



Antioxidant Activity and Hepatoprotective Potential of Flavonoids from *Arbutus pavarii* against CCl₄ Induced Hepatic Damage

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Authors' contributions

This work was carried out in collaboration between all authors. Author RA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author SE performed the experiments, author MEN performed the statistical analysis, author AHE managed the analyses of the study and revised the manuscript, author ME managed the analyses of the study. Author MAAG managed the analyses of the study and revised the manuscript and author NA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Flavonoids have been shown to have antioxidant factors and effective against hepatotoxicity. This *in vivo* study aimed to evaluate the efficacy of flavonoids rich extracts in a model of chemical-induced liver cell injury.

Materials and Methods: Flavonoids were extracted from leaves and flowers of *Arbutus pavarii* using Microwave assisted extraction method. Different concentrations of extracted flavonoids (200, 500, 1000, 2000 and 5000mg/kg bw) were evaluated up to two weeks on mice model. The

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hepatoprotective effects of the extracts were examined using mice pretreated orally with 200 and 400 mg/kg bw of flavonoids extracted from leaves and flowers as well as their combination (200 mg/kg; 1:1) for 28 days. At day 28, the mice were received orally a single dose of 1ml/kg CCl₄ in corn oil. Forty-eight hours after Carbon tetrachloride (CCl₄) treatment, the animals were sacrificed and their liver and blood samples were collected for determination of biochemical parameters (Alkaline phosphatase (ALT), Aspartate-aminotransferase (AST) and Alanine-aminotransferase (ALP)), histopathological investigation and antioxidant status.

Results: Treatment of the mice with a daily dose of flavonoids extracts up to 5 g/kg bw did not cause mortality and did not show hepatotoxicity. Pretreatment with extracts decreased the increased serum levels of ALT, AST, and ALP, decreased lipid peroxidation and maintained the levels of glutathione and antioxidant enzymes status in the CCl₄ treated mice, especially in the group treated with combined extracts. The hepato-protctive effects were confirmed by histopathological examinations.

Conclusion: The results shown by the extracted flavonoids need further investigation.

Keywords: Medicinal plants; Flavonoids; oxidative stress; antioxidant activity.

1. INTRODUCTION

Despite the continuous and rapid advances in modern medicine, liver dysfunction is still a worldwide health problem. Thus, exploring more therapeutic alternatives without severe undesirable side effects is necessary, and medicinal plants should be utilized and reevaluated because of their abundant resources.

There is accumulating evidence that oxidative stress plays a role in the mediating hepatocyte damage, which represents a spectrum of clinical illness, and morphological changes that range from fatty liver to hepatic inflammation to progressive fibrosis and ultimately cirrhosis [1]. Oxidative stress occurs when there is an imbalance between the over generation of reactive species (ROS) and the antioxidant defence system resulting in an overwhelmed balance status [2]. The ROS contribute to liver damage through a variety of mechanisms including inactivation of antioxidant enzymes, depletion of reduced glutathione, alteration of the breakdown of fat molecules, and lipid peroxidation [3]. Collectively all these alterations lead to hepatitis including inflammation and apoptosis of hepatocytes [4].

Medicinal plant extracts exhibit protective mechanism against oxidative stress by enhancing antioxidant enzyme activities and averting GSH depletion [5]. Flavonoids as secondary metabolites are widely available in the herbal extracts, which are found to be responsible for antioxidant functions [6]. Enriched foods with flavonoids, natural treatments from food or medicinal plants are considered to be

effective and safe for hepatotoxicity [7]. Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hepatoprotective activities [8]. Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon tetrachloride (CCl₄) induced injury in primary cultured neonatal rat hepatocytes and in rats with hepatic damage.

Lipid peroxidation is used model for evaluating natural antioxidants and clarifying their mechanisms of action [9]. CCl₄ induced hepatotoxicity has been a widely used in animal model for investigation of liver injuries induced by xenobiotic. Lipid peroxidation (LPO) is one of the initial pathways in liver damage induced by CCl₄. Metabolism of CCl₄ by phase I cytochrome P450 produces reactive free radicals, decreases hepatic glutathione content and initiates a chain of lipid peroxidation in the hepatocyte membrane [10,11]. A previous study showed that total flavonoids from *A. pavarii* leaves and flowers and their combined extracts have significant free radical scavenging *in vitro* [12]. This study was therefore designed to further investigate - *in vivo*- the Lipid peroxidation inhibition and hepatoprotective activities of flavonoid-rich extracts from *arbutus pavarii*.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh leaves and flowers of *Arbutus pavarii* (*Ericaceae*) were collected in January 2015 from Al Marj, Libya, washed thoroughly with water, and shade-dried. The plant was authenticated by the Botany Department, Faculty of Science, University of Tripoli, Tripoli, Libya.

2.2 Chemicals and Reagents

Tris HCl, Ethylene diamine tetra acetic acid (EDTA), N-(1-Naphthyl)-ethylene diamine dihydrochloride (NED), 2,4-Dinitrophenylhydrazine (DNPH), 5,5'-Dithiobis (2-nitro benzoic acid) (DTNB), Tri-chloroacetic acid (TCA), Thio-barbituric acid (TBA), phosphate buffered saline (PBS) and glutathione (GSH) were obtained from Sigma-Aldrich, USA. , Diagnostic kits for aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total protein, Urea, and Creatinine were obtained from Biomagreb, Tunisia. Superoxide dismutase (SOD) and catalase were purchased from Sigma-Aldrich, USA.

