Inhibitory Effect of Estrogens, Phytoestrogens, and Caloric Restriction on Oxidative Stress and Hepato-toxicity in Aged Rats[1]

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Objective To investigate the protective effect of 17β-estradiol (E2), peganum harmala extract (PHE) administration and calorie restriction (CR) treatment (60%) on oxidative stress and hepato-toxicity in aged rats. **Methods** Eighteen months old animals that were treated at the age of 12 months were divided into 4 groups: normal control group with free access to food, E2 treatment group, PHE treatment group and CR treatment group of the food given to control group. Six male rats at the age of 4 months were used as a reference group. **Results** Aging significantly decreased superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), and increased lactate deshydrogenase (LDH), gamma-glytamyl transferase (GGT), phosphatase alkalines (PAL), aspartate and lactate transaminase (AST and ALT) activities in the liver. Aging also induced an increased lipid peroxidation level, histological changes and a decreased E2 level. However, treatment with E2, PHE, and CR increased 17β-estradiol, and decreased hepatic dysfunction parameters and lipid peroxidation as well as histological changes in the liver of aged rats. **Conclusion** The antioxidant and hepatoprotective activity of PHE and CR is possibly attributed to its ability to increase E2 level, which as an antioxidant, acts as a scavenger of ROS. Further studies on the pharmaceutical functions of E2 in males may contribute to its clinical application.

Key words: Aging; Phyto (estrogens); Caloric restriction; Male; Lipid peroxidation; Liver dysfunction; Histological changes; Antioxidant

INTRODUCTION

It has been demonstrated that aging is accompanied with a reduction of steroidogenesis which is associated with the imbalance between prooxidant and antioxidant activities, leading to oxidative damage to cellular processes[1-2]. This oxidative damage is induced by free radicals which are highly reactive molecules generated during cell metabolism. These radicals exert deleterious effects on proteins, lipids and DNA according to the age[3], cause numerous diseases, such as inflammation, cancer, arteriosclerosis, hypertension, diabetes mellitus, and also accelerate aging[4]. The process of aging induces several changes in the structure and function of different organs and tissues, and the liver is primarily affected[5]. Epidemiological studies have shown that the rate of progression of chronic hepatic disease is higher in men than in women, suggesting a possible protective effect of estrogens[6]. Furthermore, estrogens exert positive effects on hepatic functions[7-8] and have anti-oxidant properties[2]. It has been shown that caloric restriction inhibits a number of age-associated pathological and biological changes[9-10]. Moreover, isoflavones including aglycones, glycosides and β-glycosides have been found in many plants, such as *Peganum harmala*[11], which are known to be able to scavenge free radicals and to modulate the expression of genes encoding antioxidant enzymes[12]. Despite their beneficial effects, little is known about the positive action of herb extract and caloric restriction on the liver of aging subjects.

This study was to investigate the effect of

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estrogens, *peganum harmala* extract administration and caloric restriction treatment on several parameters related to age-induced oxidative injury in the liver of old male rats.

MATERIALS AND METHODS

Plant Materials

The aqueous extract was prepared as previously described[13]. Briefly, local *Peganum harmala* was collected and air dried, powdered and extracted with double distilled water by refluxing for 36 h (12×3) at 80 ℃. The extract thus obtained was vacuum evaporated until it was completely powdered and re-dissolved in doubly distilled water just before administration

Treatment of Animals

Male Wistar rats aged 12 months were used. The animals were maintained in the animal house facility (Faculty of Sciences, Sfax, Tunisia) at a constant temperature of 25±3 ℃ in a 12 h light/dark cycle. The animals (6 per group) were fed with standard chow with free access to tap water. The rats were divided into normal control group with fre access to food, 17β-estradiol treatment group (1 μg/kg body weight/daily)[7], ethalonic Peganum harmala extract (PHE) treatment group (50 mg/kg body weight/daily)[9] by gastric gavage, and calorie restricted (CR) diet group receiving 60% of the food given to control group (equivalent to 413 kCal/kg body weight/day). Six male rats at the age of 4 months were used as a reference group. The handling of animals was approved by the local Ethical Committee for the Care and Use of Laboratory Animals. At the age of 18 months, the animals were weighed and sacrificed by decapitation with their trunk blood collected. The serum was prepared by centrifugation at 1 500×g for 15 min at 4 ℃. The liver was removed with its fat cleaned and weighted. All samples were stored at -80 ℃ until use.

