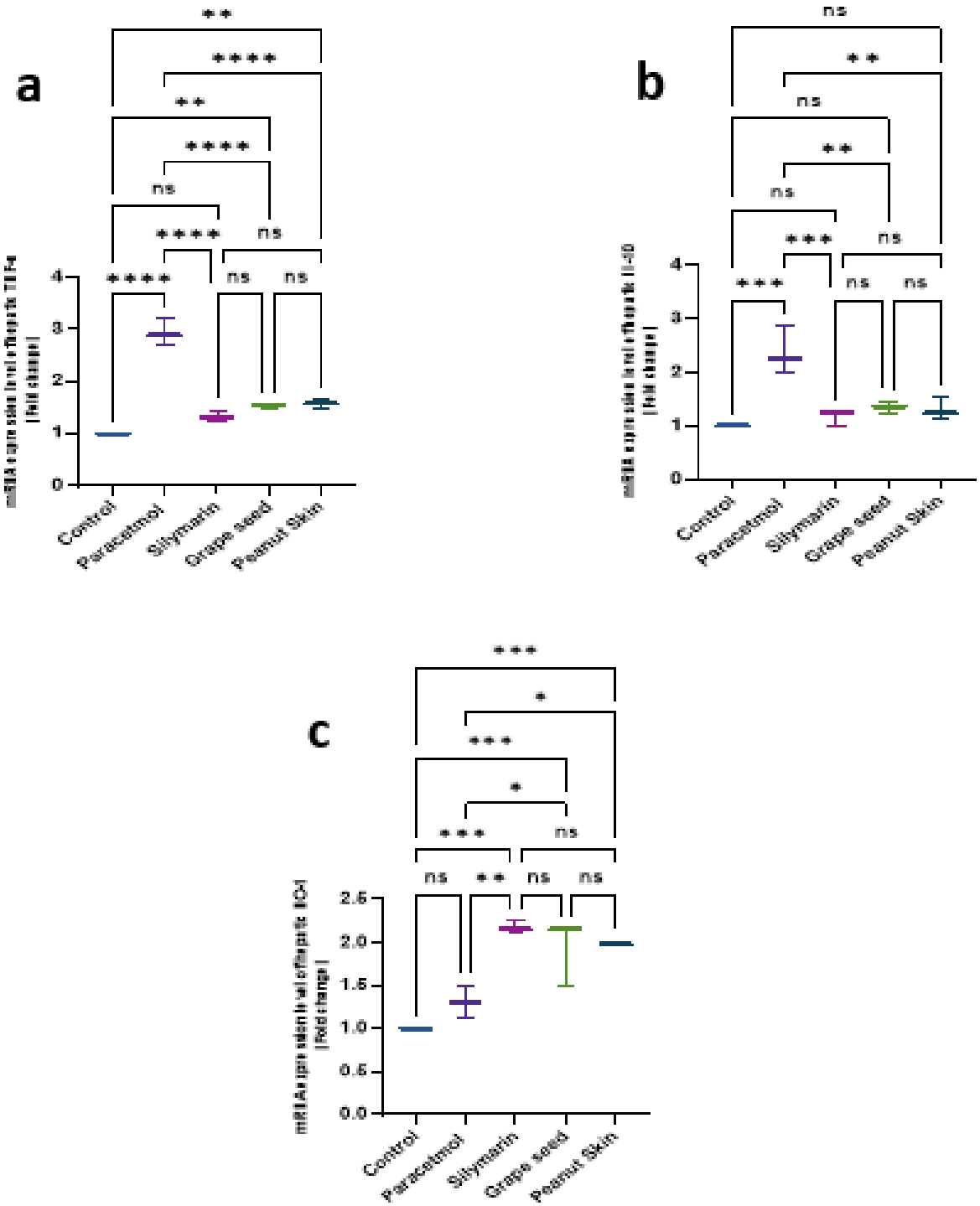

Fig. 1: Effect of both grape seeds and peanut skins ethanolic extracts *versus* silymarin on the levels of mRNA expression of hepatic TNF-α (a), IL-10 (b), and HO-1 (c) in paracetamol - overdosed rats. Results are expressed as mean ± SEM (n= 4), ns= not significant, *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, and ****= $P \leq 0.0001$.

