

# Hyaluronan as a mediator for the hepatoprotective effect of Diosmin/Hesperidin complex

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**Abstract:** Daflon is a phlebotonic drug widely used in chronic venous or lymphatic insufficiency. This study designed to investigate the relation of daflon with hyaluronan as a mediator for the hepatoprotective effect against Carbon tetrachloride (CCl<sub>4</sub>) and/or  $\gamma$ -radiation induced liver damage. Animals of this study were administered CCl<sub>4</sub> (1 ml/kg b.wt.), exposed to  $\gamma$ -radiation (1Gy) and treated with daflon (100 mg/kg/day). Results: Our results showed the ameliorative effect of daflon on cytochrome P450 (CYT P450), lipid peroxidation (MDA), liver enzymes (aspartate amino transferase; AST, alanine aminotransferase; ALT and gamma glutamyl transferase;  $\gamma$ -GT), antioxidant capacity (reduced glutathione; GSH and glutathione per oxidase; GPx), inflammatory markers (C-reactive protein; CRP and interleukin- 6; IL-6), alpha-fetoprotein (AFP) and extra cellular matrix proteins (hyaluronan; HA and hyaluronidase; HAase) which was supported by histopathological examination of liver sections compared to the damage induced in CCl<sub>4</sub> and/or rats exposed to radiation. It could be concluded that the hepatoprotective effect of daflon is mediated via antioxidant and anti-inflammatory activity in addition to preserving native tissue hyaluronan by preventing its degradation.

**Keyword:** Carbon tetrachloride;  $\gamma$ -radiation; Daflon; Hyaluronan; IL-6; hyaluronidase.

## INTRODUCTION

Fruits and vegetables are rich of polyphenolic compounds namely flavonoids. They have many natural properties including antibacterial, antiviral, anticancer, immunostimulant and antioxidant effects (Hosseinimehr *et al.*, 2009). Flavonoids are currently used as therapeutic agents. For example, diosmin and hesperidin, besides being natural constituents of foods of plant origin, are present in high amounts in phlebotonic preparations such as Daflon® (Quintieri *et al.*, 2010) which is widely used for the management of hemorrhoidal disease and chronic venous disease in addition to radiation protection for the patients undergo medical practice such as radiotherapy as regarded in cancer patients (Hosseinimehr *et al.*, 2009). Hesperidin is hydrolyzed by intestinal microflora to its aglycone hesperetin (the flavanone analogue of diosmetin) (Quintieri *et al.*, 2010). In addition, diosmetin is partly converted *in vivo* to hesperetin (Spanakis *et al.*, 2009). Both diosmetin and hesperetin have inhibitory effect on cytochrome P450 (CYT P450) activity (Quintieri *et al.*, 2010).

Hyaluronan (HA) is a high molecular weight polysaccharide that is distributed in all body tissues and fluids (Rostami and Parsian, 2013). The generation of lower molecular weight forms of HA (LMW-HA, ~100-500 kDa) from native high-molecular-mass HA molecules (HMW-HA, ~4,000 kDa) takes place either by hyaluronidase-mediated degradation or oxidative hydrolysis under pathological conditions (Yang *et al.*,

2012). Under these conditions, LMW-HA is more polydisperse, fragmented and stimulates inflammatory cells (Bonafè *et al.*, 2014). The liver is the most important organ involved in the synthesis and degradation of HA. HA is synthesized by special enzymes that are located on the inner surfaces of plasma membranes in some tissue, such as the synovial lining cells and hepatic stellate cells (HSCs). These enzymes are called hyaluronic acid synthases (Rostami and Parsian, 2013).

CCl<sub>4</sub> is a well-documented hepatotoxin used to induce acute and chronic toxic liver injury in a many of laboratory animals. The toxicity mechanism of CCl<sub>4</sub> takes place through biotransformation of CCl<sub>4</sub> by the hepatic microsomal CYT P450 to produce hepatotoxic metabolites, namely trichloromethyl free radicals (CCl<sub>3</sub> and CCl<sub>3</sub>OO $\cdot$ ). These highly reactive compounds reacts with sulfhydryl group, protein-thiol and reduced GSH, thus covalently bind to the cell membrane and leads to disruption of hepatocellular membrane, via lipid peroxidation and this end at necrosis of the cell. This is followed by chloromethylation, saturation, peroxidation and progressive destruction of the unsaturated fatty acid of the endoplasmic reticulum membrane phospholipids (Lee *et al.*, 2007, Noori *et al.*, 2009 and Al-Rasheed *et al.*, 2014). Also, CCl<sub>4</sub> metabolism involves the activation of tissue macrophages which is accompanied by the production of inflammatory and profibrogenic mediators such as transforming growth factor- $\beta$  and interleukin-6 (Al-Rasheed *et al.*, 2014).

Exposure of human to natural background radiation represents a major health dilemma which results in an

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increase of radiation burden in humans as a result of technological advancements, since exposure to low level radiation has become common during a vast amount of human activities such as diagnostic and medical procedures, as well as occupational exposure in radiology workers (Godekmerdan *et al.*, 2010). According to the Nations Scientific Committee on the effects of Atomic Radiation (UNSCEAR 1986) Report, acute doses above 2 Gy, between 2 and 0.2 Gy, and below 0.2 Gy are regarded as high, intermediate and low doses, respectively. Low radiation leads to stimulation of the immune system, while high radiation dose has an immunosuppressive effect (Farooqu *et al.*, 2011).

We aimed to evaluate the hepatoprotective efficiency of daflon against liver damage in personal might actively or passively exposed to intermediate dose of radiation. Accordingly, this aim was achieved through CCL<sub>4</sub> as a model of liver damage in irradiated rats. Hence, cytochrome P450 (CYT P450), lipid per oxidation (Malondialdehyde, MDA), liver enzymes (aspartate amino transferase; AST, alanine aminotransferase; ALT and gamma glutamyl transferase;  $\gamma$ -GT), antioxidant capacity (reduced glutathione; GSH and glutathione peroxidase; GPx), inflammatory markers (C-reactive protein; CRP and interleukin- 6; IL-6), alpha-fetoprotein (AFP) and extra cellular matrix proteins (hyaluronan; HA and hyaluronidase; HAase) were assayed to investigate this effect accompanied with histopathological examination of liver tissue.

