

Hepatoprotective Effect of Carob Pods Extract (*Ceratonia siliqua* L.) against Cyclophosphamide Induced Alterations in Rats

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ABSTRACT

Drugs used for treatment of cancerous diseases by mean of chemotherapy are often limited due to their severe undesirable side effects in multiple organs. Cyclophosphamide (CYP) belongs to class of the cytotoxic bifunctional alkylating agents. In the current study, it was revealed that CYP caused significant ($P < 0.05$) elevation in levels of different biochemical measurements. Carob extract exhibited beneficial effect through lowering of all elevated parameters. Furthermore, CYP caused decline in total antioxidant capacity (TAC) level in association with elevation of lipid peroxidation (LPO) level and significantly ($P < 0.05$) in liver tissue. Carob extract restored TAC and lowered LPO level. CYP caused several histopathological alterations in the hepatocytes and carob extract minimized severity of these alterations. The similarity index percent (SI %) in the native electrophoretic protein, lipoprotein and calcium moiety of protein patterns was represented by lowest values (87.50, 66.67 and 70.59, respectively) with the CYP-treated group. The SI % values increased in all carob treated groups. In all electrophoretic isoenzymes, the SI % value was represented by the lowest value (60.00, 66.67, 66.67 and 53.33 respectively with catalase (CAT), peroxidase (GPx), α - and β -esterase (EST) patterns) in CYP-treated group. While in all carob treated groups, the SI % value reached the highest value (100.00). Furthermore, CYP induced cleavage of the genomic DNA and the carob extract maintained the DNA integrity. The study concluded that carob showed ameliorative effect against alterations induced by CYP at biochemical, histopathological and molecular levels in liver tissue of rats.

Keywords: Cyclophosphamide, Liver, *Ceratonia siliqua*, Electrophoresis, Protein, Isoenzymes, DNA.

INTRODUCTION

Drugs used for treatment of cancerous diseases by mean of chemotherapy are often limited. They exhibited severe undesirable side effects in multiple organs¹. Thus, there are new strategies to minimize side effects of the chemotherapeutic agents while it is necessary to maintain their chemotherapeutic efficacy. Cyclophosphamide (CYP) belongs to class of the nitrogen mustard. It is known as a cytotoxic bifunctional alkylating agent that is used to treat various autoimmune, neoplastic and cancerous diseases².

Although administration of CYP alone or in conjunction with other drugs has proved an effective for treatment of different cancerous tumors, it was found that it exhibited various undesirable side effects on the liver tissue³. It is well absorbed orally and still inactive until it is metabolized in the liver through metabolic pathway by hepatic cytochrome P450 system⁴.

In 2010, Ray *et al.*⁵ postulated that the active CYP metabolites carried to tumor cells where it cleaves spontaneously giving two active cytotoxic metabolites

called acrolein and phosphoramidate mustard. It is well known that phosphoramidate exhibits anti tumor effects, while acrolein causes gastrointestinal bleeding and hemorrhagic cystitis. Also, it alkylates DNA and modifies proteins⁶.

CYP exhibited teratogenic activity after metabolic activation⁷. It exerts cytotoxic effect and accelerates the cellular proliferation by interaction with DNA and subsequently limiting DNA synthesis leading to defective DNA, alteration of cell function and hence cell death^{8,9}.

It was reported that CYP exhibited a prooxidant character and enhanced production of the free radicals which affect the cellular biomacromolecules (i.e. protein, lipids, carbohydrates and DNA)^{10,11}. Moreover, it causes elevation of lipid peroxidation (LPO) in liver tissues as a result of lowering in activities of the antioxidant enzymes¹². Also, it caused glycation of the non-enzymatic protein which may lead to disruption of cellular functions and oxidative damage to membranes¹¹. As a result of the toxicities associated with CYP, it was necessary to search for effective natural compounds to reduce severity of

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these adverse effects and to protect against organ toxicity induced by CYP during chemotherapy¹³. Kumar *et al.* (2011)¹⁴ postulated that hesperidin exhibited cardioprotective effect against CYP induced alterations in heart tissue of rats. The ameliorative effect of hesperidin is probably related to its membrane stabilizing activity and scavenging of free radicals generated by CYP. Carob (*Ceratonia siliqua* L.) seeds and pods are a particularly rich source of flavonoids such as proanthocyanidin, ellagitannin and gallotannin. These phytochemicals exhibited scavenging activities against numerous diseases caused as a result of free radical attack^{15,16}. Furthermore, it was suggested that carob pods extract exhibited a potential gastro-protective effect and ameliorative effects against oxidative damage in different tissues induced by alcohol or carbon tetrachloride in rats^{17,18}. In 2011, Agrawal *et al.*¹⁹ mentioned that the aqueous *C. siliqua* extract possesses health benefits for humans due to the antiproliferative and antioxidant efficiency which suggested the potential development of carob pods as a pharmaceutical product. This may be attributed to its antioxidants properties²⁰. It was found that *C. siliqua* pod extract showed antioxidant activity higher than many other foods rich in polyphenols, such as blueberries, grapes or red wine²¹. Although Temiz *et al.* (2015)²² documented in their recent studies that *C. siliqua* extract exhibited a hepatoprotective effect and antioxidant capacity in rats against liver toxicity induced by alcohol, there were no reports about effect of *C. siliqua* extract on markers of the oxidative stress in the literature. The present study was postulated in the fact that carob pod extract delayed progression of liver injury and oxidative stress induced by CYP in rats and for correlation of these findings to human beings.

MATERIALS AND METHODS

Materials: Chemicals, Reagents and Kits

Acrylamide, Bis-acrylamide, Ammonium persulfate (APS), N,N,N,N-Tetramethylethylenediamine (TEMED), Bovine serum albumin (BSA), Tris buffer, Coomassie Brilliant Blue G-250 (CBBR-250), Sudan Black B (SBB) and Alizarin Red 'S' were procured from Sigma-Aldrich. Hematoxylin and Eosin stains were obtained from SRL, India and the chemicals used for in-gel esterase staining including α - and β -naphthylacetate, Fast Blue RR were purchased from Qualigens Fine Chemicals, India. Cyclophosphamide (CYP), Folin Ciocalteu reagent, Gallic Acid, 2-Thio-barbituric Acid (TBA) and Benzidine were purchased from Sigma Chemicals Company (London, UK). Kits for Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Urea, Creatinine, Total Protein, Albumin, Cholesterol, Triglycerides, Creatine Phosphate Kinase (CPK) and Lactate Dehydrogenase (LDH) were obtained from Spectrum Diagnostics Egyptian Company for Biotechnology (Cairo, Egypt). The PCR kit and primers were purchased from Promega. All the chemicals and reagents used were of analytical grade and of highest purity.