2.3 Microwave Assisted Extraction (MAE) of Flavonoid

Flavonoids were extracted using Microwave assisted extraction (MAE) method [13]. Experiments were carried out in a domestic microwave oven system (Black & Decker, Model No. MZ3000 PG, SL13YD, England). Twenty-five grams of the powdered plant materials were mixed with solvents (ethanol or methanol 80%) at a suitable ratio (500 mL). An intermittent microwave irradiation method was used to keep the temperature of the extraction mixtures below 80 °C. The suspension was irradiated in microwave oven at regular intervals (30 sec radiation and 30 sec off). Extraction was stopped when the TLC spot test indicated maximum yield for the tested samples. The infusions were allowed to cool down to room temperature, filtrated and stored at (4°C) for further analysis.

2.4 Determining the Hepatoprotective Activity of Flavonoid Extracts *In vivo*

2.4.1 Animals

This animal experiment was carried out in accordance with the European Union Directive 2010/63/EU for animal experiments. Laboratory-bred adult male Albino mice were used for this study. The protocol of the study was approved by the Ethical committee of Biotechnology Research Center (Tweasha, Tripoli, Libya) on 2015.

2.4.2. Experimental design

2.4.2.1 Acute and sub-acute toxicity tests

Acute toxicity tests were performed according to OECD-423 guidelines (Organization for

Economic Co-operation and Development, Guideline- 423) [14]. The mice were fasted overnight and then orally administered with flavonoid extracts, with graded doses of up to 5000 mg/kg body weight. The animals were evaluated daily for mortality, toxic symptoms, weight, and behavior abnormality for the following two weeks.

2.4.2.2 CCl₄-Induced Hepatotoxicity in mice

The healthy male mice weighing 18-20 g were housed in cages with free access to food and water, and allowed to acclimate to animal room conditions at 20–22°C, relative humidity 50–60%, and 12 hr light and dark cycle for one week prior to the experiment. After adaptation, the mice were randomly divided into five groups (six mice per group). Group 1 (normal control) received corn oil (1 ml/kg body weight), Group 2 (CCl₄-intoxicated) received corn oil (20%v/v) daily and CCl₄ (2 mL/kg bw) ratio 1:1 on day 28. Group 3 (positive group) was treated daily with Vitamine E (400 mg/kg bw), groups (Group 4, Group 5) were treated daily with flavonoids extracted from leaves (200, 400 mg/kg), groups (Group 6, Group 7) were treated daily with flavonoids extracted from flowers (200, 400 mg/ kg bw), and group 8 was received 200 mg/kg of combined flavonoid extracts (flowers and leaves; 1:1). Groups 2, 3, 4, 5, 6, 7 and 8 were received a single dose of CCl₄ (2 mL/kg bw) on day 28. Twenty fours hr after the last treatment, the animals were sacrificed, then the collected blood was separated by centrifugation at 3000 rpm for 5 min, stored at -20°C for biochemical analysis. The whole liver was excised rapidly, blotted dry and immediately weighed. One part of tissue was used to prepare 10% of tissue homogenate while the other part was preserved in 10% buffered formalin for the histopathological examination.

2.4.3 Preparation of tissue homogenates

A 10 % of liver homogenate (W/V) was prepared with ice-cold phosphate-buffered saline using homogenizer (IKA, RW 20.n, Germany). Then the homogenate was centrifuged at 3000 rpm for 10 min using refrigerated centrifuge. The pellet was discarded and the supernatant obtained was stored at -20 C until analysis.

2.4.4 Determination of the liver function *in vivo*

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline

phosphatase (ALP) activities, and total protein content were determined by spectrophotometric procedures, using the commercially available kits from Biomagreb, Tunisia.

2.4.5 Measurement of lipid peroxidation (LP)

Thiobarbituric acid-reactive substances (TBARS) were measured in the liver using the method of Zhang [15]. In brief, the homogenate was incubated with TCA-TBA-HCL reagent (0.37% Thiobarbituric acid, 0.24 N Hydrochloric acid and 15% Trichloroacetic acid) for 20 min at 90°C. After cooling, samples were centrifuged (Sigma 2K15, Germany) at 3000 rpm for 15 min, the supernatants absorbance were read at 532 nm. 1,1,3,3-tetramethoxy-propane was used as standard and MDA was expressed as nmol/ mg protein.

2.4.6 Measurement of nitric oxide (NO)

Nitric oxide in the homogenate was determined according to Griess method with some modification [16]. A 1.0 mL of the homogenate was mixed with an equal volume of Griess reagent containing 1% sulphanilamide and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid. The mixture was then allowed to stand at room temperature for 30 min. The absorbance of the mixture was measured against the corresponding blank solutions at 546 nm. Sodium nitrate solution was used to obtain a standard curve. The amount of nitrite was expressed as nmol/ mg protein.