6. Liver Histopathology:

Microscopic pictures (Bar= 50 µm) of liver sections stained with eosin and haematoxylin in rats from the control group focused on the centrilobular area (Fig. 2 a) where the normal architecture of hepatic lobules with hepatocytes having a polyhedral shape, grouped in a cord-like pattern and divided by blood sinusoids in addition to central vein was noticed. Moreover, the portal area of the liver of control rats (Fig. 2 b) shows hepatic artery and portal vein with

healthy looks. PCM overdose led to obvious abnormal changes in both the centrolobular and the portal areas of rat liver (Fig. 2 c, d, and Table 6). Both of them showed loss of hepatic architecture with severely shrank hepatocytes (+++), severely dilated (+++) and mildly congested (+) blood sinusoids, in addition to elevation of interlobular fibrosis, scored as severe (+++). PCM-overdosed rats that received silymarin concurrently showed a marked improvement in both centrolobular and portal areas of the liver. In the centrolobular area, normal hepatic architecture with mild congested central vein (+), mild vacuolar degeneration of hepatocytes in the perivascular area (+), mild dilated and congested blood sinusoids (+) were observed (Fig. 3 a, and Table 6). The portal area of the same group also showed normal hepatic architecture with mild congested portal blood vessels (+), and mild shrinkage of some hepatocytes with mild dilated and congested blood sinusoids (+) (Fig. 3 b, and Table 6). Like silymarin, the used plant extracts (GSEE and PSEE) showed cytoprotective effects in PCM-overdosed rats. In the centrilobular area of the liver of GSEE group (Fig. 3 c, and Table 6), normal hepatic architecture with the mild congested central vein (+), mild vacuolar degeneration of hepatocytes (+) and mild dilated blood sinusoids (+) was noticed, while the portal area showed normal hepatic architecture with mild congested portal blood vessels (+), mild vacuolar degeneration of hepatocytes (+), mild dilated blood sinusoids (+), in addition to mild fibrosis (+) (Fig. 3 d, and Table 6). The hepatic parenchyma of PSEE group (Fig. 3 e, and Table 6) showed normal hepatic architecture with mild congested hepatic vessels (+), moderate vacuolar degeneration of hepatocytes (++), and mild dilated blood sinusoids (+), while the portal area showed normal hepatic architecture with moderate vacuolar degeneration of hepatocytes (++), mild shrank hepatocytes (+), as well as mild dilated and congested blood sinusoids (+) (Fig. 3 f, and Table 6).