## MATERIALS AND METHODS

### Materials

All Chemicals and CCl<sub>4</sub> were obtained from Sigma-Aldrich Chemical Co., USA. Daflon<sup>®</sup> (Servier Egypt Industries Limited, 6<sup>th</sup> October City, Giza, Egypt), consisting of 90% diosmin and 10% hesperidin, was dissolved in isotonic (0.9% NaCl) saline solution immediately before use.

### Experimental animals

Male albino rats (120-150g), 6 weeks old obtained from the Egyptian Holding Company for Biological Products and Vaccines were used as experimental animals. The animals were housed in well ventilated rooms with controlled temperature (25°C), humidity (45-75%) and photoperiod (12-h/12-h light/ dark cycle). All animals had free access to chow and water. The animals were acclimated for one week before experiment. This experiment was carried out according to recommendations in the Guide for the Care and Use of Laboratory. Animal procedures were performed in accordance with the National Institute of Health (NIH No.85:23, revised 1996) and in compliance with the regulations of National Center for Radiation Research and Technology (NCRRT). All experimental procedures were done avoiding animal suffering.

### Irradiation procedure

Whole body gamma-irradiation of rats was performed with a Canadian gamma cell-40, (<sup>137</sup>Cs) at the NCRRT, Cairo, Egypt at a dose rate of 0.45 Gy /min. Rats exposed to 1Gy of whole body  $\gamma$ -radiation on the 20<sup>th</sup> day.

### Induction of hepatotoxicity by CCl<sub>4</sub>

CCl<sub>4</sub> (1ml/kg b.wt.) was administered intraperitoneally 1:1 diluted with olive oil, for two successive days of the experimental course to induce hepatotoxicity (Althnaian *et al.*, 2013). Induction of liver toxicity in rats by CCl<sub>4</sub> was the zero time of experiment.

### Animal groups

Rats were divided into eight equal groups (6rats/group): (1) Control group: animals received 0.5ml olive oil via oral tube for 21 days, (2) Radiation group (R): rats received 0.5 ml olive oil via oral tube during 21 days and exposed to 1Gy, (3) Carbon tetrachloride intoxicated group (CCL<sub>4</sub>): rats received CCL<sub>4</sub>, (4) carbon tetrachloride intoxicated and irradiated group (CCl<sub>4</sub>+R): rats received CCl<sub>4</sub> and exposed to 1Gy, (5) Daflon (D) group: rats received 100mg/kg/day of daflon via oral tube (Sezer *et al.*, 2011) for 16 consecutive days which corresponds to 0.5 ml D suspension were administered via oral tube to the rats started from 5<sup>th</sup> day until the end of experiment, (6) Daflon treated and irradiated group (D+R): rats received D and exposed to 1Gy, (7) Carbon tetrachloride intoxicated and daflon treated group (CCl<sub>4</sub>+D): rats received CCl<sub>4</sub> and D, and (8) Combination group (CCl<sub>4</sub>+D+R): rats received CCl<sub>4</sub>, D and exposed to 1Gy. Animals were sacrificed after one day post exposure to  $\gamma$ -radiation. Serum and liver tissue were collected for biochemical and histopathological examinations.

### Biochemical assays

The GSH content was determined photometrically at 412 nm using 5, 5-dithiobis-2-nitrobenzoic acid (Ellman, 1959). The activity of GPx was assayed according to the method of Gross *et al.* (1967). AST and ALT were determined according to Reitman and Frankel, (1957) and  $\gamma$ -GT according to Szasz, (1974). The extent of lipid peroxidation was assayed by the measurement of MDA according to the procedure described by Yoshioka *et al.* (1979). All photometric determinations were done using (Thermo Electron UV-Visible spectrophotometers U.S.A.). CRP was measured using a test reagent kit according to the modified method of Schultz and Arnold, (1990). AFP was measured by ELISA (provided by Diagnostic Systems Laboratories, Inc., Webster, Texas, USA). IL6 was measured by ELISA kit provided by (Quantikin R&D system USA). Hyaluronan (HA) concentration was measured using an ELISA kit supplied by (MyBiosource USA) according to manufacturer's instruction. HAase activity was measured by ELISA kit supplied by Usen Life Science Inc. according to manufacturer's instruction (Bansal *et al.*, 1982) using

ELISA microplate reader (DV 990 BV 4/6; Gio De Vita and Co., Rome, Italy).

#### Detection of CYT P450

CYT P450 was detected by gene expression using real time PCR (RT-PCR) according to method stated by Livak and Schmittgen, (2001).

**RNA extraction:** In liver tissue homogenate, total RNA was isolated using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to the manufacturer's instruction. Liver homogenate was mixed with 1ml chloroform for 15 s. The suspension was then incubated at room temperature for 15min and centrifuged at 3000g for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol and air-dried. Then, it was dissolved in 200µl RNase-free water. The purity (A260/A280 ratio) and the concentration of RNA were detected using spectrophotometry (Gene Quant 1300, Uppsala, Sweden).

**cDNA synthesis:** The yielded RNA in a volume of 4µg was utilized for cDNA synthesis using an Oligo (dT)12-18 primer and Superscript™ II RNase Reverse Transcriptase. Afterwards, the reaction mixture was incubated at 42°C for 1h (the kit was supplied by SuperScript Choice System (Life Technologies, Breda, the Netherlands)).