2.4.7 Measurement of protein carbonyl content

Protein carbonyl content in the liver and kidney homogenates was measured spectrophotometrically [17]. Homogenized tissue sample the (final concentration of total protein less than 10 mg/mL) were incubated with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl for 1 hr at room temperature in dark place. A control sample (tissue homogenate and 2.5 M HCl) was also incubated in dark place for 1 hr with 15 min interval of vortexing. The protein is precipitated with 20% trichloroacetic acid followed by a washing step with ethanol-ethyl acetate (1:1) and dissolved in 6 M guanidine hydrochloride. The dinitrophenyl (DNP) hydrazone derivative absorbance was measured at 370 nm. Bovine serum albumin was used as standard.

2.4.8 Measurement of reduced glutathione (GSH)

Reduced Glutathione was determined using Ellman's reagent. Formations of yellow complexes after oxidation of GSH by 5,5'-dithio bis-2-nitrobenzoic acid (DTNB) were measured at 412 nm with spectrophotometer [18]. A 0.5 mL of homogenate was mixed with 0.1 mL of 25% trichloroacetic acid to precipitate proteins and centrifuged at 4,000 rpm for 5 min. Then 0.3 mL of the supernatant was mixed with 0.5 mL of 0.1 M phosphate buffer (pH 7.4) and 0.2 mL of 10 mM DTNB. After 10 min, the absorbance of yellow complex was measured at 412 nm. The amount of glutathione was determined using its molar extinction coefficient of 13600/m/cm and expressed in terms of $\mu\text{mol}/\text{mg}$ of protein.

2.4.9 Measurement of Total thiols

The total Thiols were determined according to Sedlak and Lindsay [19]. Each tissue supernatant (0.2 mL) mixed with 0.36 mL of 0.1 M phosphate buffer (pH 7.4), 40 μL of 10 mM DTNB and 1.4 mL of methanol. After 10 min, the absorbance was measured at 412 nm against a reagent blank. The concentration of free thiols was calculated from L-cysteine standard and expressed as $\mu\text{mol}/\text{mg}$ protein.

2.4.10 Measurement of Superoxide dismutase (SOD)

Estimation of SOD was performed using a SOD Assay kit-WST (Sigma Aldrich, USA) according to the manufacturers' protocol (Dojindo, Gaithersburg, MD, USA). Xanthine-xanthine oxidase system was used to generate a superoxide flux, and nitroblue tetrazolium (NBT) was used as an indicator of superoxide production.

2.4.11 Measurement of catalase (CAT)

CAT activity was measured by the method of Sinha [20], in which 100 μL of homogenate was incubated with 1000 μL hydrogen peroxide for 3.0 min at 37°C. After which the reaction was terminated by 2000 μL of dichromate/ acetic acid solution. The intensity of the yellow complex formed by dichromate and hydrogen peroxide was measured at 570 nm against the reagent blank.

2.4.12 Measurement of glutathione peroxidase (GPx)

Glutathione peroxidase activity was determined according to the method of Hafeman et al, [21]. A

100 μ L of GSH (5 mM), 100 μ L of H₂O₂ (1.2 mM), 100 μ L of NaN₃ (25 mM) and phosphate buffer (1.0 M, pH 7.0) were incubated with 100 μ L homogenate 37°C for 6 min. After that, the reaction was stopped by adding 2 mL of H₃PO₄ (1.65%) and the reaction mixture was centrifuged at 3000 rpm for 10 min. 2.0 mL of the supernatant was mixed with 2.0 mL of Na₂HPO₄ (0.4 M) and 1.0 mL of DTNB (1.0 mM) and incubated for 10 min at 37°C. The absorbance of the yellow colored complex was measured at 412 nm. Negative control tubes containing all reagent except the homogenate under the same conditions were prepared, and the absorbance was measured. GPx activity was calculated as follow:

$$\text{The GPx activity (U/mg protein)} = \frac{A(\text{blank}) - A(\text{sample}) \times 1}{0.001 \times \text{mg protein} \times 10}$$

The result was expressed as U/mg protein.

2.4.13 Histopathological observations

Livers were removed from the sacrificed animals and fixed in 10% neutral buffered formalin. Following fixation, specimens were dehydrated, infiltrated and embedded in paraffin wax, and then sectioned to 5 μ m thicknesses. Sections were stained with haematoxylin and eosin dye and viewed under light microscope [22].

2.5 Statistical Analysis

Data were expressed as means \pm standard deviations (SD) of triplicate determinations.

Statistical analyses were carried out using SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). The statistical differences between the treatments and the control were evaluated by One Way ANOVA followed by Least Significant Difference (LSD) test. Differences were considered statistically significant when $P < 0.05$. Principal component analysis (PCA) was applied to the mean values of the measured traits. Analyses were performed using Statistical Version 10 (StatSoft, Krakow, Poland).

3. RESULTS AND DISCUSSION

The leaves and flowers of *A. pavarii* proved as a source of flavonoids antioxidants against CCl₄ induced toxicity in Swiss albino mice. Treatment of mice with flavonoid extracts at different concentrations 250, 500 and 1000 mg/kg bw/day up to four weeks did not show any mortality or morbidity. Administration of higher doses was not feasible. The present study was undertaken to assess the *in vivo* antioxidant effects of flavonoid extracts on CCl₄ induced toxicity in mice. The microscopic examination revealed that all the organs from the extract treated mice did not show any alteration in cell structure or any unfavorable effects when viewed under the light microscope using multiple magnification power. The structure or coordination of cells in extract treated organs more or less similar compared with the control organs.

