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BIOCHEMICAL STUDIES ON HEPATOCELLULAR CARCINOMA IN MALE RATS: THE PROTECTIVE ROLE OF PURSLANE SEEDS EXTRACT

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ABSTRACT

Background: Purslane (Portulaca oleracea L., Portulacaceae) has been traditionally used in folk medicine to afford protection against liver injury, although its actual efficacy remains uncertain. **Objective:** To investigate the hepatoprotective and antioxidant effect of purslane seeds extract against hepatocellular carcinoma (HCC) induced by Diethylnitrosamine (DENA). **Materials and Methods**: A total of 50 male Sprague–Dawley rats were randomly divided into five groups (10 rat/group) analyzed for a total experimental period 8 weeks. The first group was maintained as normal control, second group were treated with Purslane seeds extract (50 mg/kg of body weight) daily. Rats of remain groups were injected intraperitoneally with freshly dissolved in sterile 0.9% saline DENA (200 mg/kg body weight) and two weeks later, they received a subcutaneous injection of CCl_4 (3 ml/kg body weight/week) for 6 weeks. Rats of HCC + Purslane group were injected orally with Purslane seeds extract at a daily dose level of (50 mg/kg of body weight) two weeks ***after*** DENA injection while in Purslane + HCC group were injected orally with Purslane seeds extract ***before*** DENA injection. Serum and hepatic liver enzymes, blood reduced glutathione (GSH) and malondialdehyde (MDA) in red blood cells were determined and superoxide dismutase (SOD) enzyme also assayed. Caspases-3 was analyzed using flow cytometric

analysis. **Results**: Serum liver enzymes, AFP, level of MDA and liver caspase 3 were increased in HCC group, while hepatic enzymes, tissue SOD and blood glutathione level were reduced. In groups treated with purslane seeds extract AFP, liver caspase 3, level of MDA and activity of liver enzymes were reduced and the activity of the antioxidant parameters were increased. **Conclusions:** Purslane seeds extract enhanced innate antioxidant activity and ameliorate the DENA-induced HCC and therefore can be used as a hepatoprotective drug in the future.

KEYWORDS: Purslane, antioxidant, hepatoprotective drugs, DENA, carbon tetrachloride, HCC.

1. INTRODUCTION

Liver plays a key role in various pathological disorders such as fatty liver, hepatic virus infection, chemical hepatotoxins, and toxicity cases.[1, 2] Chronic liver diseases are worldwide health problems causing approximately 800,000 deaths per year.[2] Of these, liver fibrosis is caused by inflammation and the excessive accumulation of the extracellular matrix. Subsequently, cirrhosis occurs and can cause hepatocellular carcinoma (HCC). [3] HCC is the sixth most commonly diagnosed cancer with high mortality in the world.[4] The median survival period of HCC is less than 12 months from diagnosis. [5] Several kinds of treatments might be beneficial for HCC, such as local ablative therapy, liver transplantation, resection, hepatic artery transcatheter treatment and surgical therapy. However, limited effective chemotherapy agent available for HCC patients has been found.[6] Thus, there is an urgent need to search for novel therapeutic strategies and agents. Several hepatotoxicants including polycyclic aromatic hydrocarbons, nitrosamines and carbon tetrachloride (CCl_4) are transformed into intermediate reactive oxygen species that have hepatotoxic effects in humans and experimental animal models.[7] Carbon tetrachloride (CCl_4) is widely used as experimental model of liver damage. CCl_4 is activated in the liver to highly reactive trichloromethyl radicals that initiate the free radical-mediated lipid peroxidation of membrane phospholipids, causing functional and morphological changes in the cell membrane, which stimulate hepatotoxicity, fibrosis[8], cirrhosis and HCC in animal species.[9] Moreover, Diethylnitrosamine (DENA) is a well-known hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agriculture chemicals and cosmetics and pharmaceutical products.[10-12] DENA is known to induce liver cancer in experimental animal models through inhibition of many enzymes involved in DNA repair mechanism.[13]