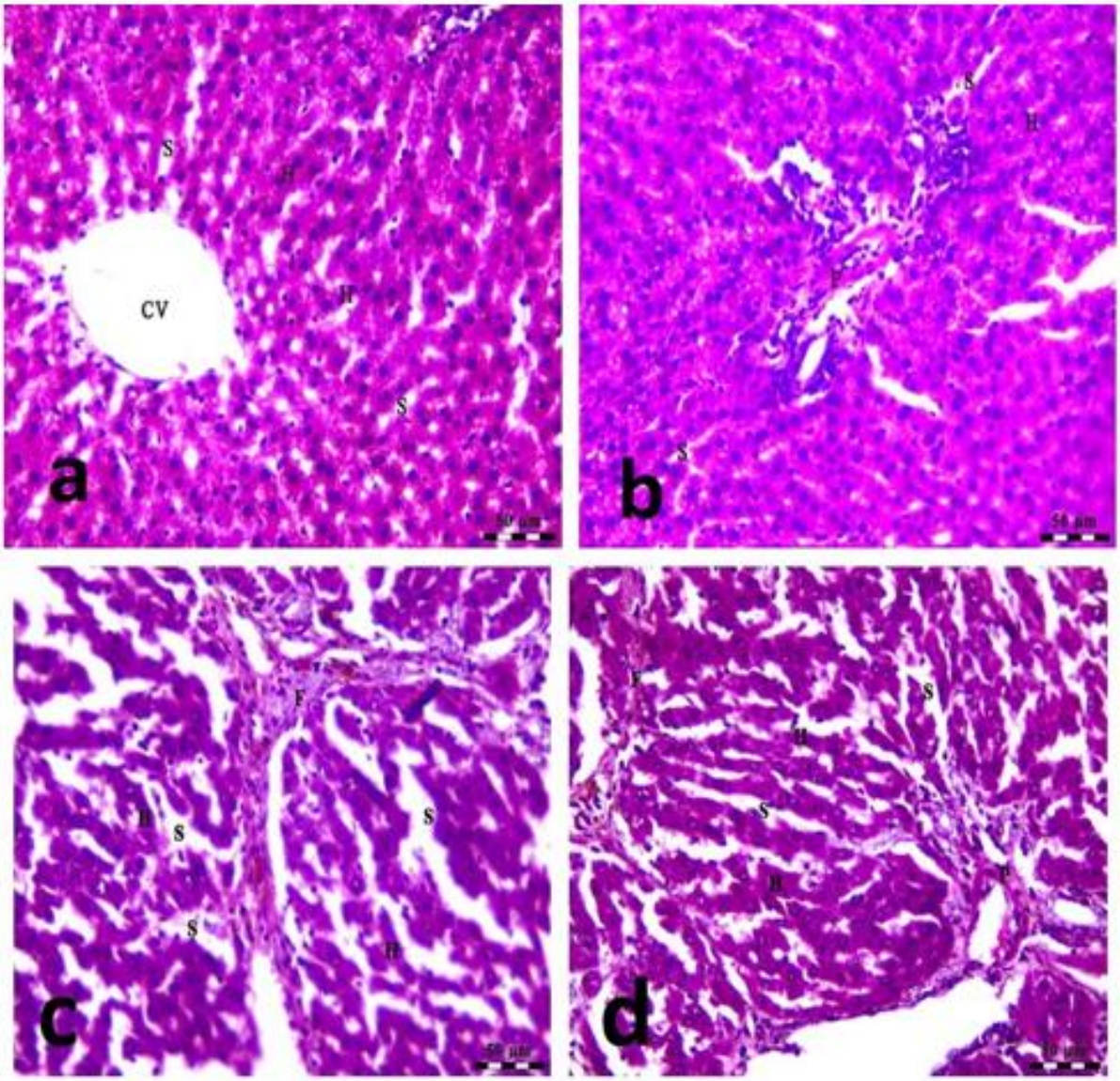

Fig. 2 (a-d): Photomicrographs of sections of rat liver from control and PCM groups stained with H&E (Bar= 50 µm). The centrolobular area of liver of control rats (Fig. 2a) showing normal architecture of hepatic lobules with a polyhedral shaped hepatocytes (H), grouped in a cord-like pattern and divided by blood sinusoids (S) in addition to central vein (CV). The portal area (P) of liver of control rats (Fig. 2b) showing hepatic artery and portal vein in addition to a polyhedral shaped hepatocytes (H), grouped in a cord-like pattern and divided by blood sinusoids (S). Both of the centrolobular and the portal areas of liver of PCM -overdosed group (Fig. 2 c, d) showing loss of hepatic architecture with severely shrank hepatocytes (H), severely dilated and mildly congested blood sinusoids (S) in addition to severely increased inter lobular fibrosis (F).