Carbon tetrachloride (CCl₄) is a common hepatotoxin used in the experimental study of liver diseases [23]. In the current study, the oral administration of CCl₄ (2 mL/kg body weight) was

Table 1. The effect of flavonoids (from leaves and flowers) on the body and Liver weights of the mice intoxicated by CCl₄

Group	Treatment	Body weight (g)		Liver weight (mg/g bw)
		Initial	Final	Liver
G1	Intact Control	23.6 \pm 2.5	29.3 \pm 2.2	50.1 \pm 3.7
G2	Intact+CCl ₄	20.2 \pm 0.5	25.5 \pm 1.8 ^{**a}	46.4 \pm 3.5 ^a
G3	Intact+ Vit. E	25.6 \pm 2.9	30.0 \pm 2.4 ^{**b}	50.1 \pm 2.9 ^b
G4	Intact+L200	20.1 \pm 1.9	25.4 \pm 1.5	51.7 \pm 4.7 ^{**b}
G5	Intact+L400	25.9 \pm 2.5	29.3 \pm 1.8 ^{**b}	50.2 \pm 3.1 ^b
G6	Intact+F200	20.6 \pm 1.9	28.3 \pm 1.6 ^{**b}	52.6 \pm 2.2 ^{**b}
G7	Intact+F400	20.1 \pm 2.5	27.8 \pm 1.4	53.4 \pm 3.6 ^{**b}
G8	Intact+L:F(2:1)	26.3 \pm 2.4	29.0 \pm 2.7 ^{**b}	50.7 \pm 3.1 ^b

Values are expressed as Mean \pm SD, One-Way ANOVA followed by Tukey test, n=six in each group; ^aCCl₄ control vs normal control group; ^btreated groups vs CCl₄ control group, * $P < 0.05$; ** $P < 0.01$. G1: control group; G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: mice treated with leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: mice treated with flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

significantly induced liver oxidative injury. On the other hand, pretreated mice with flavonoids extracted from leaves and flowers and their combination as well as vitamin E for four weeks offered a good protection to a considerable extent especially in the groups treated with 400 mg/kg bw flavonoid extracts and their combined extract. The body weights were slightly altered in the animals used in this investigation especially in G2. The group (G2) which received only CCl₄ significantly decrease body and liver weights compared with normal control group (G1) ($P<0.05$). However, treated groups represented significant ($P<0.05$) and dose-independent weight intake in comparison with CCl₄ intoxicated group (G2) (Table 1).

The effects of the oral treatment of flavonoid extracts on the serum AST, ALT, and ALP levels of hepatic-damaged mice are shown in Fig. 1. G2 which received only CCl₄ was found to develop significant hepatic damage indicated by elevated levels of AST, ALT, and ALP as compared to normal animals (G1) ($P<0.001$). Pretreatment with the extracts of flavonoid at 200 and 400 mg/kg bw and their combination for 4 weeks reduced the CCl₄ induced elevation of the AST,

ALP and ALT levels, to a substantial extent in a dose-dependent manner ($P<0.01-0.001$). The combined flavonoid extracts showed substantial effect on liver enzymes as the elevated levels were reduced (Fig. 1).

These results showed that flavonoids improved the CCl₄-induced liver injury, which was in agreement with previous finding [24]. In the group pretreated with alpha-tocopherol (400 mg/kg bw), there was a noticeable decrease in the values.

The flavonoid extracts showed a little bit less effective compared with alpha-tocopherol.

The reactive oxygen species can cause oxidative damage to DNA, proteins and lipids. Therefore, a biomarker of oxidative stress is biological substance whose chemical structure has been modified by ROS. These products are too stable to evaluate more easily than direct evaluation of ROS [25]. In the present study, the status of oxidative stress biomarkers as protein carbonyl, NO radicals and lipid peroxidation were measured in liver homogenate (Fig. 2).