Preparation of aqueous carob extract

As mentioned by Rtibi *et al.*²⁰, the mature carob pods were collected and dried in an incubator at 50 °C for 72 h. The dried plant material was crushed into powder in an electric blender and dissolved in nanopure water then filtered.

Determination of total polyphenols and antioxidant activity

Concentration of the total polyphenols was estimated in the aqueous carob pod extract by folin ciocalteu reagent using gallic acid as standard according to method described by Singleton and Rossi (1965)²³. The total reducing power was determined using ascorbic acid as standard according to method of Oyaizu (1986)²⁴. Moreover, percentage of the antioxidant activity was assessed by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical²⁵ (Brand-Williams *et al.*, 1995).

Administration of aqueous carob extract

The aqueous carob extract was administered orally by stomach tube at dose equivalent to 2 g/kg b.w. (1/10 of LD₅₀) according to the experiment carried out by Rtibi *et al.* (2015)²⁰.

Animals and treatment

Healthy thirty six adult male Wistar rats (weighting 120 - 150 g) were housed in six per cage. The animals were provided with water *ad libitum* and standard food and maintained under normal environmental conditions at 25 ± 2°C. The experimental procedures were carried out according to the ethical protocol and guidelines approved by the institutional animal care of National Research Centre, Dokki, Giza, Egypt.

Experimental Design

The rats were randomly divided into six groups. Group I (Control group): Rats were fed with normal diet as *ad libitum* and received distilled water for 7 days. Group II (Carob pod extract treated group): Rats were fed with normal diet associated with the treatment with aqueous carob extract for 7 days. Group III (CYP- treated group): Rats were treated with CYP only interperitoneally (i.p.) at a dose equivalent to 0.3 mg/kg body weight for 7 days as documented by Patel *et al.* (2012)²⁶. Group IV (Simultaneous treated group): Rats received CYP i.p. and administered with carob extract in parallel manner for 7 days. Group V (Pre-treated group): Rats were administered with carob extract for 7 days and followed by receiving CYP i.p. for another 7 days. Group VI (Post-treated group): Rats received CYP i.p. for 7 days then treated with carob extract for another 7 days.

Collection of samples

Seven days after the experimental period (i.e., on the 8th day), all the animals were anaesthetized and killed by decapitation. Blood was collected from retro orbital plexus of the animals into tubes. Serum samples were prepared for determination of the biochemical measurements by centrifuging blood samples for 15 min at 4000 rpm. The liver tissues were excised and washed in ice-cold saline. The tissues were homogenized in 0.01 M Tris -HCl buffer (pH 7.4) and aliquots of this homogenate were used for measuring the oxidative stress

markers and for undergoing the different electrophoretic patterns.

Biochemical Estimations

Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Urea, Creatinine, Total Protein (TP), Albumin (Alb), Cholesterol, Triglycerides (TGs), Creatine Kinase (CPK) and Lactate Dehydrogenase (LDH) were measured in all experimental animals by using commercially available kits obtained from Spectrum Diagnostics Egyptian Company for Biotechnology (Cairo, Egypt).

Markers of oxidative stress

The lipid peroxidation (LPO) was determined in the liver tissue homogenate spectrophotometrically at wave length 535 nm using a UV-vise spectrophotometer (Shimadzu uv-2401 pc) according to method suggested by Ohkawa *et al.* (1979)²⁷. The total antioxidant capacity (TAC) was measured based on capacity of the sample to inhibit production of thiobarbituric acid reactive substances from sodium benzoate under the influence of the free oxygen radicals derived from Fenton's reaction as mentioned by Koracevic *et al.* (2001)²⁸.

Statistical Analysis

All data were statistically evaluated by the Statistical Package for Social Sciences (SPSS for windows, version 11.0). The results were expressed as mean $\pm$ standard error (SE). Significant difference between the groups were statistically analyzed by one-way analysis of variance test (one-way ANOVA) followed by least significant difference (LSD) test and confirmed by Benferoni test. A "P" value of less than 0.05 was considered to indicate statistical significance.

Histopathological examination

As documented by Banchroft *et al.* (1996)²⁹, autopsy specimen was taken from liver tissues of different groups after sacrifice. All samples were immediately fixed in 10% formal saline for 24 hr and washed in tap water then dehydrated in serial dilutions of alcohol solutions. Tissue fragments were then cleared in xylene and embedded in paraffin and used for histopathological examination. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The tissue sections were collected on glass slides and deparaffinized then stained by hematoxylin & eosin (H&E) stain for examination through the light electric microscope.

Preparation of samples

The liver tissues were freezed rapidly with liquid nitrogen and ground then homogenized in 1 ml water-soluble extraction buffer. The homogenates were centrifuged at 10,000 rpm at 4°C for 15 min. The supernatants containing water-soluble proteins were transferred to new eppendorf tubes. All samples of each group were pooled together and used as one sample. Protein concentration was assayed in all pooled samples according to method of Bradford (1976)³⁰ using bovine serum albumin as standard. Quantities of protein must be equal in all wells during the electrophoretic analysis.

Electrophoretic patterns

The polyacrylamide gel electrophoresis was carried out using Mini-gel electrophoresis (BioRad, USA) at the concentration 10 % according to method documented by Laemmli (1970)³¹ with the modification that samples, gels and running buffers were lacking sodium dodecyl sulphate³² (Darwesh *et al.*, 2015). Bands of the native protein were visualized by staining with Coomassie Brilliant Blue G-250. The relative mobility (Rf) and band percent (B %) of the electrophoretically separated proteins were determined in addition to the molecular weight (Mwt) which was estimated in comparison to marker of standard molecular weights with regularly spaced bands ranging from 6.458 to 195.755 KDa. Furthermore, the native gels were stained by mean of isoelectrophoresis for lipids and calcium moieties with Sudan Black B (SBB)³³ and Alizarin Red 'S'³⁴, respectively.

Electrophoretic localization of in-gel enzyme activity

The non-denaturing gel was stained for electrophoretic catalase (CAT) pattern according to method of Siciliano and Shaw (1976)³⁵. For electrophoretic peroxidase (GPx) pattern, it was stained with benzidine stain prepared according to method described by Rescigno *et al.* (1997)³⁶. It was processed for localization of in-gel α - and β -esterase (EST) activities according to method modified recently by Ahmad *et al.* (2012)³⁷ who postulated that the gel was incubated in reaction mixture containing α , β -naphthyl acetate (5.58×10^{-3} mM, pH 7.5) as substrates along with dye coupler Fast Blue RR in dark.