**Real-time quantitative polymerase chain reaction (PCR):** Real-time PCR (RT-PCR) (Step One Plus Applied Biosystem, USA) amplification was carried out using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Relative expression of studied gene was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes. The primer used to specifically amplify the gene of CYT P450 was forward: 5'-ACTTCTACCTGCTGAGCAC-3' and reverse: 5'-TTCAGGTCTCATGAA CGGG-3'. The internal control gene of GAPDH was amplified using the primers: forward: 5'-CTCCATTCTTCCACCTTTG-3' and reverse 5'-CTTGCTCTCAGTATCCTTGC-3'.

#### Histopathological study

Autopsy samples of liver were taken from different animals groups (3 samples/group) and fixed in 10% formaldehyde for 24hrs. Serial dilutions of methanol, ethanol and absolute ethanol were used for dehydration after washing by water. Then samples were cleared in xylene and fixed in paraffin at 56°C in hot air oven for 24hrs. Blocks of paraffin bees wax tissue were organized for sectioning at 4 microns thickness by sledge microtome. The tissue sections were fixed on glass slides, deparaffinized and stained by hematoxylin and eosin stain for histological investigation (Banchroft *et al.*, 1996).

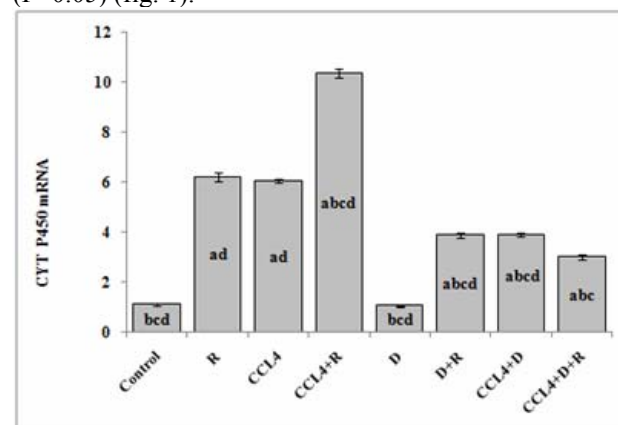
#### Analysis of data

Statistical analyses of all data were presented as the mean ± Standard Error (SE). Statistical analyses were performed by one-way ANOVA test followed by Duncan's post hoc test for multiple comparisons. Differences were considered statistically significant for values of P<0.05. All data were analyzed by SPSS PC-software version 21 for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

## RESULTS

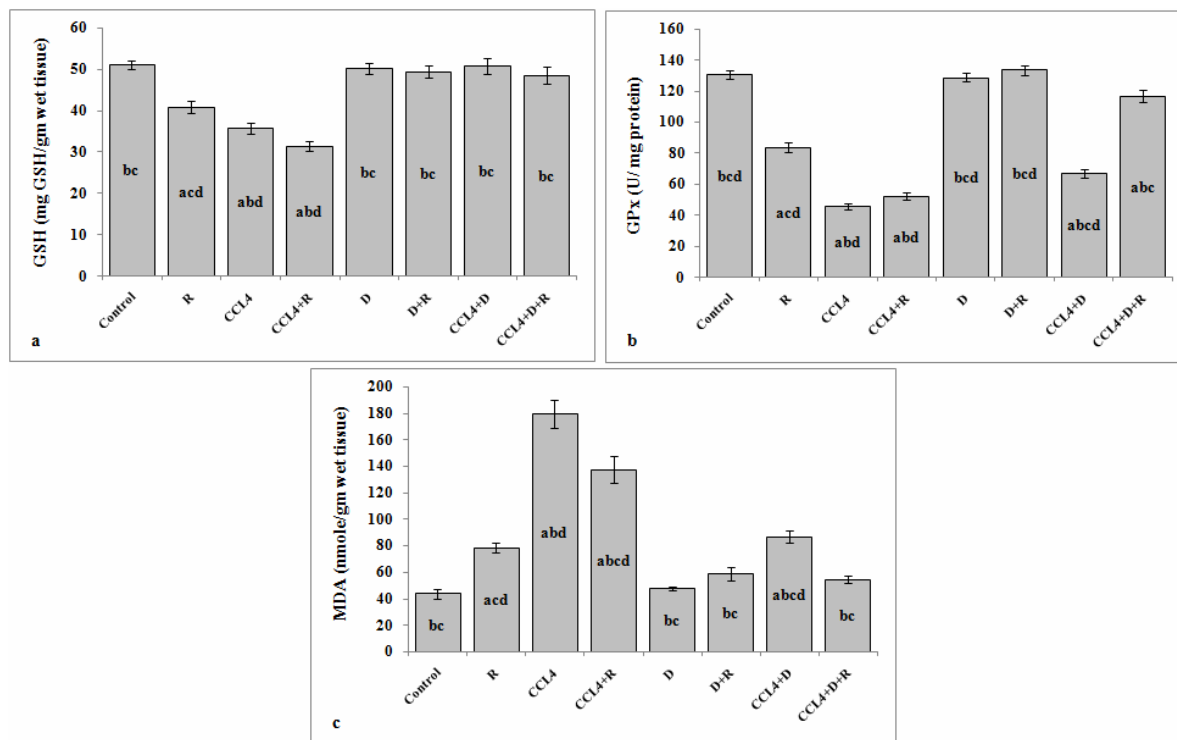
#### Biochemical assays

In the present study, the expression of liver CYT P450 mRNA of control group was 1.12±0.07 folds. In group injected with CCL<sub>4</sub> and/or exposed to R, a significant increase in CYT P450 mRNA was observed as compared to control group. Also, the CYT P450 mRNA results showed a significant elevation in CCL<sub>4</sub>+R compared to R group and CCL<sub>4</sub> group. The experimental data showed no significant change in the expression of liver CYT P450 mRNA in group administered with D compared to control group (P <0.05). Meanwhile, the results revealed significant improvement in CCL<sub>4</sub> and/or R groups after administration of D compared to CCL<sub>4</sub> and/or R groups (P<0.05) (fig. 1).