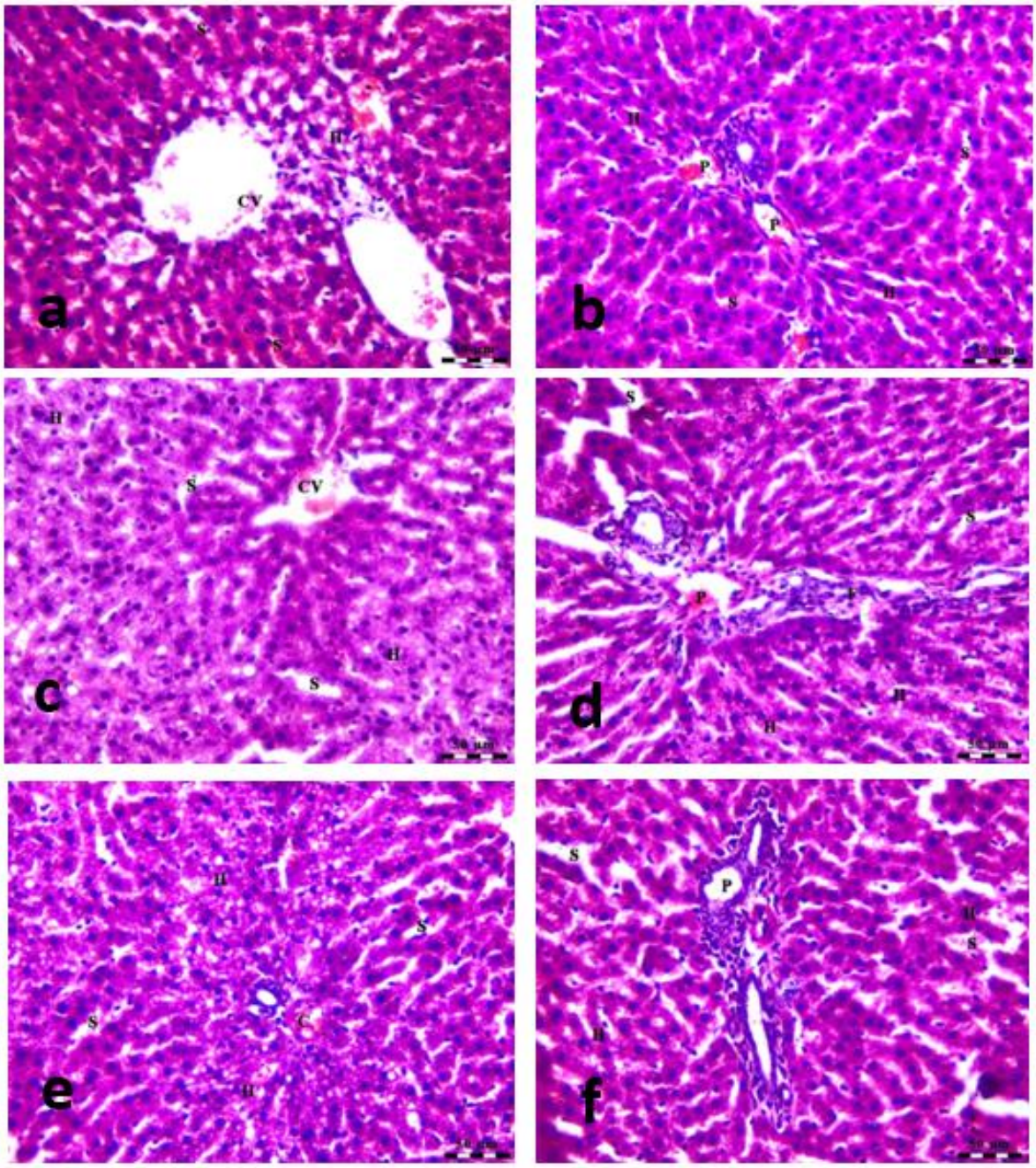

Fig. 3 (a-f): Photomicrographs of rat liver sections from silymarin, GSEE and PSEE groups stained with H&E (Bar= 50 µm). The centrolobular area of liver of silymarin group (Fig. 3a) displaying a typical liver architecture with mild central vein (CV) congestion, mild hepatocyte vacuolar degeneration (H) in perivascular area, mild dilated and congested blood sinusoids (S). The portal area of liver of silymarin group (Fig. 3b) also displaying a typical liver architecture with mild congested portal blood vessels (P), mild shrinkage of some hepatocytes (H) with mild dilated and congested blood sinusoids (S). The centrolobular area of liver of GSEE group (Fig. 3c) displaying a typical liver architecture with mild central vein (CV) congestion, mild hepatocyte vacuolar degeneration (H), and mild dilated blood sinusoids (S). The portal area of liver of GSEE group (Fig. 3d) also displaying a typical liver architecture with mild congested portal blood vessels (P), mild vacuolar degeneration of hepatocytes (H), mild dilated blood sinusoids (S), in addition to mild fibrosis (F). The hepatic parenchyma of PSEE group (Fig. 3e) displaying a typical liver architecture with mild congested hepatic vessels (C), moderate hepatocyte vacuolar

Table 6. Hepatic lesion scores in the studied groups**.**

Semi-quantitative scoring of histopathological lesion of liver					
Groups / Lesions	Control	PCM	Silymarin	GSEE	PSEE
Fibrosis	-	+++	-	+	-
Hepatocytes shrinkage	-	+++	+	-	+
Congestion	-	+	+	+	+
Dilated sinusoids	-	+++	+	+	+
Hepatocytes vacuolar degeneration	-	-	+	+	++