Steroids and Biochemical Determinations

The estradiol level was measured by RIA using highly specific antibodies from P.A.R.I.S (Compiègne, France). The intra- and inter-assay coefficients of variation were 8% and 5%, respectively, for 17β-estradiol. After homogenization of the liver in a phosphate buffer (1 g/2 mL), the lipid peroxidation was determined in the homogenized liver from the control and treated rats by quantifying the thiobarbituric acid reactive substances (TBARS) as previously described[14]. The protein level was measured with the method of Lowry *et al.*[15]. The superoxide dismutase activity in the sample from control and treated rats was assayed as previously described[16]. The glutathione peroxidase and catalase activities were measured as previously described[17-18]. The levels of hepatic lactate deshydrogenase (LDH), aspartate and lactate transaminase (AST & ALT), gammaglytamyl-transferase (GGT), and phosphatase alkaline (PAL) were measured using commercial kits from Sigma Munich (Munich, Germany) and Boehringer-Mannheim (Mannheim, Germany).

For histological studies, pieces of liver were fixed in a Bouin's solution for 24 hours and embedded in paraffin. Five μm thick sections were stained with blue toluidine borate and examined under Olympus CX41 light microscope.

Statistical Analysis

Data are presented as mean±SEM. The determinations were performed from six animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher's test. $P \leqslant 0.05$ (StatView) was considered statistically significant.

RESULTS

Body and Liver Weight

As shown in Fig. 1, body and liver weights increased significantly with aging. However, the body and liver mass of rats in the three age groups were significantly decreased after treatment with E2, PHE and caloric restriction ($P<0.05$) compared with the controls at the age of 18 months.

Plasmatic 17β-estradiol Level

The 17 β-estradiol level in plasma of young and old rats is shown in Fig. 2. The 17 β-estradiol level was 40 pg/mL in 4 the month-old rats and 27 pg/mL in the 18-month-old male rats. The 17β-estradiol level was significantly higher in the 18-month-old rats after treatment with E2, PHE or CR than in the untreated animals ($P<0.05$).

Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX) Activities

As shown in Fig. 3, SOD, CAT, and GPX activities were lower in the 18-month-old untreated rats than in the young animals ($P<0.05$). However,

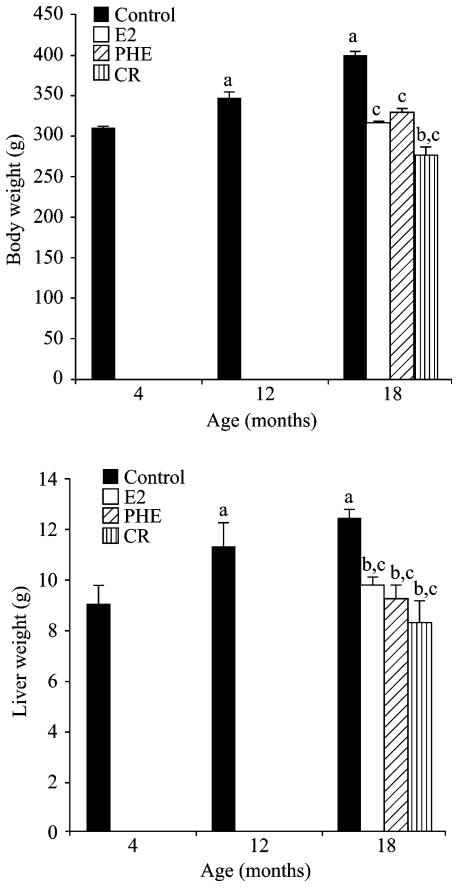


FIG. 1. Effect of aging and E2, PHE, and CR on body and liver weights. Data are expressed as $\bar{x} \pm s$ (n=6). [a]P<0.05, [b]P<0.05, [c]P<0.05 *vs* young control rats (4 months of age), 12- month-old control group, and 18-month-old control group, respectively.