**Fig. 1:** Effect of Daflon on gene expression of liver CYT P450 mRNA in CCL<sub>4</sub> and/or R-induced hepatic damage in rats. Each value represents the mean ± SE (n=6). Mean values represent at (P<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub> and <sup>d</sup>CCL<sub>4</sub>+D+R.

In the control group, GSH level and GPx activity of liver tissue were 51.08±51.08 and 130.72±2.59 respectively, while lipid peroxidation (MDA) was 43.78±3.77 (fig. 2). In groups treated with CCL<sub>4</sub> and/or R, the results showed significant decrease in liver antioxidant (GSH and GPx) (fig. 2a&b) whereas MDA showed significant increase compared to control (fig. 2c) (P<0.05). In CCL<sub>4</sub>+R group, liver antioxidant showed significant decrease compared to R group and no significant change compared to CCL<sub>4</sub> group (fig. 2a&b). In addition, in CCL<sub>4</sub>+R group, liver MDA revealed significant increase compared to R group and significant decrease compared to CCL<sub>4</sub> group (fig. 2c)



**Fig. 2:** Effect of Daflon on liver (a) GSH (mg GSH/ gm wet tissue) (b) GPx (U/ mg protein) and (c) MDA (nmole/gm wet tissue) in CCL<sub>4</sub>and/or R-induced hepatic damage in rats. Each value represents the mean  $\pm$  SE (n=6).Mean values represent at (p<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub>and <sup>d</sup>CCL<sub>4</sub>+D+R.

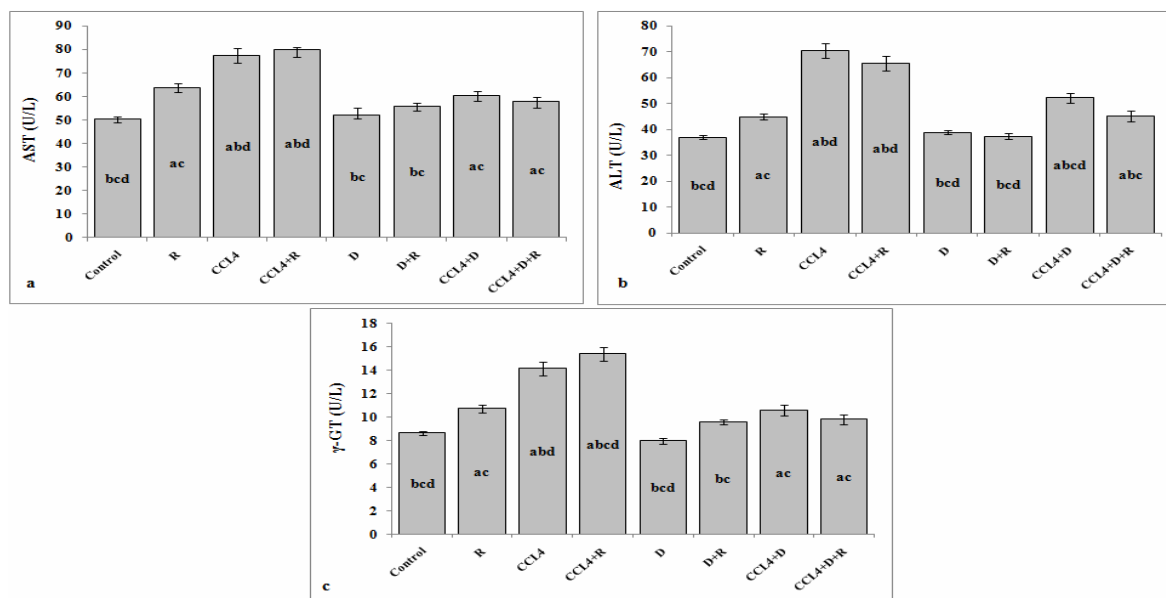
(P<0.05). The data showed no significant change in liver GSH, GPx and MDA in D group compared to control group (P<0.05) (fig. 2). Administration of D in group injected with CCL<sub>4</sub> and/or exposed to R revealed significant increase in GSH compared to CCL<sub>4</sub> and/or R and no significant change observed compared to control (fig. 2a) (P<0.05). Furthermore, administration of D to CCL<sub>4</sub> and/or R groups ameliorates significant disorder in liver GPx and MDA compared to CCL<sub>4</sub> and/or R (figs. 2b&c) (P<0.05). The experimental results observed that, GPx activity and MDA content significantly much improved in CCL<sub>4</sub>+D+R group compared to CCL<sub>4</sub>+D group (figs. 2b&c) (P<0.05).

The serum levels of liver enzymes (AST, ALT and  $\gamma$ -GT) of control group were 50.27 $\pm$ 1.05, 36.98 $\pm$ 0.72 and 8.65 $\pm$ 0.17, respectively (fig. 3). In CCL<sub>4</sub> and/or R groups, significant elevation in these enzymes was observed compare to control group. The results showed in CCL<sub>4</sub>+ R significant increase in  $\gamma$ -GT compared to CCL<sub>4</sub> group (fig. 3c) and significant raise in all liver enzymes compared to R group (fig. 3) (P<0.05). In addition, serum levels of liver enzymes (fig. 3) revealed that no significant changes in D group compared to control group (P<0.05). Also, the data showed an amelioration in liver enzymes of CCL<sub>4</sub> and/or R group after administered of D compared to CCL<sub>4</sub> and/or R groups (P<0.05) (fig. 3).

The inflammatory markers (IL-6 and CRP) of control group were 29.65 $\pm$ 0.98 and 1.09 $\pm$ 0.035, respectively (figs.4a&b). In groups treated with CCL<sub>4</sub> and /or R, the data revealed significant increase compared to control group. Whereas in CCL<sub>4</sub>+R group, the results showed significant elevation in serum levels of IL-6 and CRP compared to R group and CCL<sub>4</sub> group. The experimental data indicated the serum levels of IL-6 and CRP in D group and showed no significant change compared to control group (fig. 4) (P<0.05). However, there were significant improvement in IL-6 and CRP serum levels in CCL<sub>4</sub> and/or R groups administered with D compared to CCL<sub>4</sub> and/or R (fig. 4) (P<0.05). Also, the results of CRP displayed more amelioration in CCL<sub>4</sub>+D+R group compared to CCL<sub>4</sub>+D group (fig. 4b) (P<0.05).