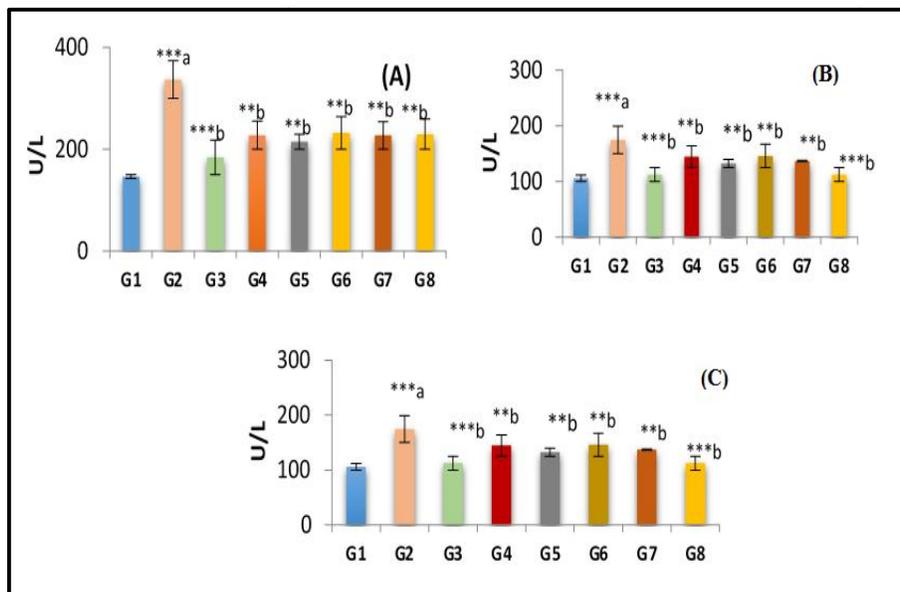


Fig. 1. The effect of flavonoids (from leaves and flowers) on AST(A), ALT (B) and ALP (C) on CCl₄ intoxicated mice

Values are represented as mean±SD, One way ANOVA followed by Tukey test, n=6. ^aCCl₄ control vs normal control group; ^btreated groups vs CCl₄ control group, * $P<0.05$; ** $P<0.01$, *** $P<0.001$. G1: control group; G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

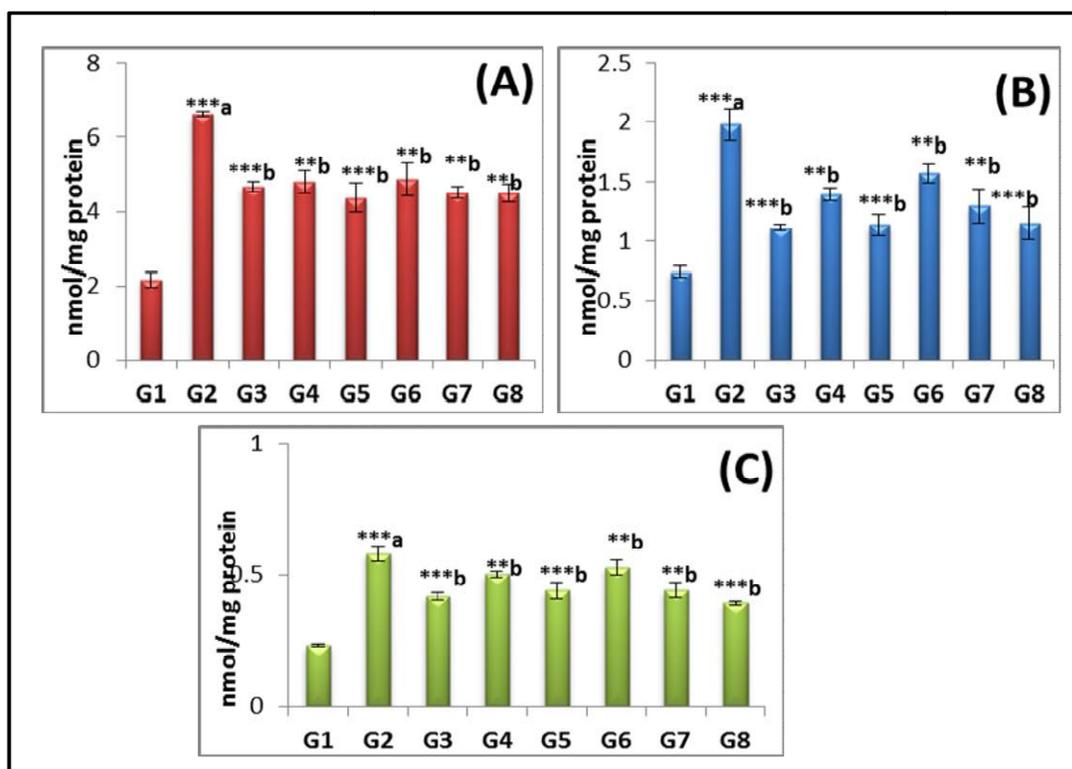


Fig. 2. Effect of Flavonoids on liver tissue; on the concentrations of Nitric oxide (A), Protein Carbonyl (B), lipid peroxidation (C) of the mice intoxicated by CCl₄.

values are represented as mean±SD, One way ANOVA followed by Tukey test, n=6. ^aCCl₄ control vs normal control group; ^btreated groups vs CCl₄ control group, *p<0.05; **p<0.01; ***P<0.001. G1: control group; G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: mice treated with leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: mice treated with flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

The levels of liver MDA, NO and PC in CCl₄ - intoxicated group were significantly higher than in the control group (P<0.001). The increase in oxidative stress marker levels in liver tissue suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant mechanisms to prevent the production of excessive free radicals. In contrast, pretreatment with flavonoids significantly reduced the oxidative stress markers especially in G3, G4, G5 and G8 (P<0.001). Our results have shown that flavonoids possess a noticeable hepatoprotective activity against CCl₄ induced damage in albino mice. Similar results were previously reported regarding liver tissues [26].