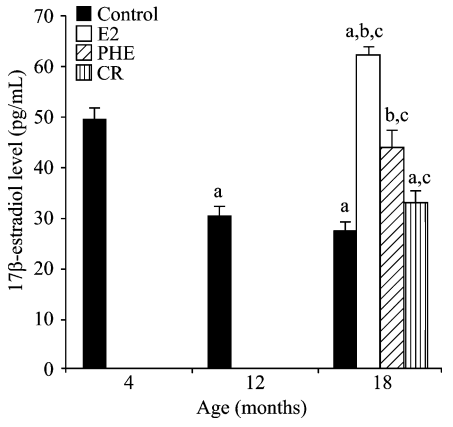
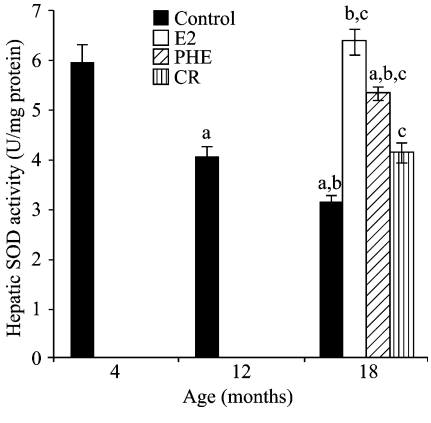
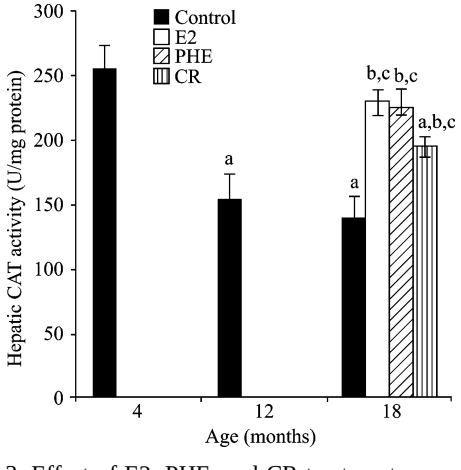


FIG. 2. Effect of aging, E2, PHE, and caloric restriction on 17β-estradiol level. Data are expressed as $\bar{x} \pm s$ (n=6). 17β-estradiol level is presented as pg/mL plasma. [a]P<0.05, [b]P<0.05, [c]P<0.05 *vs* young control rats (4 months of age), 12-month-old control group, and 18-month-old control group, respectively.





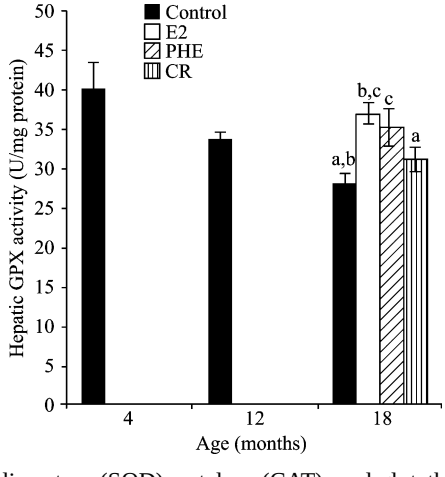


FIG. 3. Effect of E2, PHE, and CR treatment on superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities in the liver of aged rats. Rats were pre-treated with E2 (1 μg/kg bw daily), PHE (50 mg/kg bw daily) or caloric restriction caloric (40%) for six months. The animals were sacrificed by decapitation. Data are $\bar{x} \pm s$ (n=6). [a]P<0.05, [b]P<0.05, [c]P<0.05 *vs* young control rats (4 months of age), 12-month-old control group, and 18-month-old control group, respectively.

the activities of hepatic SOD, CAT, and GPX were significantly increased in the aged rats after treatment with E2, PHE or CR (P<0.01). The SOD activity was augmented by 103%, 70%, and 31% in the liver after treatment with E2, PHE, and CR, respectively. Moreover, the CAT activity was increased by 66%, 63%, and 39%, respectively, in rats after treatment with E2, PHE and CR compared with the untreated 18-month-old rats. Also, the hepatic GPX activity in aged rats after treatment with E2, PHE, and CR was augmented by 31%, 25%, and 11%, respectively, compared with the untreated 18-month-old rats.