The serum AFP level of control group was 0.25 $\pm$ 0.023 (fig.5). In rats treated with CCL<sub>4</sub> and/or R showed significant increase in serum AFP compared to control. The experimental data demonstrated that no significant change in AFP in D group as compared to control group (fig. 5) (P<0.05). The rats treated with D in CCL<sub>4</sub> and/or R revealed a significant improvement in AFP levels compared to CCL<sub>4</sub> and/or R (fig. 5) (P<0.05).

The liver HA and HAase activity of control group were 15.39 $\pm$ 0.08 and 16.2 $\pm$ 0.11, respectively (figs. 6a&b). In rats injected with CCL<sub>4</sub> and/or exposed to R groups,



**Fig. 3:** Effect of Daflon on serum levels (U/L) of (a) AST, (b) ALT and (c)  $\gamma$ -GT in CCL<sub>4</sub> and/or R-induced hepatic damage in rats. Each value represents the mean  $\pm$  SE (n=6). Mean values represent at (P<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub> and <sup>d</sup>CCL<sub>4</sub>+D+R.

significant increase in liver HA and HAase activity (fig. 6) were observed compared to control rats. The results showed that, there was no significant change in HA level and HAase activity of D group compared to control group (fig. 6) (P<0.05). In addition, HA and HAase results revealed a significant attenuation in CCL<sub>4</sub> and/or R groups after administered of D compared to CCL<sub>4</sub> and/or R group (P<0.05). In CCL<sub>4</sub>+D+R group, the data revealed a more prominent modulation in HA content and HAase activity more than CCL<sub>4</sub>+D group at (P<0.05) (fig. 6).

#### **The histological examination of the liver**

In group of rats kept as control, histological examination to liver sections showed that there was no histopathological alteration and the normal histological structure of central vein and portal area with the surrounding hepatocytes were recorded as in (fig. 7A).

In groups of rats injected with CCL<sub>4</sub> and/or exposed to R-induced hepatic damage, the histopathological examination observed that Kupffer cells were proliferated in between the hepatocytes associated with dilatation in the central vein in R group (fig. 7B), whereas the portal area was infiltrated by inflammatory cells associated with congestion in the portal vein and degeneration in the hepatocytes of CCL<sub>4</sub> group (fig. 7c) and the microscopic examination of liver section of CCL<sub>4</sub>+R revealed hyperplasia in the bile ducts with dilatation and congestion in the portal vein as well as fibrosis, inflammatory cells infiltration and oedema in the portal area (fig. 7D).

After administrated of D, the microscopic examination showed that the central vein was dilated in D group (fig.

8A), the Kupffer cells were proliferated in diffuse manner between the hepatocytes in D + R group (fig. 8B), diffuse kupffer cells proliferation in CCL<sub>4</sub>+D group (fig. 8c) and the portal vein was congested in CCL<sub>4</sub>+D+R (fig. 8d).

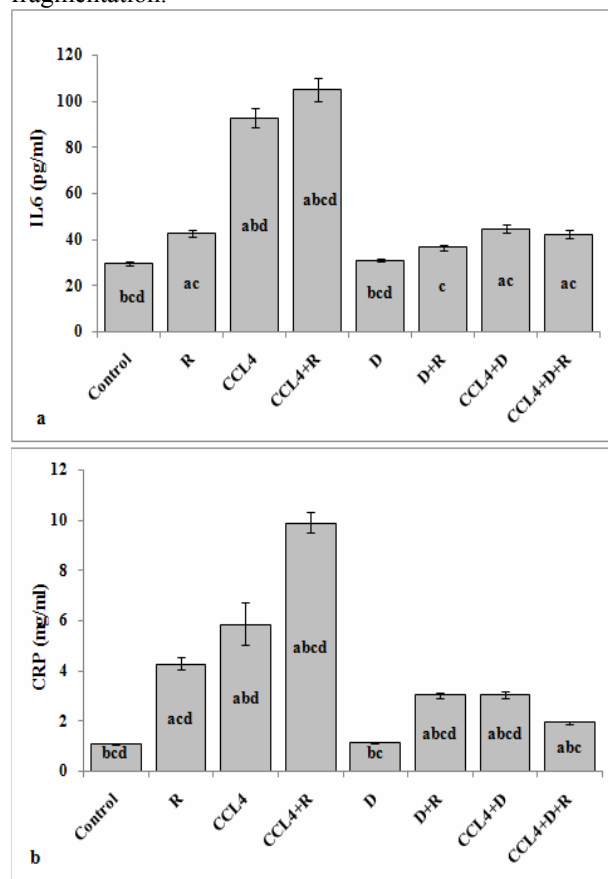
#### **DISCUSSION**

During a hepatocellular injury, the serum HA level rises. Transformation of stellate cells to myofibroblasts, release of various extracellular matrix (ECM) components such as elastin, collagens, glycoproteins and proteoglycans and HA is a later event. Furthermore, HAase hydrolyses the  $\beta$  1-4 glycoside bond between N-acetyl-D-glucosamine and D-glucuronic acid in HA, and makes fragments of different sizes (Rostami and Parsian, 2013). The HA-LMW stimulation is responsible for inflammatory cell migration and homing in damaged tissues but the HA-HMW have contradictory effect as it has anti-angiogenic properties and block the pro-inflammatory effect of LMW. The results obtained after daflon administration display modulation as revealed by a significant elevation reduction in liver HA level and HAase activity in daflon treated rats compared to CCL<sub>4</sub> and/or R rats. This may be attributed to the daflon inhibitory effect on HAase and HA degradation (Martinez *et al.*, 2011) to its fragmented LMW-HA which results in accumulation of HMW-HA in damaged tissue and elicits its protective effect. Subsequently, the HMW-HA interacts with CD44 receptor and prevents extensive liver damage and massive fibrosis (Bonafè *et al.*, 2014). Beside this view, the daflon metabolites, diosmetin and hesperetin, are potent inhibitors of CYT P450 (Quintieri *et al.*, 2010). Interestingly, the chemical structure of hesperidin might be responsible for antioxidant behaviour through direct