Genomic DNA Fragmentation

The genomic DNA was extracted from liver tissue using method described by Barker *et al.* (2004)³⁸. As mentioned by Rapley (1998)³⁹, the polymerase chain reaction was performed for amplification of the genomic DNA with 8 random primers of different nucleotide sequences (OPA-04 (5'-AATCGGGCTG-3'), OPA-05 (5'-AGGGGTCTTG-3'), OPA-07 (5'-GAAACGGGTG-3'), OPA-10 (5'-GTGATCGCAG-3'), OPA-11 (5'-CAATCGCCGT-3'), OPA-12 (5'-TCGGCGATAG-3'), OPA-14 (5'-TCTGTGCTGG-3') and OPA-15 (5'-TTCCGAACCC-3')) using thermal cycler (Progeny 30, Techno, Cambridge Ltd. Dux ford Cambridge, UK). The amplification process was carried out through the thermal program initial denaturation at 94°C for 5 min. followed by 40 cycles of DNA amplification. Each cycle was consisting of denaturation at 94°C for 2 min. then annealing at 40°C for 2 min. and extension at 72°C for 7 min. The amplified fragments were separated on 2% agarose gel visualized on a UV transilluminator and photographed by Gel Documentation System.

Data analysis

The native bands in the polyacrylamide gel and the DNA fragments in agarose gel were analyzed using Quantity One software (Version 4.6.2). Percent of the similarity index (SI) was calculated according to equation suggested by Nei and Li (1979)⁴⁰ to compare all treated groups to control group.

RESULTS

It was revealed that concentrations of the total polyphenols, total reducing power and free radical

scavenging activity were about 0.578 ± 0.006 mg gallic acid / 100 gm, 6.374 ± 0.742 mg / 1 ml and 9.74 %, respectively.

Biochemical measurements

As presented in Table 1, it was found that CYP caused significant ($P < 0.05$) elevation in all biochemical measurements (liver enzymes (ALT and AST), protein profile (TP and Alb), renal functions (urea and creatinine), lipid profile (cholest. and T.Gs) and heart enzymes (CK and LDH)) as compared to control. Carob extract lowered levels of all elevated measurements significantly ($p < 0.05$) in all carob treated groups. It is worth to be notable although carob extract alone caused significant ($P < 0.05$) increase in levels of protein profile, its combination with CYP lowered its levels significantly ($p < 0.05$) with respect to CYP-treated group.

As reported in Table 2, it was postulated that CYP caused significant ($P < 0.05$) decline in TAC level in association with significant ($P < 0.05$) elevation in LPO level in liver tissue. Carob extract restored the TAC to normalcy and lowered LPO concentration in all carob treated groups significantly ($P < 0.05$).

Histopathological examination

As illustrated in Figs. 1a&b, the liver tissue appeared with normal histological structure of the central vein (CV) and surrounding hepatocytes (h) in the hepatic parenchyma in control and carob extract treated groups. In the CYP-treated group, several histological alterations were represented by appearance of fatty changes in the hepatocytes (black arrow) with diffuse kupffer cells proliferation (k) in between. In another microscopic field, multiple numbers of double nuclei hepatocytes (red arrow) were detected in the parenchyma. Furthermore, the portal area showed dilatation in the portal vein with oedema and cystic bile ducts (Fig. 1c). In the simult-treated group, it was observed that there was congestion in both central (CV) and portal veins (PV) associated with diffuse kupffer cells proliferation (k) in between the hepatocytes (Fig. 1d).

Focal hemorrhage (h) was noticed in the hepatic parenchyma surrounding the dilated central vein in the pre-treated group. Moreover, the portal area showed dilatation in the portal vein (PV) as well as few inflammatory cells infiltration (m) in portal area surrounding the bile ducts (bd). Fatty changes (black arrow) were detected in some hepatocytes associated with pyknotic nuclei (P) in others (Fig. 1e). In the post-treated group, dilatation was observed in both central (CV) and portal veins (PV). In another field, there were degenerative changes (d) in the hepatocytes surrounding the central vein. Furthermore, there was few pericudtal inflammatory cells infiltration (m) surrounding the bile ducts (bd) (Fig. 1f).

parenchyma associated with congestion in portal vein (PV) (H&E, X 40) and fatty changes (black arrow) in some hepatocytes (H&E, X 80) and f) post-treated group with dilation of central vein (CV) and portal vein (PV) with degeneration (d) in adjacent surrounding hepatocytes (H&E, X 40).

The representative electrophoretic profile of the native protein (Fig. 2a) showed that protein molecule with high molecular weight migrates slowly and find close to the well comb while the protein molecule with low molecular weight migrates rapidly to end the gel plate. Six common bands were identified at Rfs 0.09, 0.39, 0.58, 0.68, 0.86 and 0.94 (Mwts 167.60, 30.09, 18.04, 15.35, 7.85 and 5.45, respectively). There were no characteristic bands. The SI % was represented by the lowest value (87.50 %) in the CYP-treated group. While in the simult-treated, pre-treated and post-treated groups, the SI % values were represented by 94.12, 100.00 and 94.12, respectively.

The electrophoretic lipoprotein pattern (Fig. 2b) verified that 5 common bands were identified at Rfs 0.15, 0.51, 0.59, 0.67 and 0.98 (B% 15.42, 14.22, 14.22, 13.55 and 8.77, respectively). One characteristic band was noticed in CYP-treated group at Rf 0.05 (B % 16.25). It was emphasized that CYP caused no quantitative variations but it caused qualitative disturbances represented by disappearance of 3 normal bands with notification of 2 abnormal bands at Rfs 0.05 (B % 16.25) and 0.31 (B % 15.53). The SI % was represented by the lowest value (66.67 %) in the CYP-treated group. It reached the highest value (100.00) in all carob treated groups.

In electrophoretic calcium moieties of native protein pattern, it was revealed that 6 common bands were notified at Rfs 0.19, 0.30, 0.38, 0.47, 0.62 and 0.97 (B % 11.07, 11.94, 11.36, 12.06, 9.90 and 9.90, respectively). Two characteristic bands were identified in CYP-treated group at Rfs 0.20 and 0.54 (B % 13.58 and 12.80, respectively). CYP caused disturbances represented by disappearance of 3 normal bands with existence of 2 unique characteristic bands. The SI % was represented by the value 70.59 % in the CYP-treated group. It was recorded equal (94.12) in all carob treated groups (Fig. 2c).