Although there are many strategies for the treatment of liver cancer, its therapeutic outcome remains very poor.[14] Therefore, prevention seems to be the best strategy for lowering the incidence of this disease. *Portulaca oleracea L*, is commonly known as purslane. It is a warm climate, annual and green shoot.[15] Research indicates that purslane offers better nourishment than the major cultivated vegetables due to its shoot that is a rich source of X9-3-fatty acids, α-tocopherols, ascorbic acid, β-carotene and glutathione.[15] These features contribute to the anti-oxidative properties of purslane which derive from the following pharmacologically active substances, including: 28% flavonoids; 8% terpenoids; 6– 12% organic acids; and > 0.5% proanthocyanidins defined as flavonoid-based polymers.[16] Purslane is effective as an antioxidant agent as well as providing nourishment for the liver.[15] Other authors reported that the purslane contains many compounds, including alkaloids, omega-3 fatty acids, coumarins, flavonoids, polysaccharide, cardiac glycosides, anthraquinone glycosides and containing β-sitosterol.[17] Based on these issues and concerns, the present study was designed to investigate the preventive effect leaves extract of purslane on DENA mediated liver damage.

2. MATERIALS AND METHODS

2.1. Chemicals

Diethylnitrosamine (DENA) and carbon tetra chloride (CCl_4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DENA was freshly dissolved in sterile 0.9% saline and given to rats at a single dose of 200 mg/kg body weight.[18] CCl_4 was given to rats at a dose of 3 ml/kg/body weight/week to initiate the effect of DENA.[19, 20]

2.2. Plant materials

Seeds of purslane were purchased from El-Nekity market, Mansoura Dakahlia Governorate, Egypt. The seeds were dried in the shade and powdered by an electric grinder then, they were exhaustively extracted with 80% ethanol. The solvent was removed by evaporation under reduced pressure using Bucchi Rotary Evaporator.[21]

2.3. Experimental animals

Fifty male Sprague–Dawley (SD) rats weighing 180-200 g were obtained from Meladco Feed Company (Aubor City, Cairo, Egypt) and were allowed acclimatization period of 14 days in an ambient temperature of 25 ± 32°C on light/dark cycle of 12/12 hours. Animals received human care and the present study complies with the instruction's guidelines. The local

committee approved the design of the experiments and the protocol conforms to the guidelines of the National Institutes of Health (NIH). All rats were kept in clean polypropylene cages and administered food and water ad libitum. This was approved by the Animal House of Zoology Department, Faculty of Science, Mansoura University, Egypt.

2.4. Hepatocellular rat's model

Experimental hepatocellular carcinoma rats were subjected by a single intraperitoneal injection of freshly prepared DENA (200 mg/kg body weight), then 2 weeks later received a subcutaneous injection of CCl_4 once every week (3 mL/kg body weight) for 10 weeks to promote the carcinogenic effect of DENA.[20]

2.5. Experimental design

At the end of the acclimatization period, the experimental 50 male Sprague–Dawley (SD) rats were divided into five groups (10 rats/group) analyzed for a total experimental period 8 weeks as follows: Group 1(Control): rats of this group did not receive any treatments; Group 2 (Purslane): rats of this group are treated with Purslane seeds extract at a daily dose level of (50 mg/kg body weight); Group 3 (HCC): rats treated with a single intraperitoneal injection of freshly dissolved in sterile 0.9% saline DENA (200 mg/kg body weight) and two weeks later, they received a subcutaneous injection of CCl_4 (3 ml/kg/week) for 6 weeks to promote the carcinogenic effect of DENA; Group 4 (HCC + Purslane): rats were injected orally with Purslane seeds extract at a daily dose level of (50 mg/kg/kg) two weeks ***after*** DENA injection and Group 5 (Purslane + HCC): rats were injected orally with Purslane seeds extract at a daily dose level of (50 mg/kg/kg) two weeks ***before*** DENA injection.