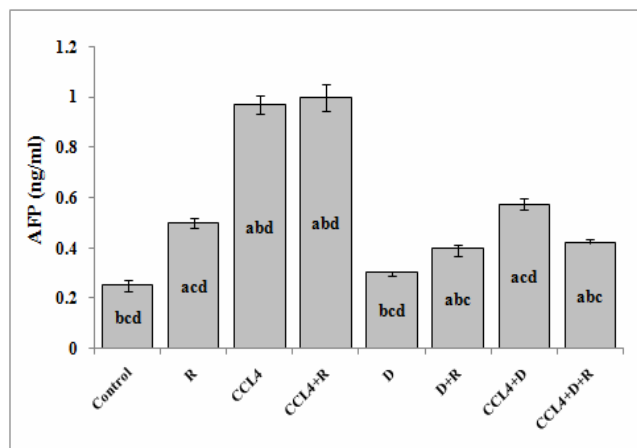
scavenge of ROS and activation of antioxidant enzymes (Hussein and Othman, 2010 and Kuntić *et al.*, 2010). Moreover, hesperidin could augment cellular antioxidant defence capacity through the induction of hemoxygenase-1 (HO-1) via erythroid 2-related factor 2 (Nrf2) dependent manner and extra cellular signal-regulated protein kinases 1 and 2 (ERK ½) signalling pathway (Parhiz *et al.*, 2015). Pradeep *et al.* (2008) elucidated the hepatoprotective effect of hesperidin against radiation induced liver damage and attributed this effect to antioxidant efficacy and reduction of liver enzymes. Also, Tamilselvam *et al.* (2013) reported that hesperidin reduced MDA level and enhanced activity of GPx with recovered levels of reduced GSH. So from our data, daflon administration to rats showed cell membrane stabilizing property and hepatoprotective efficacy. This was confirmed with histological observation in D liver sections revealed diffuse kupffer cells proliferation and the portal vein was congested (fig. 8). These results are in agreement with the findings of Tahir *et al.* (2013). Meanwhile, a marked reduction in serum IL-6, CRP and AFP level have shown after administration of daflon (fig. 4, 5). This elicited anti-inflammatory effect of daflon could be attributed to the up-regulation of peroxisome proliferator activated receptor gamma (PPARγ) and down-regulation of nuclear factor- kappa B (NF- κB) by hesperidin (Mahmoud, 2014). As elucidated in previous studies, daflon reduced IL-6 (Tamilselvam *et al.*, 2013, Tahir *et al.*, 2013, Mahmoud, 2014 and El-Marasy *et al.*, 2014), CRP (Rizk and Sabri, 2009) and AFP levels (Fathy *et al.*, 2014) which are in agreement with our data.

The metabolites of CCl<sub>4</sub> induces hepatic toxicity through a significant increase in CYT P450 activity (Bahashwan *et al.*, 2015), depletion of antioxidant, GPx activity (Yeh *et al.*, 2013) and GSH levels (Lee *et al.*, 2007). Depletion of antioxidants results in enhanced MDA (Saad *et al.*, 2014) as shown in our results (fig. 2). So that, the increase in serum levels of hepatic enzymes (AST, ALT and γ-GT) observed in the present study (fig. 3) attributed to the liver injury by CCl<sub>4</sub>, because these enzymes are placed in cytoplasmic area of the cell and released into circulation after cellular damage (Althnaian *et al.*, 2013). Furthermore, CCl<sub>4</sub> intoxication significantly increased ALT, AST, γ-GT, CRP (Sehrawat and Sultana, 2006 and Sadek and Saleh, 2014) and IL-6 in serum of rats (Zhao *et al.*, 2013). These finding are supported by the experimental data as shown in (fig. 3 and 4). The histopathological observation is consistent with the previous finding where the inflammatory cells infiltrated with congestion in the portal vein in portal area accompanied by degeneration of hepatocytes (fig. 7c). The increase levels of IL-6 and CRP in present study (fig. 4) might be related to the activation of nuclear factor-κB (NF-κB) by CCl<sub>4</sub> which contributes to the production of inflammatory cytokines. IL-6 triggers the activation of transcription factors that bind to DNA elements and