The electrophoretic CAT pattern (Fig. 3a) presented that 3 common bands were identified at Rfs 0.13, 0.66 and 0.91 (B % 21.04, 23.06 and 14.70, respectively). Two characteristic bands were noticed in CYP-treated group at Rfs 0.05 and 0.83 (B % 22.19 and 13.37). CYP caused disappearance of one normal band in addition to presence of the characteristic bands. The lowest SI % value (60.00 %) was observed in CYP-treated group. While in all carob treated groups, the SI % value reached the highest value (100.00).

As illustrated in Fig. 3b, 3 common bands were identified in the electrophoretic GPx pattern at Rfs 0.23, 0.68 and 0.86 (B % 20.13, 21.73 and 20.13, respectively). No characteristic bands were observed. CYP caused disappearance of the 1st normal band with shifting the 3rd to be identified at Rf 0.56 (B % 25.30). The lowest SI % value was observed in the CYP-treated group (66.67 %). While in simult-treated, pre-treated and post-treated groups, values of the SI % were represented by 80.00, 100.00 and 100.00, respectively.

As revealed in Fig. 4a, the study which was concerned with identification α -EST showed that there were 3 common bands identified at Rfs 0.16, 0.50 and 0.78 (B % 18.10, 20.99 and 18.92, respectively). One characteristic

Table 1: Effect of aqueous carob pods extract against cyclophosphamide induced changes in biochemical functions in rats.

	C	Carob	CYP	Simult	Pre-treated	Post-treated
ALT (U/L)	58.09 ± 0.96	60.22 ± 0.48	198.50 ± 2.20 ^a	179.29 ± 1.06 ^{ab}	76.44 ± 0.85 ^{ab}	68.99 ± 0.41 ^{ab}
AST (U/L)	86.57 ± 1.36	86.65 ± 0.35	291.03 ± 1.13 ^a	267.65 ± 1.03 ^{ab}	95.14 ± 0.93 ^{ab}	94.40 ± 0.90 ^{ab}
TP (g/dl)	5.49 ± 0.08	5.79 ± 0.05 ^a	8.85 ± 0.03 ^a	7.78 ± 0.04 ^{ab}	6.35 ± 0.04 ^{ab}	6.56 ± 0.02 ^{ab}
Alb (g/dl)	2.86 ± 0.02	3.00 ± 0.03 ^a	3.85 ± 0.02 ^a	3.66 ± 0.02 ^{ab}	3.11 ± 0.02 ^{ab}	3.00 ± 0.03 ^{ab}
Urea (mg/dl)	54.07 ± 0.81	56.93 ± 0.37	86.13 ± 0.91 ^a	74.88 ± 0.58 ^{ab}	57.93 ± 0.35 ^{ab}	61.10 ± 0.66 ^{ab}
Creat. (mg/dl)	0.84 ± 0.01	0.85 ± 0.01	3.24 ± 0.02 ^a	2.88 ± 0.01 ^{ab}	0.90 ± 0.01 ^{ab}	0.87 ± 0.01 ^b
Cholest. (mg/dl)	41.19 ± 0.39	42.92 ± 0.33	84.97 ± 0.70 ^a	74.61 ± 0.52 ^{ab}	50.11 ± 0.26 ^{ab}	46.00 ± 0.70 ^{ab}
T.Gs (mg/dl)	75.00 ± 0.91	73.59 ± 0.31	128.68 ± 0.89 ^a	111.63 ± 1.67 ^{ab}	85.48 ± 0.71 ^{ab}	84.59 ± 0.61 ^{ab}
CK (U/L)	127.06 ± 0.40	124.27 ± 0.40	293.27 ± 1.34 ^a	246.88 ± 0.86 ^{ab}	175.67 ± 1.69 ^{ab}	138.31 ± 0.90 ^{ab}
LDH (U/L)	264.27 ± 1.20	266.35 ± 1.20	512.57 ± 1.76 ^a	421.30 ± 2.76 ^{ab}	289.09 ± 1.05 ^{ab}	288.62 ± 1.76 ^{ab}

a: values compared to control group ; b: values compared to CYP-treated group (significant p<0.05).

Table 2: Effect of aqueous carob pods extract against cyclophosphamide induced oxidative alterations in liver tissue in rats.

	C	Carob	CYP	Simult	Pre-treated	Post-treated
TAC (mM/L)	1.06 ± 0.01	1.06 ± 0.01	0.63 ± 0.01 ^a	1.02 ± 0.01 ^{ab}	1.06 ± 0.01 ^b	1.05 ± 0.01 ^b
LPO (nmol/g wet tissue)	18.75 ± 0.21	20.02 ± 0.05	51.79 ± 0.64 ^a	35.42 ± 0.74 ^{ab}	28.07 ± 0.05 ^{ab}	22.04 ± 0.13 ^{ab}

a: values compared to control group ; b: values compared to CYP-treated group (significant p<0.05)

band was noticed in CYP-treated group at Rf 0.02 (B % 25.78). It was displayed that CYP caused absence of one band in addition to presence the characteristic band. In the CYP-treated group, the SI % value reached the lowest value (66.67 %) while in the carob treated group, it reached the highest value (100.00 %). The electrophoretic β -EST pattern (Fig. 4b) displayed that 4 common bands were identified at Rfs 0.30, 0.42, 0.74 and 0.84 (B % 11.88, 12.64, 12.64 and 12.64, respectively). Three characteristic bands were identified in CYP-treated group at Rfs 0.14, 0.37 and 0.63 (B % 14.07, 12.44 and 13.71). It was observed that CYP caused absence of 3 normal bands with existence of the characteristic bands. The lowest SI value (53.33 %) was observed in the CYP-treated group and it reached the highest value (100.00 %) in the carob treated group.