Hepatic lesions scoring represented as: (-) nil, (+) mild, (++) moderate and (+++) severe

DISCUSSION

Globally, PCM is frequently used as an anti-fever and analgesic medication (Saccomano, 2019). Although it is safe and effective when taken as directed, an overdose may result in hepatotoxicity and acute liver failure (ALF) (Michaut *et al*., 2014). For COVID-19 patients, PCM has been recommended as the first-line antipyretic and analgesic according to the World Health Organization (WHO) pain ladder, and recent health reports approved the high rate of its accidental toxicity in Egypt hospitals (Hegazy and Elfiky, 2016; Abdelhamid, 2021; Mostafa *et al*., 2022). So, there was a bad and continuous need for natural dietary interventions have the ability to face the mechanisms of toxic actions of PCM. In the present study, the hepatoprotective effects of both grape seeds and peanut skins ethanolic extracts *versus* silymarin in PCM-overdosed rats were studied.

Herein, the chemical investigation revealed that GSEE had somewhat lower total phenolics and flavonoids as well as radical DPPH scavenging ability than PSEE. Previous research has indicated that the main polyphenolic compounds found in grape seed extract are 15; these include 11 flavane-3-alcohols [(+)-catechin, epigallocatechin gallate, (−)-epicatechin, proanthocyanidins B1, B2, B3, B4, B1-3-O-gallate, B2-3 '-O-gallate, and gallate C1], 3 flavonols (kaempferol, myristin, and quercetin), and phenolic acid (gallic acid) (Zhao and Lou, 2001; Rodriguez-Perez *et al*., 2019). On the other hand, epigallochatequin, catechin, epicatechin and procyanidins were found to be the most important phenolic compounds in peanut skins (Francisco and Resurreccion, 2008; Taha *et al*., 2012; Chen *et al*., 2018). In general, phenolic and flavonoid compounds were suggested to be responsible for a large number of biological and pharmacological attributes of these byproducts, such as anti-oxidation, anti-inflammation, etc. (de Camargo *et al*., 2017; Chen *et al*., 2020; Kyei *et al*., 2021).

PCM overdose-induced liver dysfunction evidenced, in the present study, through this significant increase in liver enzyme activities in serum along with the significant reduction of serum protein levels especially albumin, was in harmony with many literature *in vivo* and *in vitro* studies suggested increased oxidative stress and inflammation outputs as main and intermediate promoters (Abu Ahmed *et al*., 2021; Bouhlali *et al*., 2021; Gokkaya *et al*., 2022; Singh *et al*., 2022; Ahmed *et al*., 2023).

Hepatotoxicity caused by paracetamol is intimately associated with oxidative stress, inflammatory response, and apoptosis (Brown *et al*., 2014). This explains why GSH level and SOD activity, in the present work, were significantly decreased, while there was a significant increase in malondialdehyde concentrations in liver tissue homogenates of the PCM group. Simply, at therapeutic doses, 80%-90% of paracetamol is conjugated with glucuronic acid or sulfate and excreted via the kidneys. On the other hand, the remaining paracetamol is acted upon by cytochrome P450 enzymes like Cyp2E1 and Cyp1A2, forming the reactive metabolite N-acetyl-p-benzoquinone

imine (NAPQI) (Mazaleuskaya *et al*., 2015). When someone overdoses on paracetamol, increased amounts of NAPQI are produced in excess of glutathione (GSH) detoxification capacity, i.e. only part of NAPQI can be conjugated with GSH and detoxified, while the other part binds to the liver proteins and causes excessive reactive oxygen species (ROS) production, along with mitochondrial injury and necrotic cell death (El Faras and Elsawaf, 2017; Yan *et al*., 2018).