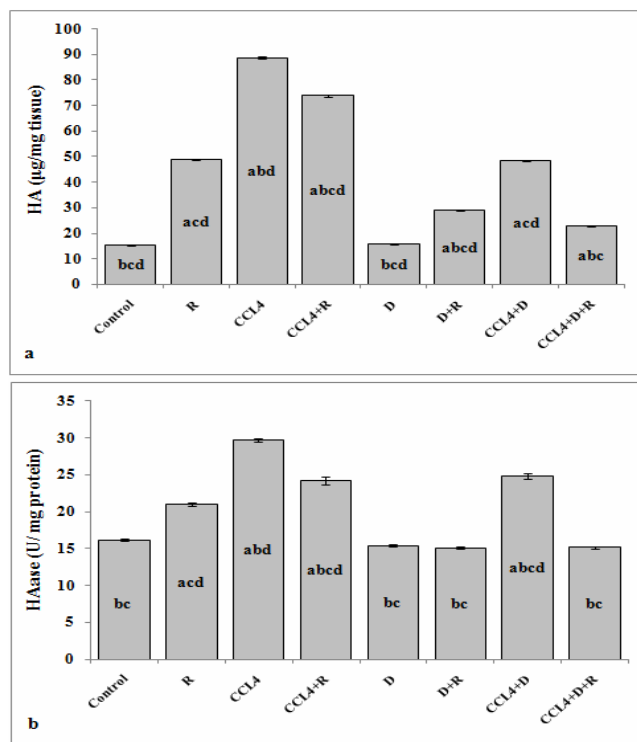
stimulate CRP transcription (Al-Rasheed *et al.*, 2014). The elevated serum level of AFP observed in rats treated with CCl<sub>4</sub> (fig. 5) could be due to formation of ROS and oxidative DNA damage by CCl<sub>4</sub> (Sarhan *et al.*, 2012). AFP is a fetal glycoprotein, following birth its levels decrease rapidly and increase significantly in certain pathologic conditions (Mousa *et al.*, 2012). Similarly, Engelhardt *et al.* (1967) reported that AFP level was increased significantly after the exposure to CCl<sub>4</sub>. The observed elevation in HA level and HAase activity as shown in (fig. 6) might be due to inflammatory response of liver cells resulted from induction of ROS by CCl<sub>4</sub> (Saad *et al.*, 2014). Particularly, degradation of the normally protective HMW- HA into the pro-inflammatory LMW-HA fragments is initiated by ROS, which in turn propagate the inflammatory response *via* the induction of inflammatory chemokines and cytokines by monocytes, macrophages and dendritic cells migrate and home in damaged tissues (Eberlein *et al.*, 2008). Also, Monzon *et al.* (2010) observed that exposure to ROS results in increased HAase activity which promote and orchestrate inflammatory responses associated with HA fragmentation.



**Fig. 4:** Effect of Daflon on serum (a) IL-6 (pg/ml) and (b) CRP (mg/ml) in CCL4 and/or R-induced hepatic damage in rats. Each value represents the mean ± SE (n=6). Mean values represent at (p<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub> and <sup>d</sup>CCL<sub>4</sub>+D+R.



**Fig. 5:** Effect of Daflon on serum AFP (ng/ml) in CCL<sub>4</sub> and/or R-induced hepatic damage in rats. Each value represents the mean  $\pm$  SE (n=6). Mean values represent at (p<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub> and <sup>d</sup>CCL<sub>4</sub>+D+R.

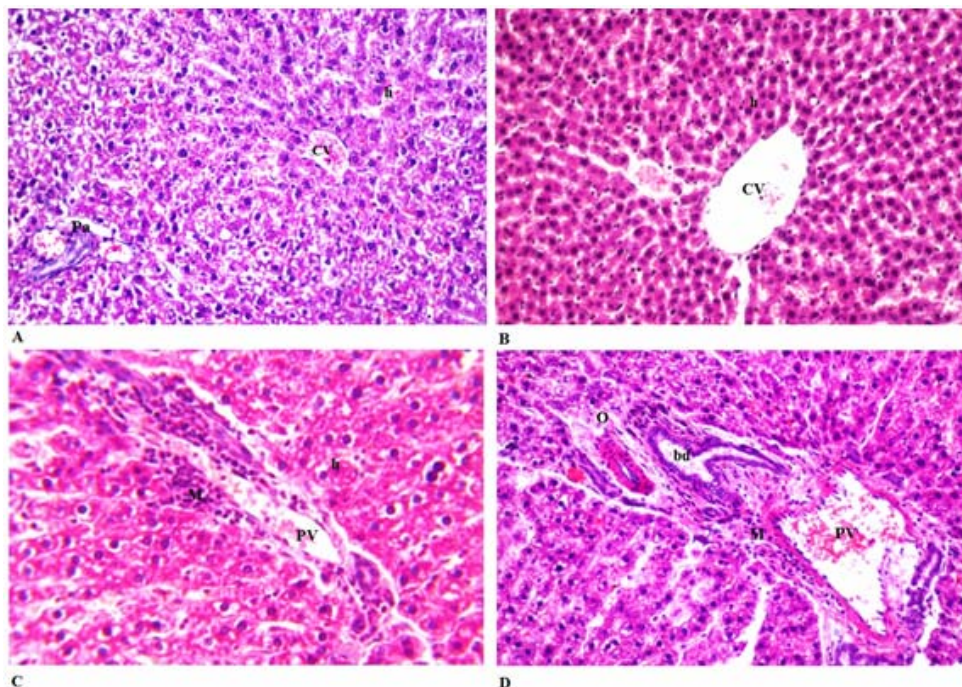


**Fig. 6:** Effect of Daflon on liver (a) HA ( $\mu$ g/mg tissue) and (b) HAase (U/ mg protein) in CCL<sub>4</sub> and/or R-induced hepatic damage in rats. Each value represents the mean  $\pm$  SE (n=6). Mean values represent at (p<0.05) significant differences from <sup>a</sup>control, <sup>b</sup>R, <sup>c</sup>CCL<sub>4</sub> and <sup>d</sup>CCL<sub>4</sub>+D+R.