As illustrated in Fig. 5a, the CYP exhibited various genomic qualitative and quantitative alterations detected electrophoretically using various primers with different nucleotide sequences. The qualitative abnormalities may be represented by disappearance of one or more of normal bands and / or existence of some abnormal bands. Otherwise, the bands may be identified at the same relative mobilities of the control bands but with different quantities (quantitative mutation). The amplicon with the

the primers OPA-04 and OPA-05 verified that CYP caused qualitative mutagenicity represented by shifting 3 normal bands to be identified at Rfs 0.16, 0.57 and 0.66 (Mwts 1586.29, 920.85 and 833.16 Bp, respectively). With the primers OPA-07 and OPA-10, CYP exerted mutagenic effect represented by absence of one normal band with appearance of 2 abnormal bands at Rf 0.45 (Mwts 1119.31) and Rf 0.52 (Mwts 1025.40 Bp). The amplification with the primers OPA-11 and OPA-12 showed that CYP caused qualitative effect by disappearance of some normal band with existence of 6 abnormal bands (Rfs 0.23, 0.40, 0.48, 0.54, 0.65 and 0.70 with Mwts 1493.80, 1160.43, 1012.75, 915.08, 788.21 and 735.49 Bp). With the primers OPA-14 and OPA-15, in the CYP-treated group, the abnormalities occurred at the qualitative level through absence of one normal band with appearance of 4 abnormal bands at 0.09, 0.17, 0.33 and 0.55 (Mwts 1788.60, 1593.13, 1277.89 and 932.17 Bp, respectively). Furthermore, CYP caused variation at the quantitative level represented by changing quantity of the qualitatively normal bands. From averages of the SI % values with all primers as compared to control, the lowest SI % value was noticed in CYP-treated group (SI % 44.70) and the carob extract increased the SI % values in simult-treated, pre-treated and post-treated groups (SI

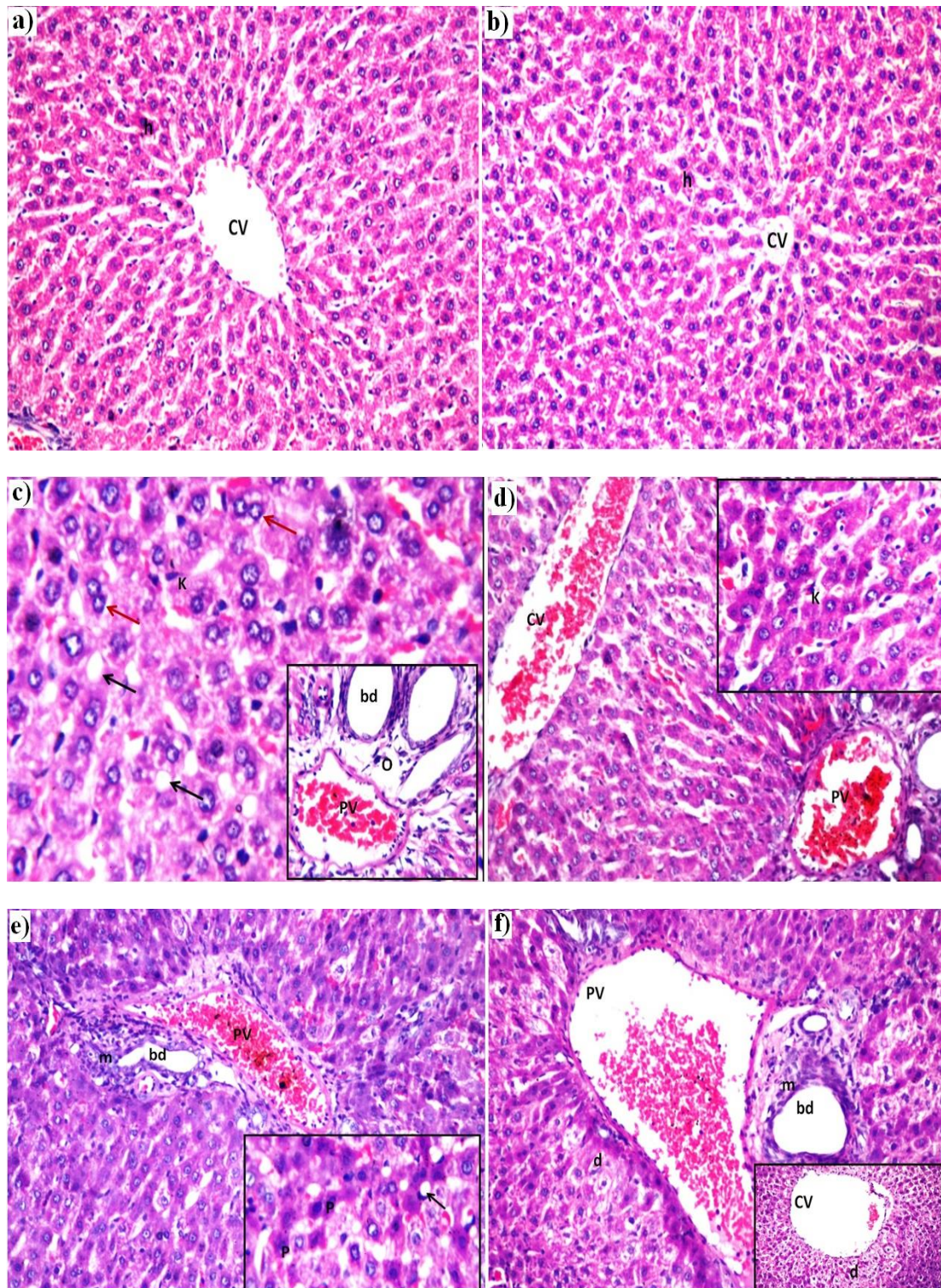


Figure 1: Liver tissue of rats showing a) control group with normal histological (H&E, X 40), b) carob treated group without deviation from normal histological structure (H&E, X 40), c) CYP-treated group with histopathological changes in the hepatocytes (H&E, X 80), d) simult-treated group with congestion in central (CV) and portal (PV) veins (H&E, X 40), e) pre-treated group with focal hemorrhages (h) in hepatic

% values 76.55, 67.48 and 72.45, respectively) through restoring the absent bands and hiding the abnormal bands

DISCUSSION

Hepatotoxicity occurred as a result of CYP has been a limitation due to its use as a successful anticancer

chemotherapeutic drug⁴¹. CYP remains the first line therapy for treatment of metastatic breast cancer and large granular lymphocyte leukaemia⁴². Numerous studies were concerned with studying the various liver lesions caused by CYP⁴³. The microsomal enzymes (cytochrom P450 peroxidases and lipooxygenases) play a vital role in liver