Moreover, paracetamol at the used dose (640 mg/kg body weight/day) promoted inflammation obviously. This can be attributed to increased ROS production. ROS has the ability to promote an inflammatory response by activating nuclear factor-kappa B (NFκB), which controls how genes involved in inflammation and tissue injury are expressed (Shen *et al*., 2014). Findings of gene expression assay approved this hypothesis, as a highly significant upregulation of the expression of TNF-α and IL-10 mRNAs in the liver of PCM-treated rats, which was in agreement with a number of previously published studies (Wan *et al*., 2020; Woolbright *et al*., 2022). TNF-α is considered the "top regulator" of inflammatory cytokine synthesis (Parameswaran and Patial, 2010). Transcription of its gene is a subsequent of NF-κB activation (Bieghs and Trautwein, 2013). Conversely, IL-10 is a cytokine synthesized by M2 macrophages with potent anti-inflammatory properties. IL-10 was proven to protect against APAP toxicity by downregulation of inducible nitric oxide synthase (iNOS) expression and peroxynitrite formation (Bourdi *et al*., 2002). However, despite being negligible, PCM-treated rats' hepatic HO-1 mRNA expression was upregulated in comparison to the control group, which was consistent with Wan *et al*. (2020). HO-1 expression was reported to be undetectable in the liver under normal conditions; however, it is upregulated under stress, since it was revealed to exert anti-oxidative and anti-inflammatory functions (Loboda *et al*., 2016; Salerno *et al*., 2019).

Overall, the histopathological alterations observed in the liver matter of the group that had taken an overdose of paracetamol were consistent with the findings of numerous studies (Gad *et al*., 2013; Mahmoud *et al*., 2014; Rivera *et al*., 2017; Muhammad-Azam *et al*., 2019). Paracetamol's capacity to enhance ROS as well as pro-inflammatory cytokines production, as displayed herein, is the mechanism by which it gives rise to these anomalies in liver structure, as suggested in many studies. Kupffer cells were reported to have a role in hepatic injury induced by various toxic agents, including paracetamol through its pro-inflammatory/anti-inflammatory effects (Ju *et al*., 2002).

On the contrary, silymarin, GSEE and PSEE, in the existing study, corrected lipids and protein contents of liver tissue homogenates, thus improving the levels of liver enzymes in serum. Besides, they regulated the mRNA expression of the hepatic genes and improved both antioxidant and anti-inflammatory defense systems in liver tissue homogenate. Due to these actions, structure abnormalities disappeared quietly. Numerous investigations using various models of hepatotoxicity (Kumar *et al*., 2019; Shareef *et al*., 2022; Wang *et al*., 2023) rather than paracetamol (Islam *et al*., 2021) not only boosted the preventive qualities of silymarin but also used it as a standard safe agent. Across these investigations, the mechanistic insights into the hepatoprotective role of silymarin were studied. It was reported that it induces antioxidant and anti-inflammatory properties, and these properties are in turn attributed to its mixed composition of three isomer flavonolignans: silybin, silychristin, and silydianin. Of them, silybin only represents 50% of the silymarin structure and has been considered the most prominent bio-active (Bijak, 2017; Wadhwa *et al*., 2022).

According to the present study, GSEE is the closest to silymarin. The hepatoprotective activity of grape seed extracts was also cited in many animal studies (Osuntokun *et al*., 2020; Abu Hafsa and Hassan, 2022). The phenolic compounds found in grape seeds function as antioxidants

by stabilizing radical intermediates and preventing oxidation in addition to being hydrogen and electron donors. One of the most efficient radical-scavenging components in GSEs is procyanidin B1. Proanthocyanidins of GSE shield glutathione's sulfhydryl groups from oxidative damage (Ishige *et al*., 2001; Sgorlon *et al*., 2005; Guendez *et al*., 2005). Additionally, it has been demonstrated that grape seed proanthocyanidins reduce oxidative stress by improving detoxification pathways, modifying metabolic processes, and blocking xenobiotic interactions with biological molecules (Hassan *et al*., 2013). Besides, grape seed phytochemicals were found to induce anti-inflammatory and lipid-lowering effects (Chen *et al*., 2020). In the same range, PSEE induced its protective effects compared to the paracetamol group. In fact, studies on the biological effects of peanut skins are somewhat few. Kyei *et al*. (2021) reviewed the potential pharmacological activities of phytochemicals from peanut skin extracts. *In vitro* and *in vivo* studies revealed that they exhibited anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer/anti-tumor, anti-cardiovascular, and weight-lowering properties. The findings of Tamura *et al*. (2012) indicated that phenolic compounds derived from peanut skins exhibited hypocholesterolemic effects in an animal model. Taha *et al*. (2012) also demonstrated that phenolic compounds found in peanut skins, like catechin, epicatechin, gallic acid, and protocatechuic acid, prevented the *in vitro* growth of tumor cell lines from the colon, cervix, and liver. Moreover, the incorporation of peanut skin powder into diets of alloxan-induced diabetic rats with concentrations of 5, 7 and 10 % showed dose-dependent hypoglycemic and hepato-preventive actions (Elhardallou *et al*., 2015).