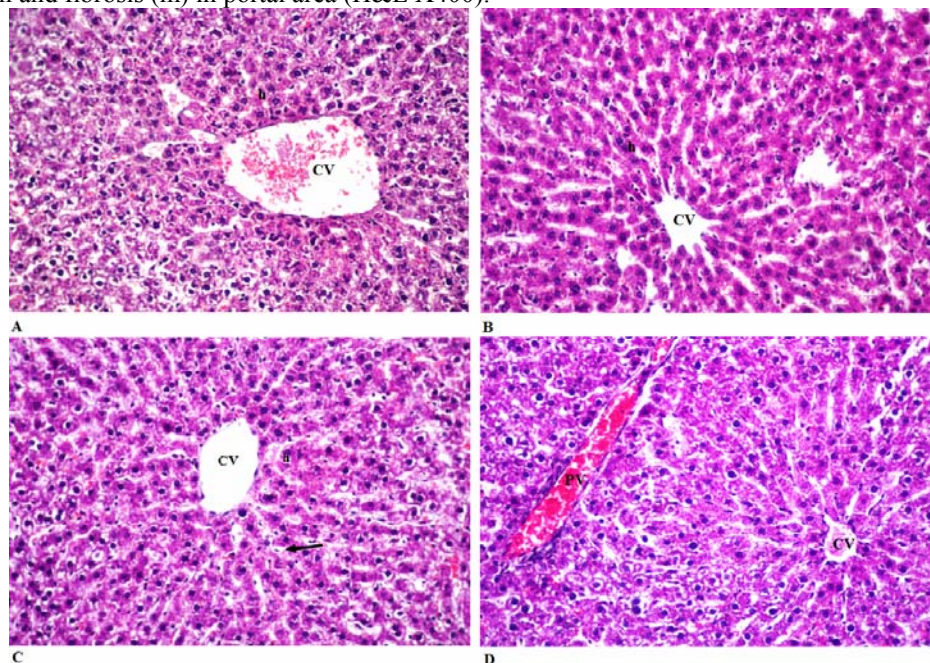
On the other hand, radiation promotes lipid peroxidation formation (MDA) via the generation of free radicals which attack fatty acid moiety of cell membrane, which cause interphase cell death. The deleterious effect provoked by radiation may extend to many enzyme

systems and DNA other than the damage of cell membranes by lipid per oxidation (El-Batal *et al.*, 2012). Data of R and R+CCL<sub>4</sub> groups displayed significant increase in MDA and decrease in GSH and GPx (fig. 2). The observed decreases in antioxidant capacity could attribute to its consumption during neutralization of the oxidative stress induced by irradiation (Shirazi *et al.*, 2013). Besides, the intermediate dose of whole body irradiation have a modulatory effect on detoxifying enzymes (CYT P450) which enhance formation of toxic substances (Rendic and Guengerich, 2012) as proved in the present study (fig. 1). The inflammatory response after radiation increases the intracellular ROS generation, which leads to the activation of stress activated transcription factor NF- $\kappa$ B. Accordingly, NF- $\kappa$ B binds to specific sequences in the promoter region of target genes which in turn enhance a variety of pro-inflammatory molecules expression such as IL-6 (Das *et al.*, 2014) Subsequently, the indicated rise in CRP level due to radiation exposure might be attributed to IL-6 which stimulates CRP transcription (Al-Rasheed *et al.*, 2014). This might explain the significant elevation of IL-6, CRP and AFP in R and R+CCL<sub>4</sub> groups (fig. 4, 5). Thus, the observed elevation of liver enzymes (fig. 3) could be attributed to oxidative stress and inflammation induced by  $\gamma$ -radiation which causes liver tissue damage and leakage of cellular enzymes (Pradeep *et al.*, 2008). The results of the present study showed a significant rise in liver HA and liver HAase post irradiation (fig. 6) this might be attributed to radiation induce generation of ROS and stimulate HAase activity which act to break down the ECM component HA into LMW fragments under oxidative conditions occurs at sites of inflammation, tissue injury and tumorigenesis (Li *et al.*, 2000 and Eberlein *et al.*, 2008). These results were also confirmed by histopathological observation of Kupffer cells which were proliferated between the hepatocytes. This was associated with dilatation in the central vein in R liver sections (fig. 7B). These results are in agreement with the findings of Pradeep *et al.*, 2008.

Worthwhile, the combination of radiation with daflon in CCL<sub>4</sub>+D+R group show augmented antioxidant and anti-inflammatory effect observed in improvement of GPx, MDA, CRP, HA and HAase data compared to CCL<sub>4</sub>+D group (fig. 2bc, 4b and 6a). Pathak *et al.* (2007), reported that the exposure to intermediate dose of gamma radiation stimulate the enzymatic GPx and non-enzymatic defence GSH which enhance the endogenous antioxidant machinery and protect the organs from the damage caused by oxidative stress. The augmented antioxidant improvement due to this combination could be responsible for decrease of cellular damage (MDA) and subsequently the observed decrease of HA, HAase and tissue damage marker (CRP).



**Fig. 7:** Histopathological examination of liver sections. A) Liver of rat in control group showing normal histological structure of central vein (CV) and portal area (Pa) with the surrounding hepatocytes (h) (H&E X400). B) Liver of rat in R group showing dilatation in the central vein with diffuse Kupffer cells proliferated in between the hepatocytes (H&E X400). C) Liver of rat in CCL<sub>4</sub> group showing inflammatory cells infiltration (M) with congestion in the portal vein (PV) in portal area with and degeneration in the hepatocytes (h) (H&E X400). D) Liver of rat in CCL<sub>4</sub>+R group showing dilatation and congestion in the portal vein (PV), hyperplasia in bile duct (bd) with oedema (o), inflammatory cells infiltration and fibrosis (m) in portal area (H&E X400).



**Fig. 8:** Histopathological examination of liver sections. (A) Liver of rat in D group showing dilation in central vein (CV) (H&E X400). (B) Liver of rat in D+R group showing normal histological structure of the hepatocytes with diffuse Kupffer cells proliferation in between (H&E X400). (C) Liver of rat in CCL<sub>4</sub>+D group showing diffuse kupffer cells proliferation in between the hepatocytes (arrow) (H&E X400). (D) Liver of rat in CCL<sub>4</sub>+D+R group showing congestion in portal vein (pv) (H&E X400).

## CONCLUSION

In conclusion, our data revealed that daflon has hepatoprotective properties against CCl<sub>4</sub> and radiation induced hepatotoxicity. These protective effects attributable to more than one mechanisms namely: Modulation of metabolizing enzymes favoring low CCl<sub>4</sub> generated free radical accumulation, antioxidant activity, anti-inflammatory effect and inhibition of hyaluronan degradation to prevent further tissue damage.

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