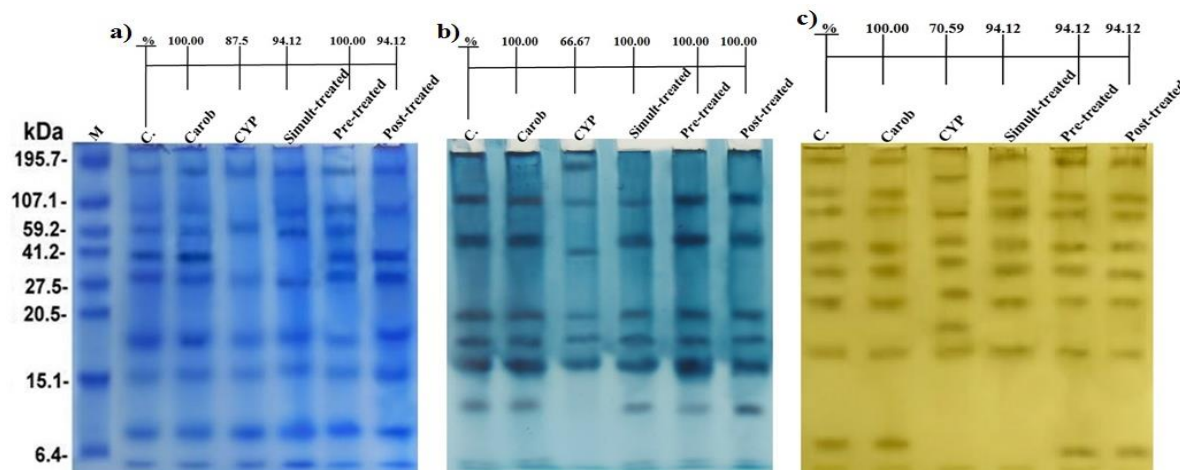


Figure 2: Electrophoretic patterns showing the curative effect of aqueous carob extract against cyclophosphamide induced alterations on a) native protein, b) lipoprotein and c) calcium moieties of the native protein in liver tissue of rats.

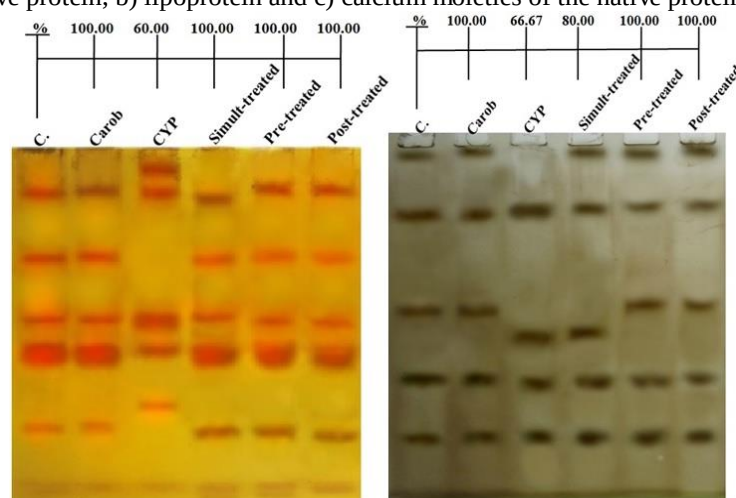


Figure 3: Electrophoretic zymogram showing the curative effect of aqueous carob extract against cyclophosphamide induced alterations on a) catalase pattern and b) peroxidase pattern in liver tissue of rats.

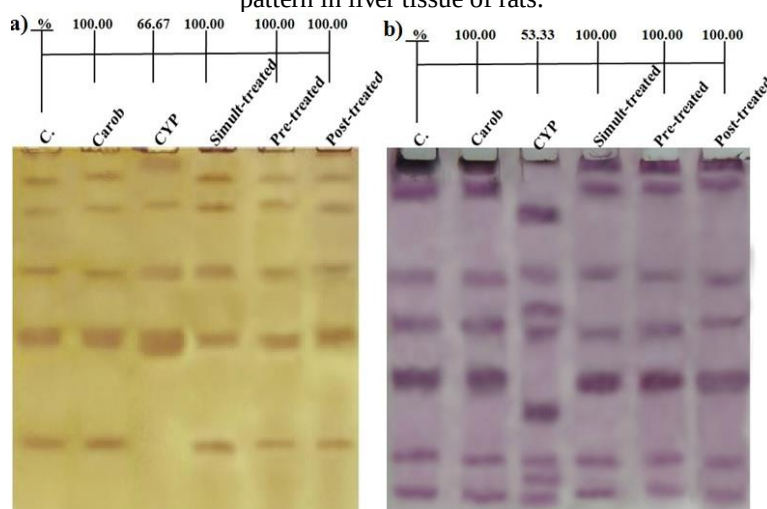


Figure 4: Electrophoretic zymogram showing the curative effect of aqueous carob extract against cyclophosphamide induced alterations on a) α-esterase pattern and b) β-esterase pattern in liver tissue of rats.

tissue by metabolism of CYP (an inactive cytostatic alkylating agent) into active metabolites (phosphoramidate mustard and acrolein)⁴⁴.

ALT is hepatospecific enzyme principally found in the cytoplasm⁴⁵. AST is considered as an enzyme abundant in cytoplasm and mitochondria of hepatocytes. In addition,