Free radical scavenging of enzymatic and non-enzymatic components such as Superoxide dismutase, Catalase, Glutathione peroxidase and Glutathione is the first line of cellular defence against oxidative liver injury and are involved in the disposal of superoxide anions, hydrogen

peroxide [27-29]. They can serve as a potential marker of susceptibility of early and reversible tissue damage, and of decreased levels of antioxidant defense [29]. The effect of flavonoids on enzymatic and non-enzymatic antioxidant levels in CCl₄ intoxicated mice are shown in Table 2.

In this study, we focused on the levels of thiol group and GSH in relation to oxidative damage to proteins. In the control group, the levels of GSH and total thiol groups were found to be 1.28±0.02, 3.50±0.06 µg/mg protein respectively. Meanwhile, the level of GSH markedly decreased in the CCl₄- treated group to 0.10±0.02, 2.54±0.01 µg/mg protein, respectively (P<0.01) indicating tissue disorder and injury [30]. The significant decrease in GSH level in different organs of the CCl₄ intoxicated mice was reported by Ohta et al, [31]. Oral administration of flavonoids (200 and 400 mg/kg bw) and their mixture (L: F; 2:1) up to 4 weeks increased the

GSH and thiol contents in a dose-dependent manner (Table 2). Treatment with the extracts at the higher dose (400mg/kg bw) and their mixture raised the GSH and thiol contents significantly as compared with CCl₄ intoxicated group ($P<0.01$). Vitamin E offered good protection and increased the GSH and thiol levels to 0.35 ± 0.02 , 2.97 ± 0.09 $\mu\text{g}/\text{mg}$ protein respectively in G8. Previous results showed that treatment with flavonoid extracts might provide a mean of recovering reduced GSH levels and to prevent tissue disorders and injuries.

SOD is one of the most important intracellular antioxidant enzyme, present in all aerobic cells and is the first line of defense against reactive oxygen species [32]. CAT is a hemoprotein, which protects cells from the accumulation of H₂O₂ by reducing it to molecular oxygen and water. Reduced glutathione and GSH-dependent enzymes, glutathione peroxidase are important factors in antioxidant defense of the body. Glutathione peroxidase GPx activity influences the concentration of H₂O₂ and other hydroperoxides because of the key role in degradation of the mentioned peroxides. Glutathione peroxidase GPx competes significantly with catalase for H₂O₂ as a substrate and is the major source of protection against low levels of oxidative stress. The level of antioxidant enzymes assessed in different berries is collectively represented in Table 2. The significant reduction ($P<0.05$) in the antioxidant enzyme activities was observed in CCl₄ intoxicated mice (G2), whereas the antioxidant

enzymes are markedly increased in the groups fed with flavonoid extracts and vitamin E. Inhibition of the generation of free radical is important in the protection against CCl₄-induced liver lesion. Increased SOD activity was observed in liver and kidney homogenates of all animals treated with both flavonoid extracts and vitamin E as compared with CCl₄ intoxicated group. The activities of both SOD and CAT were significantly higher ($P<0.05$) in the homogenates of flavonoid-treated mice than vitamin E-treated or the control groups. Similarly, the extract of leaves showed significantly ($P<0.05$) higher CAT activity in homogenates of flavonoids treated mice than alpha-tocopherol or control mice (Table 2). However, there was no significant ($P<0.05$) difference in CAT activity between alpha-tocopherol and control treated mice. It was clear that flavonoids extracted from leaves, flowers of *A. pavarii* have an effect on increasing enzymatic antioxidants in tested animals, and their effects can be magnified by increasing the extracts concentrations.

As SOD and CAT are both inducible enzymes whose production can be stimulated, increasing their activity in the present study may suggest an induction of the enzymes by the flavonoids in the mice. Earlier reports support this finding in which SOD and CAT can be induced in animals treated with plant extracts [33,34]. Elevated activity of the enzymes could enhance the anti-oxidative capacity of the animals [35].

Table 2. The effect of flavonoids on liver tissue enzymatic and non-enzymatic antioxidants in the mice intoxicated by CCl₄

Groups	Tissue enzymatic markers [‡]			Tissue non enzymatic markers ^{‡#}	
	CAT	SOD	GPx	GSH	Thiol
G1	13.47±0.40	16.21±0.05	3.61±0.18	1.28±0.02	11.50±0.03
G2	5.059±0.24 ^{**a}	2.34±0.18 ^{**a}	1.10±0.04 ^{***a}	0.10±0.02 ^{***a}	2.28±0.13 ^{**a}
G3	7.72±1.18 ^b	13.66±0.80 ^{***b}	2.76±0.10 ^{**b}	0.75±0.04 ^{***b}	6.32±0.06 ^{***b}
G4	7.64±0.25 ^b	9.88±0.34 ^{**b}	2.89±0.07 ^{**b}	0.42±0.04 ^{**b}	4.38±0.20 ^b
G5	9.20±0.54 ^{**b}	11.41±0.21 ^{***b}	2.99±0.20 ^{***b}	0.58±0.04 ^{**b}	5.19±0.13 ^b
G6	6.45±0.25 ^b	9.46±0.34 ^{**b}	2.08±0.27 ^b	0.35±0.03 ^{**b}	4.34±0.39 ^b
G7	7.71±0.61 ^b	10.29±0.81 ^{**b}	2.21±0.35 ^{**b}	0.60±0.08 ^{**b}	4.58±0.13 ^b
G8	9.56±0.69 ^{**b}	11.36±1.17 ^{***b}	2.99±0.15 ^{***b}	0.79±0.03 ^{***b}	5.79±0.09 ^{***b}