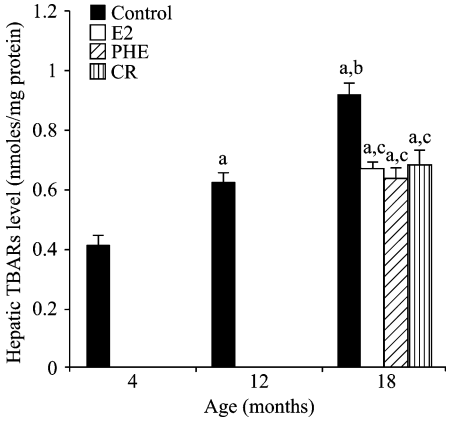


FIG. 4. Effect of aging, E2, PHE, and caloric restriction treatment on hepatic TBARs level. Data are expressed as $\bar{x} \pm s$ (n=6). TBARs level is presented as nmoles/mg proteins. [a]P<0.05, [b]P<0.05, [c]P<0.05 *vs* young control rats (4 months of age), 12-month-old control group, and 18-month-old control group, respectively.

Hepatic TBARs Level

As shown in Fig. 4, the TBARs level in the totally homogenized liver was increased by 48% in the aged rats as compared with the younger animals. However, the TBARs level was decreased by 1.35, 1.20, and 1.34 times, respectively, in aged rats after treatment with E2, or PHE, or CR compared with the untreated aged rats.

Hepatic AST, ALT, LDH, PAL, and GGT Activities

As shown in Fig. 5 homogenated hepatic AST, ALT, LDH, and PAL activities were increased significantly in the untreated aged rats by 2.56, 4, 4.4, and 4.7 times, respectively, compared with the 4-month-old control rats. Treatment with E2, extract containing phytoestrogens or caloric restriction practically reduced the rate of all indices of liver toxicity. The hepatic GGT activity was inhibited by aging and all treatment modalities significantly improved the liver-related conditions.

Histopathological Findings

As shown in Fig. 6, the antioxidative effect of E2, PHE, and caloric restriction was supported by the histopathologic examination. Fig. 6A exhibits liver tissues in young animals. Slightly damaged cells and accumulated lipids were found in liver tissue of 12-month-old rats (Fig. 6B), while clear lipid accumulation and damaged cells were observed in liver tissue of untreated 18-month-old rats (Fig. 6C). Conversely, hepatic toxicity was significantly reduced in aged rats after treatment with E2, PHE, or CR, and their liver function was as the young rats (Figs. 6D-6F).

DISCUSSION

This study revealed that aging of male rats was accompanied with a significantly decreased plasmatic 17β-estradiol level. The decreased estrogen level during aging is related to the production of free radicals synthesized mainly by the mitochondria cells[19-20]. These free radicals induced damages to cell macromolecules and consequently, decline the SOD, CAT and GPX activities and increased lipid peroxidation as observed in our study, which are consistent with the reported findings[21-22]. In addition, histological observation demonstrated a reduction in liver cells and lipid accumulation in old rats. All these variations induced by aging lead to liver dysfunction by significantly increasing the serum GGT, PAL, PAC, LDH, and bilirubin levels. 17β-estradiol, PHE and caloric restriction can protect the liver of aged animals against damage. The increased 17β-estradiol level in rats after treatment with E2, PHE, or CR significantly increased the hepatic SOD, CAT, and GPX activities in our study (P<0.05), which is in agreement with the previous findings[1-2,7-10]. This increase in antioxidant capacity leads to decreased lipid peroxidation and liver toxicity indices. Low estrogen levels are responsible for enhanced-free radical generation[7] leading to increased lipid peroxidation and reduced antioxidant enzyme activity. Treatment with E2, PHE, and CR appears to be an adaptive strategy for protecting liver integrity and function during aging. Indeed, E2 can act on the delay of onset of many age-related functional changes by providing subcellular antioxidant protection. It has been recently reported that estrogens can play an antioxidant role in scavenging free radicals[23] and thus may prevent any damage to cell protein and DNA. The reduced mitochondrial

ROS production and oxidative damage are also suggested as putative mechanisms underlying the anti-aging effects of phyto (estrogens) and caloric restriction[4, 24].