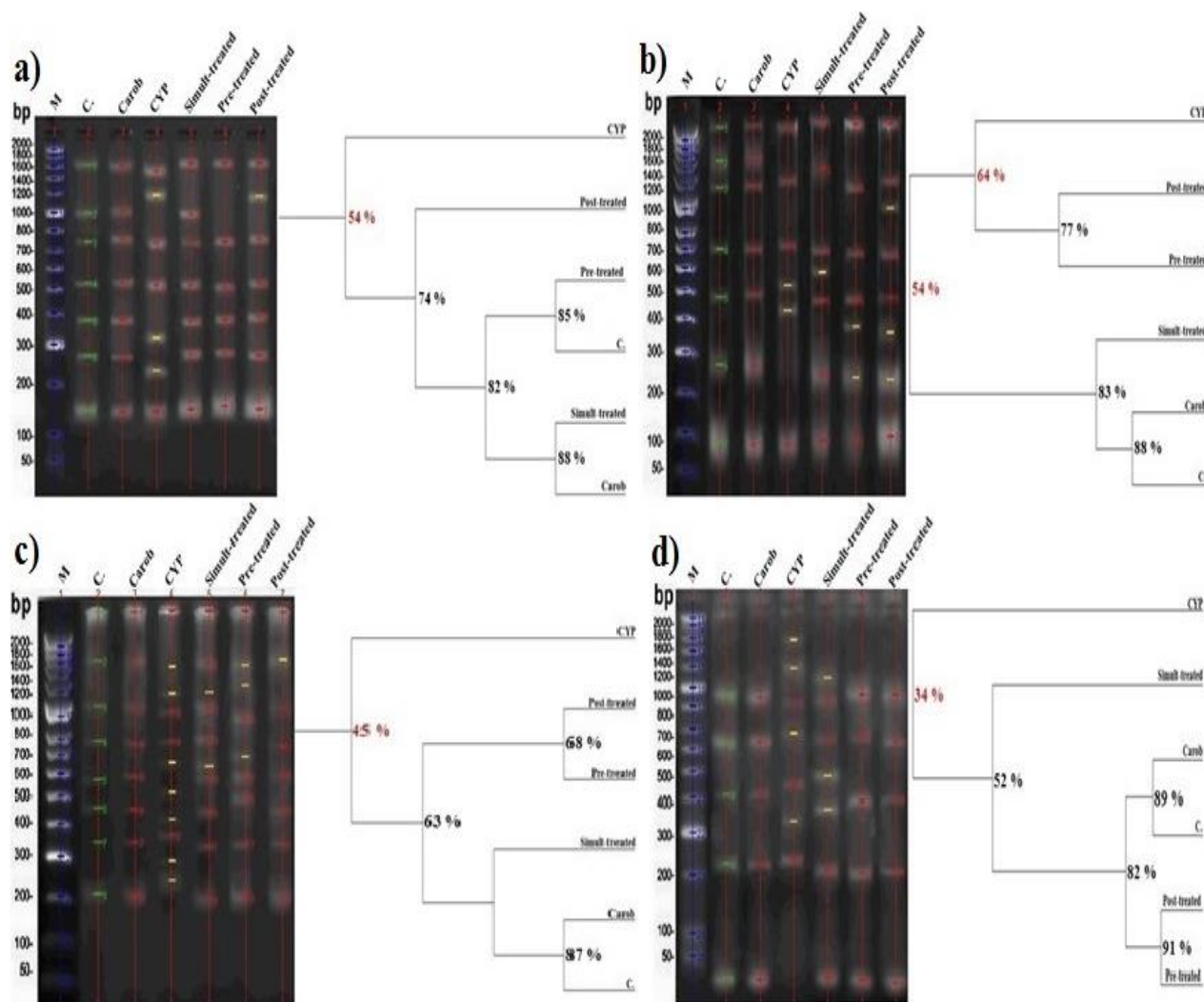


Figure 5: Genomic DNA pattern showing the alterations induced by cyclophosphamide and the curative effect of aqueous carob extract in liver tissue using the random primers a) OPA-04 and OPA-05, b) OPA-07 and OPA-10, c) OPA-11 and OPA-12 and d) OPA-14 and OPA-15.

it is present in heart, brain and skeletal muscles. Elevation of levels of these enzymes is considered as a good sign of necrosis of the parenchymal cells in the liver⁴³. During the current study, CYP enhanced levels of the liver enzymes. This was in accordance with Sreetha *et al.* (2009)⁴⁶ who documented that CYP caused generation of the free radicals which cause cellular damages and loss of functional integrity of hepatocytes membranes and hence liberation of these enzymes in the bloodstream. It is well known that the protein content inside the cells determined not only by rates of synthesis, but also by rates of degradation. Therefore, increased protein content may be due to a decrease in cell protein degradation or an increase in protein synthesis⁴⁷. Serum albumin is the most abundant blood protein and it constitutes about 50% of plasma protein⁴⁸. During the present study, CYP caused significant enhancement in protein profile (total protein and albumin). This was in agreement with Abraham *et al.* (2007)⁴⁷ who suggested that protein level elevated due to decreasing activities of all the lysosomal enzymes and this leads to accumulation abnormal amounts of proteins after CYP administration. Urea and

creatinine are the end metabolic products which required to be freely filtered by the renal glomeruli. Levels of these measurements used as indicators for screening of renal disorders⁴⁹. During the present experiment, levels of these parameters elevated in the CYP-treated group. This was in accordance with Senthilkumar *et al.* (2006)⁵⁰ who reported that increase of these measurements might be attributed due to effect of the toxic CYP metabolites on renal cells through causing intrinsic renal lesions with marked damage in functioning nephrons. In the present study, the carob extract restored levels of the hepatic and renal functions. These findings were supported by previous studies that reported that carob had ameliorative effect against hepatotoxicity⁵¹ and nephrotoxicity⁵². This may refer to the polyphenols richness in aqueous carob extract¹⁷. These active constituents are considered as primal source of the antioxidants which have the ability to scavenge the free radicals especially the hydroxyl radical ($\text{OH}^\bullet$). This radical is the major cause of lesions in the liver and kidney tissue⁵³.

In the current study, CYP enhanced levels of cholesterol and TGs. This was in agreement with the study suggested

by Loudet *et al.* (1984)⁵⁴ who mentioned that CYP is known to result in hypercholesterolemia and hypertriglyceridemia. Moreover, the cellular cholesterol accumulated due to effect of the free radicals which increase cholesterol biosynthesis and its esterification⁵⁵, decrease hydrolysis of cholesteryl ester and reduce cholesterol efflux⁵⁶. Furthermore, Enhancement of these lipid measurements might occur due to decreasing cytochrome P450 activity which subsequently leads to depressing cholesterol 7-hydroxylase activity, the key enzyme in the conversion of cholesterol to bile acids after 7 days following CYP injection⁵⁷ and / or peroxidation of the unsaturated lipids in biomembranes by attack of free radicals. Subsequently, this leads to leakage of these lipids into blood⁵⁸. The aqueous carob extract proved to reduce total cholesterol and T.Gs. This might be due to presence of an insoluble dietary fiber which comprised of 80 % insoluble polyphenol in the carob pod extract⁵⁹. This leads to improvement of endothelial function and reduction of inflammation and fibrosis and hence reduction of serum cholesterol and T.Gs consequently⁶⁰. Results of the current experiment revealed that CYP elevated levels of the heart enzymes CK and LDH. This was in accordance with Shrivastava *et al.* (2011)⁶¹ who substantiated that CYP exhibited cardiotoxicity due to generation of hydrogen peroxide which is an important reactive oxygen species (ROS) because of its ability to penetrate biological membranes when it is converted to OH•⁶².

These free radicals cause oxidation of unsaturated fatty acids in membranes of the cardiocytes causing decrease in membrane fluidity and disruption of membrane structure and hence lead to leakage of these enzymes from disrupted cardiac tissue⁶³.

In the present study, the aqueous carob extract maintained level of these enzymes to near normalcy thereby restoring the membrane integrity and function⁵². These might refer to the antioxidant properties of the carob extract by decreasing the peroxidation reaction and hydrogen peroxide contents in the heart tissues⁶⁴.