2.6. Blood collection and tissue preparation

After the end of the experimental period (10 weeks), all animals were being fasted for 12 h then sacrificed under chloroform anesthesia. Blood samples were collected from overnight rats. Whole blood was collected in clean, dry tubes with/without EDTA. Whole blood without EDTA centrifuged 15 minutes at 4000 rpm and they were kept in Eppendorf tubes and stored at -20ºC until required for assay of biochemical parameters. Glutathione reduced (GSH) was determined in whole blood. The lower erythrocyte layer in the EDTA tubes was used for determination of MDA while ALT, AST, ALP and total antioxidant capacity were assayed in rat's sera. At the same time, a certain weight of liver tissue from each rat was washed with normal saline and then it was homogenized in ice cold phosphate buffer (50 mM, pH 7.5) and the resultant homogenate (10%, w/v) was centrifuged at 12000 rpm for 20

min at 4˚C in a cooling centrifuge then the supernatant was collected and stored at -20°C for subsequent biochemical assays which include SOD and catalase.

2.7. Biochemical analysis

Alpha-fetoprotein (AFP) level was estimated by immunoenzymatic colorimetric method according to **Acosta, (1983).**[22] Alanine transaminase (ALT) and Aspartate transaminase (AST) activities in serum were assayed according to the method of **Reitman and Frankel (1957)**[23] while serum alkaline phosphatase (ALP) and GGT were determined by using kits obtained from Biodiagnostics according to the method of **Belfield and Goldberg (1971)**[24] **and Szasz (1969)**[25]**,** respectively. Assay of superoxide dismutase (SOD) activity in liver was determined according to the method described by **Nishikimi et al., (1972).**[26] Assay of liver reduced glutathione (GSH) concentration in blood was done by the method of **Beutler et al., (1963).**[27] Lipid peroxidation was evaluated on the basis of Malondialdehyde (MDA) level, MDA in RBCs was determined using the method described by **Stocks and Donnandy, (1971).**[28] Other chemicals used throughout this investigation were of the highest analytical grade available.

2.8. Determination of Caspases-3.

Fresh liver tissue specimens were transported to the laboratory in isotonic saline and prepared according to the method described by **Tribukait *et al*., (1975)**[29] For the caspases-3 assay, liver cell suspensions were prepared with a PBS/BSA buffer. One hundred microliters of the cell suspension were added to 7 µl of antibody (FITC Rabbit Anti- Active Caspase-3 (CPP32; Yama; Apopain, BD Bioscience), mixed well and incubated for 30 min at room temperature, the cells were washed with BD Perm/Wash (BD Bioscience), centrifuged at 400 rpm for 5 min and the supernatant was discarded, the cells finally were re-suspended in BD Perm/Wash and analyzed using flow cytometry **(Belloc *et al*., 2000).**[30]

2.9. Statistical analysis

The data were expressed as mean ± S.D and statistical analysis were performed by using ANOVA followed by post hoc test (Tukey). It is a parametric statistical analysis that compares between-and within-groups variance to measure differences between two or more groups. The P value less than 0.05 was considered as statistically significant. The data analysis was done with SPSS version 22.

3. RESULTS

3.1. Serum liver enzymes

As shown in Table 1, there was no significant difference (P > 0.05) in activities of serum AST, ALT, ALP and GGT enzymes in Purslane rats compared with control group. There were significant elevations of serum AST, ALT, ALP and GGT enzymes activities (p < 0.0001) in HCC rats compared with control group**.** In addition, there was decrease activity of serum AST, ALT, ALP and GGT in rats Purslane ± HCC (AST: P < 0.0001; ALT: P < 0.001; ALP: P < 0.001 and GGT: P < 0.05) and HCC ± Purslane (AST: P < 0.0001; ALT: P < 0.001; ALP: P < 0.001 and GGT: P < 0.05), respectively compared with HCC but the decrease in HCC ± Purslane more than Purslane ± HCC rats group with no significant difference between treated groups.

Table 1: Effect of Purslane administration on serum liver enzymes in rats infected with HCC alone and those treated with Purslane before/after HCC.