In conclusion, rats given an overdose of paracetamol showed encouraging results from the ethanolic extracts of grape seeds and peanut skins in preventing acute liver damage. Although peanut skin ethanolic extract showed an increase in total phenolics and flavonoids as well as antioxidant capability, grape seed ethanolic extract was the more effective treatment when compared to silymarin, a common hepatoprotective agent.

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Conflicts of Interest:

There are no competing interests to disclose.

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ARABIC SUMMARY

دور مستخلصات بعض المنتجات الغذائية الثانوية مقارنة بالسيليمارين كعوامل وقائية ضد الإصابة الكبدية الحادة في الجرذان المعرضة لجرعة زائدة من الباراسيتامول

وفاء أحمد جاب الله، أسماء عنتر فرماوي، سماح أحمد الحشاش
قسم التغذية وعلوم الأطعمة – كلية الاقتصاد المنزلي – جامعة الأزهر – نواج – طنطا – مصر

في هذه الدراسة، تمت مقارنة خصائص وقاية الكبد للمستخلصات الإيثانولية من بذور العنب وقشور الفول السوداني (GSEE وPSEE، على التوالي) مع السيليمارين في الجرذان المعرضة لجرعة زائدة من الباراسيتامول (PCM). تم قياس الكمية الإجمالية للفينولات والفلافونويدات وكذلك القدرة على إزالة جذور DPPH لـ GSEE وPSEE. للتقييم في الجسم الحي، تم تقسيم عشرين من ذكور الجرذان البيضاء البالغة بالتساوي إلى خمس مجموعات تشمل المجموعة الضابطة (GI)، في حين حصلت المجموعة (GII) المعالجة بالباراسيتامول عليه مرة واحدة يوميًا عن طريق الفم لمدة 14 يوم بجرعة 640 ملجم/كجم دون معالجة وقائية. تم إعطاء السيليمارين (50 ملجم / كجم / يوم) و 200 ملجم / كجم / يوم من GSEE أو PSEE بشكل متزامن مع PCM إلى المجموعات (GIII و GIV و GV) على التوالي.

أدت الجرعة الزائدة من PCM إلى ارتفاع كبير في أنشطة الإنزيمات الناقلة لمجموعة الأمين والفوسفاتيز القلوي (84.00 ± 10.64 و 396.00 ± 49.44 و 579.67 ± 72.00 وحدة / لتر مقابل 44.00 ± 5.72 و 178.00 ± 22.09 و 322.0 ± 40.58 وحدة / لتر في المجموعة الضابطة)، مع انخفاض واضح في بروتينات المصل. في الكبد، اختلت صورة الدهون، وكان هذا مرتبطًا بارتفاع ملحوظ في مؤشرات الأكسدة والالتهاب، وهو ما تم تأكيده أيضًا عن طريق الفحص النسيجي. تم تخفيف هذه الاضطرابات عن طريق إعطاء مستخلصات المخلفات الغذائية. على الرغم من إظهار PSEE زيادة طفيفة في إجمالي الفينولات والفلافونويدات والقدرة على إزالة جذور DPPH (604.45 و 542.05 و 618.59 مقابل 583.70 ملجم من حمض الجاليك، و503.80 ملجم من مكافئ الكاتشين، و550.44 ملجم من مكافئ الترولوكس، على التوالي / جرام من GSEE)، كان GSEE هوالمعالجة الأقرب مقارنة بالسيليمارين. تقترح هذه الدراسة GSEE وPSEE كعلاجات بديلة للتسمم الكبدي الناجم عن الباراسيتامول.

الكلمات المفتاحية: التسمم الكبدي، جرعة زائدة من العقار، المخلفات الغذائية، الخصائص المضادة للأكسدة والالتهاب، الجرذان.