Values are represented as mean±SD, One way ANOVA followed by Tukey test, n=6, [‡]:nmol/mg protein; [#] #: $\mu\text{mol}/\text{mg}$ protein. ^aCCl₄ control vs normal control group; ^btreated groups vs CCl₄ control group, * $P<0.05$; ** $P<0.01$; *** $P<0.001$. G1: control group; G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: mice treated with leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: mice treated with flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

3.1 Histopathological Effects of Flavonoid Extracts on Hepatic Damaged Mice

In light microscopic investigation, histopathological changes were observed in the livers of all experimental groups compared with those of controls. The histopathological lesions are summarized in Table 3, in which the severity of the changes was classified from slight to severe. No histological abnormalities were observed in control mice. The administration of the only CCl₄ for four weeks caused serious liver damage (a multi focal infiltration, hepatocytes swelling, vacuolization and necrosis,

which appeared in all animals of this group (Table 4).

In contrast, the histological investigation of tissue sections from mice treated with flavonoid extracts for four consecutive weeks showed an improvement of liver histomorphology, aside from mild inflammation. Necrotic cells and vacuolization are in minimal severity (Fig. 3). Furthermore, pretreating the mice with the flavonoid extracts at two different doses improved the histoarchitecture of the hepatic tissue in a dose dependent manner as shown in Fig. 3.

Table 3. Effects of flavonoid extracts on CCl₄-induced liver injury in mice

Histopathological features		Groups							
		G1	G2	G3	G4	G5	G6	G7	G8
Inflammation		-	+++	+	++	+	++	+	+
Degenerative Changes	Fatty changes	-	+++	++	++	++	++	+	+
	Hydropic changes	-	-	-	-	-	-	-	-
Necrosis		-	+++	++	+	++	+	+	+
Apoptosis		-	-	-	-	-	-	-	-

none (-), mild (+), moderate (++) and severe (+++) damage. G1: control group; G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: mice treated with leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: mice treated with flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

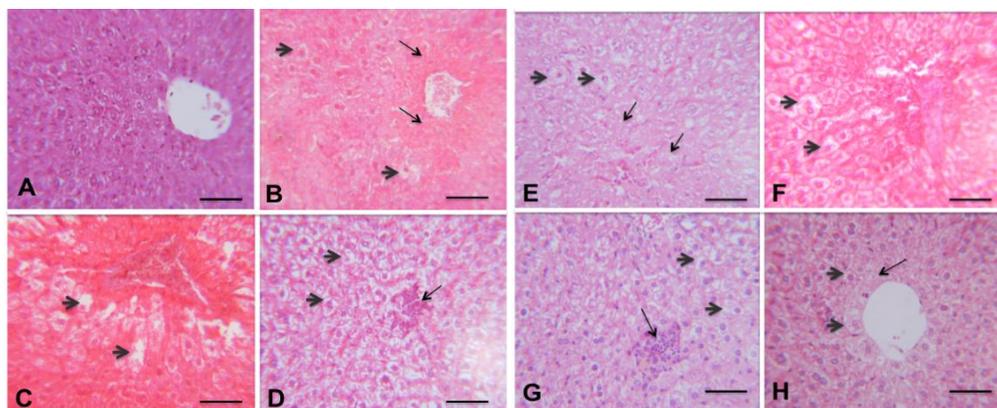


Fig. 3. Histopathologic findings in the liver of mice related to: A, Control group showed a typical microscopic pattern of the liver; B, CCl₄ treated group revealed centrilobular hepatocyte necrosis (long arrows) accompanied by hepatocyte hydropic degeneration (short arrows) and congestion; C, Vit E + CCl₄ treated group showed hepatocyte hydropic degeneration (short arrows); D, L200 + CCl₄ treated group revealed hepatocyte hydropic degeneration (short arrows) associated with focal lymphohistiocytic infiltration (long arrows); E, L400+CCl₄ treated group showed centrilobular hepatocyte necrosis (long arrows) accompanied by hepatocyte hydropic degeneration (short arrows) and congestion; F, F200+CCl₄ treated group revealed hepatocyte hydropic degeneration (short arrows) and congestion; G, F400+CCl₄ treated group revealed hepatocyte hydropic degeneration (short arrows) associated with focal lymphohistiocytic infiltration (long arrows) and congestion; H, (F200+L200)+CCl₄ treated group showed hepatocyte hydropic degeneration (short arrows) associated with minimal centrilobular hepatocyte necrosis (long arrows; HE staining; Scale bar, 50 µm)

3.2 Principal Component Analysis

Principal component analysis (PCA) was conducted to confirm any relationships among the analyzed variables from the flavonoid extracts. After the statistical analysis of all data, the PCA model retained two principal components characterized the protein carbonyl, nitric oxide, MDA, glutathione, total thiol, and antioxidant enzymes activities (CAT, SOD and GPx) of seven groups obtained from antioxidant activity in liver (Fig. 4) with a cumulative explained total variance of 97.8 %.