Groups	Serum liver enzymes			
	AST (U/L)	ALT (U/L)	ALP (U/L)	GTT (mg/dL)
Control	48.3±19.2	39.5±4.8	58.6±18.5	21.2±3.3
Purslane	45.2±23.2	34.6±7.4	59.8±18.6	22.3±4.1
HCC	153.4±47.7	118.8±51.8	185.1±27.6	35.6±9.5
Purslane + HCC	57.4±13.1	55.8±18.1	67.6±23.3	28.1±6.7
HCC + Purslane	48.2±15.6	53.6±15.21	64.1±16.8	26.8±3.9
HCC vs Control	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***
HCC vs Purslane + HCC	< 0.0001***	< 0.001*	< 0.01*	< 0.05
HCC vs HCC + Purslane	< 0.0001***	< 0.001*	< 0.001**	< 0.01*

Data are expressed as mean ± S.D. (n = 10 in each group). HCC: hepatocellular carcinoma. ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: alkaline phosphatase and GGT: gamma-glutamyl transferase.

*P > 0.05 considered not significant, * P < 0.05 considered significant, **P < 0.01 considered high significant, and ***P < 0.0001 considered extremely significant and (ns) not significant.*

3.2. Hepatic liver enzymes

As shown in Table 2, there was no significant difference (P > 0.05) in activities of hepatic AST, ALT, ALP and GGT enzymes in Purslane rates compared with control group. There were significant elevations of hepatic AST, ALT, ALP and GGT enzymes activities (p < 0.0001) for all parameters compared with control group. In addition, there was decrease

activity of hepatic AST, ALT, ALP and GGT in rats Purslane ± HCC (AST: P < 0.0001; ALT: P < 0.001; ALP: P < 0.001 and GGT: P < 0.05) and HCC ± Purslane (AST: P < 0.0001; ALT: P < 0.001; ALP: P < 0.001 and GGT: P < 0.05), respectively compared with HCC but the decrease in HCC ± Purslane more than Purslane ± HCC rats group.

3.3. Antioxidants profile

Table 3 showed that, intoxication of rats with DENA and CCl_4 (HCC group) induce significant (P < 0.0001) the level of Superoxide dismutase (SOD) induce significant (P increase in MDA level, a significant (P < 0.0001). Moreover, the administration of Purslane to HCC rats succeeded in restoring oxidative stress through decreases in MDA level and induced a significant improvement in the antioxidant biomarkers by the observed increase in GSH, GST and SOD in all the examined tissues, indicating the antioxidant activity of Purslane but the HCC ± Purslane more effective than Purslane ± HCC rats group with no significant difference between treated groups.

Table 2: Effect of Purslane administration on hepatic liver enzymes in rats infected with HCC alone and those treated with Purslane before/after HCC infection.

Groups	Parameters (U/g wet tissue)			
	AST	ALT	ALP	GTT
Control	22.8±4.0	56.4±17.8	30.8±18.5	23.5±4.3
Purslane	24.1±5.5	59.0±13.4	29.8±7.2	24.6±3.4
HCC	17.2±4.4	41.3±10.7	51.1±10.6	31.5±7.5
Purslane + HCC	19.5±3.8	50.3±10.0	36.7±16.0	25.8±6.7
HCC + Purslane	20.0±6.0	52.6±15.2	40.2±14.1	25.0±6.8
HCC vs Control	*< 0.001*	*< 0.001*	*< 0.0001*	*< 0.01*
HCC vs Purslane + HCC	*< 0.05*	*< 0.05*	*< 0.05*	*> 0.05*
HCC vs HCC + Purslane	*< 0.05*	*< 0.05*	*< 0.05*	*< 0.05*

Data are expressed as mean ± S.D. (n = 10 in each group). HCC: hepatocellular carcinoma. ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: alkaline phosphatase and GGT: gamma-glutamyl transferase.

P > 0.05 considered not significant, * P < 0.05 considered significant, **P < 0.01 considered high significant, and ***P < 0.0001 considered extremely significant and (ns) not significant.

Table 3: Effect of Purslane administration on oxidate stress and antioxidant biomarkers in the liver of control and different treated rat groups (means ± SD).