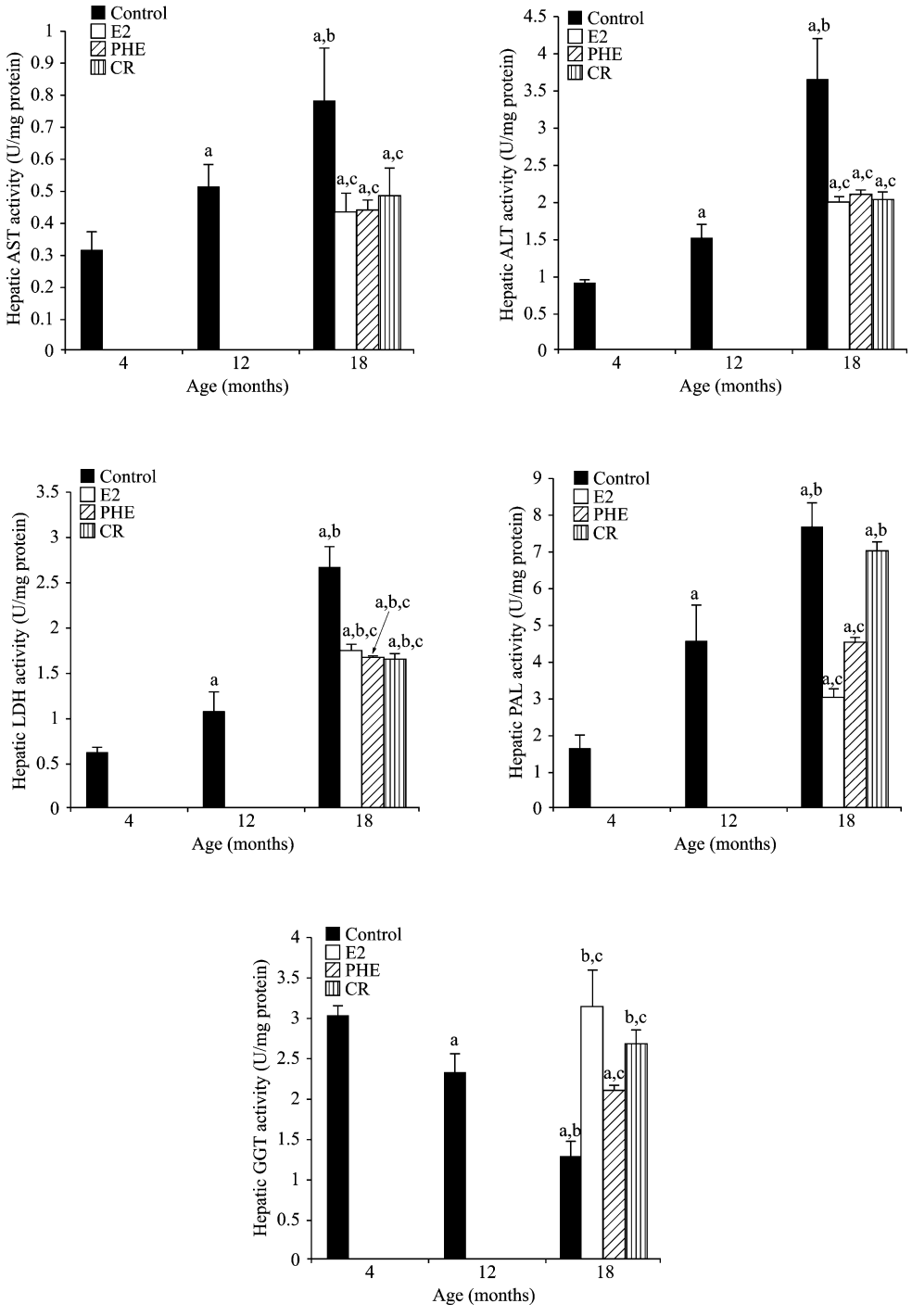


FIG. 5. Effect of aging, E2, PHE, and caloric restriction treatment on hepatic function parameters (AST, ALT, LDH, PAL, and GGT activities in liver). Data are expressed as $\bar{x} \pm s$ (n=6). AST, ALT, LDH, PAL, and GGT activities are presented as Units/L. [a]P<0.05, [b]P<0.05, [c]P<0.05 *vs* young control rats (4 months of age), 12-month-old control group, and18-month-old control group, respectively.