The oxidative stress biomarkers, LPO and TAC, were also studied during the current study. It was previously documented that there is a link between oxidative stress and tissue injuries⁶⁵. CYP is well known to have pro-oxidant characters, generating ROS resulting in depletion of cellular detoxifying thiols and antioxidant enzymes⁶⁶. In the present study, CYP intoxication declined TAC level in the liver tissue. This was in agreement with Alkan *et al.* (2012)⁶⁷ who reported that CYP declined the TAC due to decreasing activities of antioxidant enzymes and inactivation of cellular antioxidants. Activities of the antioxidant enzymes may decrease due to effect of acrolein which enhanced the LPO through production of intracellular ROS such as OH• and superoxide anion radicals⁵⁰. Moreover, decline of the TAC may be related to inhibitory effect of acrolein on microsomal enzymes resulting in subsequent elevation in free radicals generation and hence LPO⁶⁸. Furthermore, elevation of hepatic LPO in CYP-treated rats may be attributed to due to depletion of GSH⁵. During the current study, the

aqueous carob extract exhibited high reducing power. This was in agreement with Oktay *et al.* (2003)⁶⁹ who postulated that the carob extract serves as a significant reflection of the antioxidant activity. In addition, the compounds in carob extract and have reducing power, are considered as electron donors and can reduce the oxidized intermediates of LPO processes⁷⁰. Moreover, the antioxidative efficiency of carob extract may refer to presence of flavonoids of quercetin glycosides, catechin and epicatechin gallate, polyphenols of gallic acid and ellagic acid and proanthocyanidins^{71,72}. This was in addition to presence of carotenoids⁷³. All these active phenolic molecules provide the extract with the ability to scavenge the OH• which is the major cause of LPO^{17,64}.

During the current study, CYP induced severe histopathological alterations represented by microvascular fatty changes and diffusion of kupffer cells proliferation with oedema and cystic bile ducts in the liver tissue. This was in agreement with Khan *et al.* (2014)³. This might be due to the oxidative stress and the toxic ROS generated as a result of CYP and involved mainly in the injury mechanism⁷⁴. Furthermore, the liver lesions may refer to the toxic CYP metabolic products which exhibited extensively direct toxic effect on sinusoidal endothelium in the liver, thereby causing necrosis, obstruction and obliteration of hepatic veins^{75,76}. The oxidative injury in the liver tissue was minimized by the carob extract. This may be attributed to the biological benefits of its phytochemical components which possess antioxidant hepatoprotective efficacy and strong radical scavenging activity against oxidative injuries^{77,78}.

The macromolecules such as proteins, lipids and nucleic acids may interact with either of the active CYP metabolites resulting in production of unstable ROS⁷⁹. The electrophoretic pattern is the most commonly tool used to analyze stoichiometry of a specific subunit of a protein complex⁸⁰. In the current study, CYP caused alterations in the electrophoretic native protein pattern. This finding was supported by Stankiewicz and Skrzydlewska (2005)⁸¹ who showed that CYP administration resulted in modifications in the protein structure through the reactive metabolite acrolein and/or ROS generated during CYP metabolism. Also, the alterations in electrophoretic protein pattern may occur due to the LPO product that readily forms adduct with cellular proteins⁸². The carbonyl compounds produced due to combination of acrolein with LPO product. It is well known that the carbonyl compounds are very reactive and can interact with amino acids residues in protein molecules causing structural and functional changes in the antioxidant enzymes⁸³.

Lipids are the most sensitive part of the cellular macromolecules¹¹. During the present study, CYP caused alterations in the electrophoretic lipoprotein pattern. This was in accordance with Ariketh *et al.* (2004)⁸⁴ who suggested that abnormalities of the lipoprotein pattern might be attributed due to formation of acrolein-lysine adducts in the plasma lipoprotein of CYP-treated rats. Subsequently, this leads to modification of lipoproteins and disruption of the cellular lipid levels⁸⁵ (Li *et al.*,

2004). Calcium-binding proteins are low molecular weight acidic proteins. They exert inhibitory effect on formation of hydroxyapatite and, consequently, lead to alteration in the mineralization process⁸⁶. The alterations in these proteins result in abnormal mineralization of tissues⁸⁷. Calcium-binding proteins have a specific role in resistance to the bifunctional alkylating agents which include CYP⁸⁸. In the present study, CYP induced alterations in calcium-binding proteins due to role of this alkylating agent in conversion of an active hydrogen atom from these biologically active macromolecules⁸⁹. The antioxidant enzymes expressed mainly in the liver tissue which is considered as one of the highest antioxidant enzyme capacity in the body due to its major metabolic roles⁹⁰. In the present study, CYP caused alterations in the electrophoretic CAT and GPx isoenzymes. This may occur as a result of variations in rates of protein expression secondary to DNA damage initiated by free radicals and hence affecting the isoenzymes. If there was no alteration in the protein expression, enzymatic activity of these two proteins was not altered⁹¹. Moreover, the alterations in the electrophoretic isoenzymes may be attributed to effect of the free radicals which are directly targeting on the nucleic acids (DNA & RNA) responsible for biosynthesis of these enzymes¹¹. EST-like albumin activity used as prognostic markers for various diseases⁹². It can be visualized by substrate staining with using α - and β -naphthyl acetate in the presence of Fast Blue RR salt as a dye coupler³⁷. The alterations in the electrophoretic EST pattern may occur due to expression of various markers of oxidative stress and the liver toxicity which often results in the alteration of both structure and function of albumin⁹³. Due to presence of polyphenols, the carob extract exhibited antioxidative properties that could play an important role in protection of integrity of the macromolecule against the oxidative reactions⁹⁴. In addition, these polyphenols stimulate activity of the antioxidant enzymes to overcome attack of the free radicals targeting these biomacromolecules⁹⁵. During the present study, CYP caused alterations in the genomic DNA pattern. This was in accordance with many previous studies that reported that damage of the genomic DNA may occur due to the oxidative effect of hydroperoxide CYP derivatives which crossed linking of DNA's double helix and interfering with DNA replication and RNA transcription^{8,96} and / or due to effect of the active CYP metabolites which form covalent bonds with DNA molecules through cross linking purine bases causing modifications in the molecular structure of DNA molecule, thus inhibiting DNA, RNA and protein synthesis leading to formation of micronucleus and hence cell death^{83,97}. The beneficial antioxidant effects could be attributed to the polyphenol inclusion in aqueous carob extract which has antioxidant capacity⁷⁷.

CONCLUSION

The present study demonstrated that CYP induced various alterations in liver tissues at the biochemical, histopathological and molecular levels. Administration of

the aqueous carob extract exhibited protection against the tissue damage produced by CYP through lowering the elevated measurements by decreasing the oxidative stress in a way depending on the order of carob extract administration.

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