Mice treated with extracts of flavonoids from leaves; (200mg/kg) (G4), and (400mg/kg) (G5) as well as extracts of flavonoids from flowers (200mg/kg) (G6) and (400mg/kg) (G7) were very close on the fourth axe; the other two groups, mice treated with vitamin E (G3) and treated with combined (L: F; 2:1) extract (G8) groups were clearly separated. This was due to better values presented by groups 3 and 8 for all analysis, compared to other groups. Mice induced CCl₄ (G2) was distant from all other groups on the right side as a result of its high contents of

malondialdehyde (MDA), nitric oxide (NO) and protein carbonyl.

The eigenvectors indicating association between variables and PCs are presented in Table 4. The bigger the eigenvectors, the higher the correlations between variables and PCs. Principal component 1 (PC1) correlated positively with, GSH, Total Thiol, and antioxidant enzymes activities (CAT, SOD and GPx) of seven groups. Moreover, PC1 was inversely correlated with nitric oxide, MDA and glutathione peroxidase (GPx) levels.

Principal Component 2 (PC2) had high component loadings from the variables analyzed the GSH, SOD and GPx and weaker ones with MDA, while it had positive loadings from protein carbonyl, GSH, SOD and weaker ones with nitric oxide, total thiol and CAT.

GSH, total thiol, CAT, SOD and GPx were highly positively associated with PC1, whereas nitric oxide, MDA and protein carbonyl were negatively associated with PC1. Protein carbonyl, MDA, total thiol, CAT and GPx were positively associated with PC2 (Table 4).

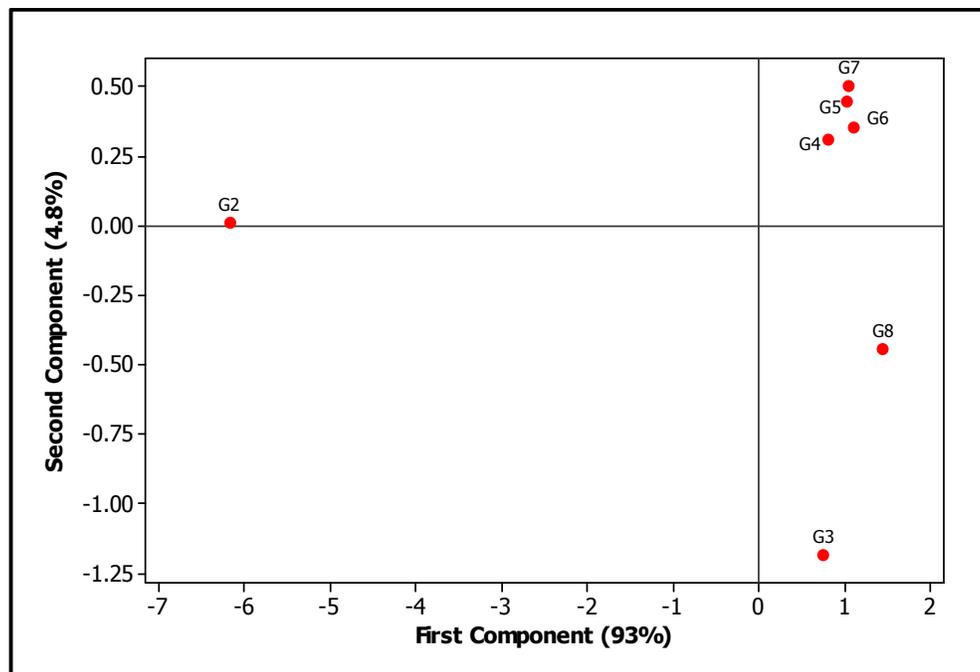


Fig. 4. Biplot (PC1xPC2) of scores and loadings for the PCA of *in vivo* antioxidant capacity.

G2: CCl₄ group; G3: vitamin E group; G4: mice treated with leaves flavonoid extract (200mg/kg); G5: mice treated with leaves flavonoid extract (400mg/kg); G6: mice treated with flowers flavonoid extract (200mg/kg); G7: mice treated with flowers flavonoid extract (400mg/kg); G8: mice treated with combination of leaves and flowers extract (2:1).

Table 4. Eigenvectors of the included variables in PCA of Fig. 4 liver on PC1 and PC2.

Component	PC1	PC2
Protein carbonyl content	0.989	-0.102
Nitric oxide content	-0.980	-0.027
MDA content	-0.981	0.018
Glutathione content	0.926	0.148
Total Thiol content	0.971	-0.042
Catalase activity	0.940	-0.071
Superoxidase activity	0.960	0.149
Glutathione peroxidase activity	-0.299	0.949

The eigenvectors indicate an association between variables and PCs

4. CONCLUSION

In conclusion, the results of this study showed that flavonoids extracted from *A. pavarri* would be safe for clinical application, and it would be effective for the prevention of xenobiotics induced hepatic injuries. The flavonoid extracts showed hepatoprotective effects in CCl₄-intoxicated mice, especially leaves extract and combined extracts due to their antioxidant activities, which most probably related to flavonoids.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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