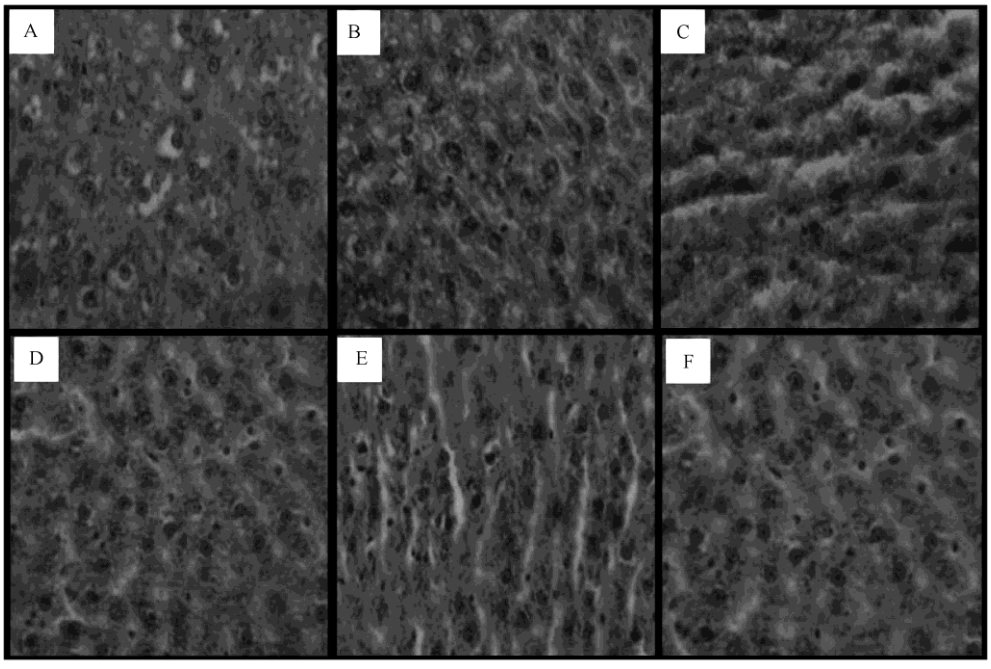


FIG. 6. Effect of aging, PHE, and caloric restriction treatment on the histological changes (100×) in the young control group with regular function of liver (A), reduced liver cells and lipid accumulations in the 12-month-old untreated rats (B), damaged cells and lipid accumulations in the 18-month old untreated rats (C), and a significant protective effect on oxidative stress and hepatic toxicity in old rats after treatment with E2, PHE, and caloric restriction (D-F).

The protective effect of PHE is related with its components that possess antioxidant properties of flavonoids in *Peganum harmala*. In fact, flavonoids isolated from *Peganum harmala*, as a powerful antioxidant, can scavenge the superoxide anion[25], singlet oxygen[26], and lipid peroxy radicals[27], and stabilize free radicals involved in oxidative processes through either hydrogenation or in combination with oxidizing species[28]. Indeed, PH flavonoids isolated from *Peganum harmala* enhance the expression of intracellular endogenous antioxidants such as SOD, catalase, and GPX[20] due to their high activities, and increase the levels of other antioxidant enzymes, such as glutathione reductase, glutathione peroxidase, glutathione-S-reductase, and quinone reductase[29-30]. In fact, phytoestrogens like estrogens bind to a specific estrogen response element (ERE) in the promoter region of target genes, leading to transcriptional activation of various antioxidant genes such as SOD, GPX[31-33]. The protective effect of E2, PHE, and CR on hepatic toxicity and dysfunction is confirmed by its low activity in AST, ALT, LDH, ALP, and GGT, which is consistent with previous findings[12, 34-35].

Furthermore, CR slows down the aging and delays the appearance of age-associated disorders in individuals without under-nutrition[9-10]. Dietary restriction decreases the rate of reactive oxygen species (ROS) generated by rat and mouse mitochondria[36]. Decrease in the ROS level suppresses the age-related oxidative damage to lipids, DNA and proteins, and increases the resistance of cells to oxidative stress[37]. These changes are likely due to a similar number of Leydig, Sertoli and germ cells in aged rats treated with CR, as reported by Chen *et al.*[36]. In addition, our early works[9-10] have confirmed that long-term CR-treatment prevents aging and causes breakdown in estrogen receptor gene expressions and 17β-estradiol level. This increase in estradiol level in aged rats treated with CR, as observed in this study, plays a role in scavenging free radicals. The expression of intracellular endogenous antioxidants such as SOD, CAT, and GPX, can prevent the deleterious action of aging on liver function.

Inconclusion, administration of E2 or *Peganum harmala* extract containing isoflavones to old male rats is able to improve the oxidative stress and liver toxicity. A low caloric diet improves the protection of cells against ROS by increasing the function of cellular antioxidant defence system in which estrogens may play an important role, as demonstrated by the efficacy of estradiol treatment.

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