Groups	SOD (% inhibition)	GSH (mMol/g Hb)	GST (mMol/gHb)	MDA (mg/dL)
Control	57.5±6.4	242.0±25.2	0.34±0.08	7.3±0.9
Purslane	55.2±6.5	253.7±49.8	0.33±0.09	7.5±0.8
HCC	32.2±4.2	145.6±14.2	0.19±0.11	10.1±1.3
Purslane + HCC	46.4±6.9	200.7±54.9	0.26±0.05	8.7±0.6
HCC + Purslane	48.2±5.7	210.7±42.1	0.29±0.07	8.0±0.7
HCC vs Control	< 0.0001***	< 0.001**	< 0.001*	< 0.0001***
HCC vs Purslane + HCC	< 0.0001***	< 0.01*	> 0.05	< 0.0001***
HCC vs HCC + Purslane,	< 0.0001***	< 0.0001***	< 0.01	< 0.0001***

Data are expressed as mean ± S.D. (n = 10 in each group).

HCC: hepatocellular carcinoma; SOD: superoxide dismutase; GSH: glutathione; MDA: malondialdehyde P > 0.05 considered not significant, * P < 0.05 considered significant, **P < 0.01 considered high significant and ***P < 0.0001 considered extremely significant and (ns) not significant.

3.4. Immunological study

The mean level of AFP was 1.01±0.23 ng/mL in control group, 1.10±0.25 ng/mL in rats treated with Purslane, 2.24±0.61 ng/mL in HCC rats group and it was 1.30±0.44 and 1.06±0.20 ng/mL in rats treated with Purslane before and after infected with HCC, respectively. Student T-test indicated that, there was no significant difference (P > 0.05) in rates treated with Purslane rats group compared with control group while, there was extremely high significant increase (P < 0.0001) HCC rats group compared with control group. In addition, there was decrease (P < 0.0001) in rats treated with Purslane before and after HCC infection compared with HCC alone but in rats treated with Purslane seeds extract after more decrease than before.

3.5. Flow cytometric hepatic caspase-3

Figure 1 showed hepatic apoptotic marker % (caspase 3) in control and different treated rat groups. In the case of HCC rats group, there was a significant increase in liver caspase 3 compared with control rats group. In contrast the treatment of HCC rats showed a significant decrease in liver caspase 3 but not significant in the case of Purslane administration in caspase 3 if compering to HCC rats group.

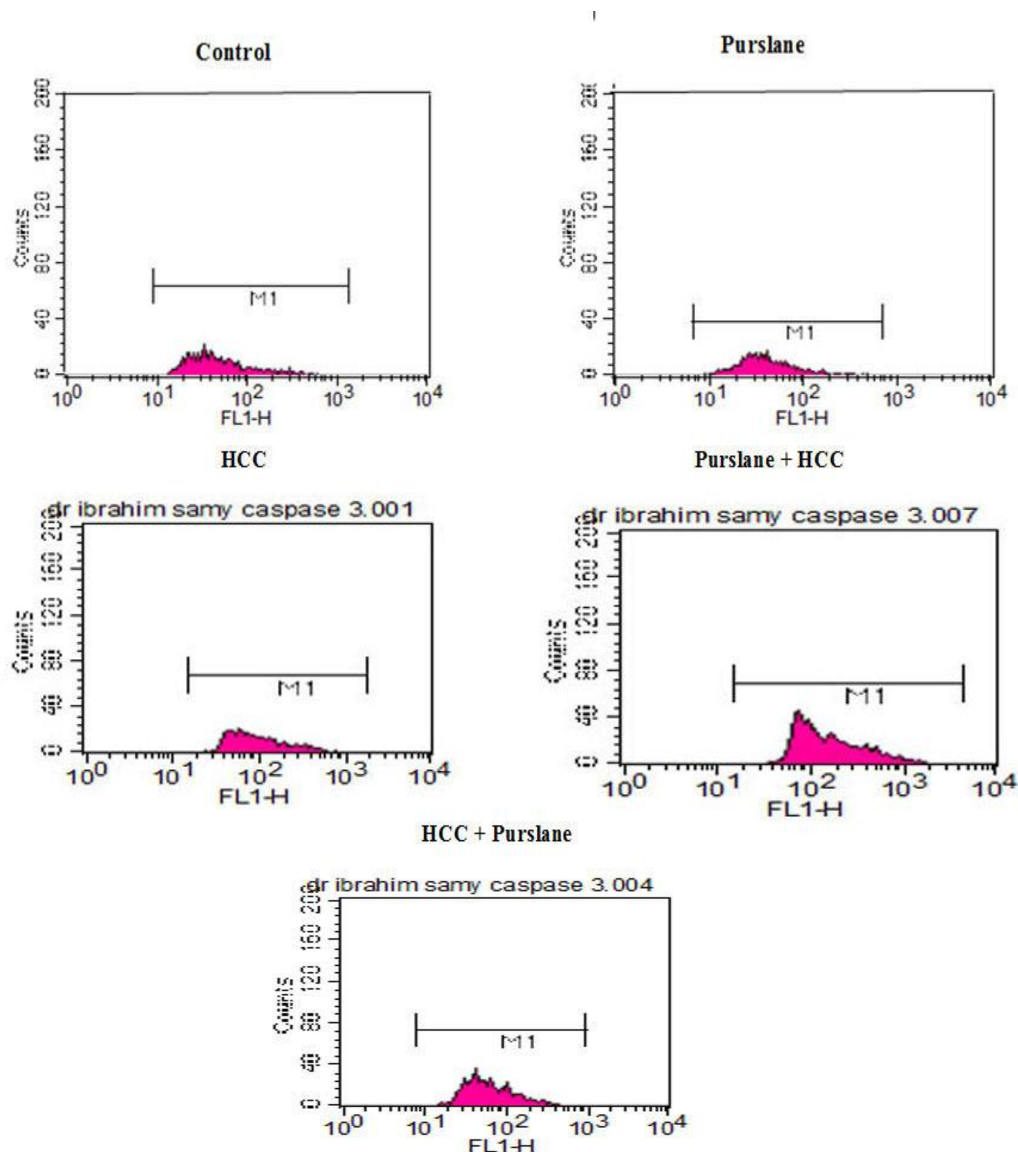

Fig. 1: Flow cytometeric hepatic caspase-3 (as an apoptotic marker) analysis showing in control and different treated rat groups.

4. DISCUSSION

There has been growing interest in dietary bioactive compounds obtained from natural sources which have a therapeutic effect against various diseases including chemoprotective properties against cancer.[31] HCC is a common disease, being the third leading cause of death worldwide.[32] The current study suggests that treatment with DENA and CCl_4 is a good model for the induction of HCC in rats.[33] In the present study, we observed elevation of serum AST, ALT and ALP in HCC rats. These findings may be due to damage to hepatocytes caused by exposure to DENA resulting in hepatic dysfunction and subsequent leakage of these enzymes from the neoplastic cell into circulation.[34] Elevated serum aminotransferase

activity is more specific for liver injury due to damage to the liver cell membrane.[35] As well, alkaline phosphatase is used as a specific tumor marker for making diagnoses in the early detection of cancer.[35] This enzyme is involved in the transport of metabolites across cell membranes, in protein synthesis, secretory activities and glycogen metabolism. It is a membrane-bound enzyme and its alteration is likely to affect the membrane permeability that produces derangement in the transport of metabolites.[37] The observed increases of serum and liver ALP in HCC rat groups may be due to altered gene expression.[38] Upon treatment with purslane extract, the levels of these markers were restored to near normal or were only slightly elevated, indicating protection against liver damage. Such a restoration of increased serum levels of hepatic enzymes to the normal range reflects protection against hepatic damage caused by hepatotoxins.[39] The obtained data were in line with those of **Venukumar and Latha, (2002)**[40] whom mentioned that the raise in the activities of ALT, AST and ALP in rats' serum was a sign of hepatocellular damage of liver similar to acute viral hepatitis. On the other hand, **Ahmida (2010)**[41] reported that the hepatoprotective effective effect of purslane was due to the phytochemical present in it, included omega-3-fatty acids, Bcarotene, flavonoids and alkaloids.

The hepatotoxicity of CCl_4 is due to reductive dehalogenation products, such as trichloromethyl (CCl3·) and trichloromethyl peroxyl (CCl3O2·) radicals **(Brattin et al., 1985).**[42] These radicals can bind to proteins and lipids or remove a hydrogen atom from unsaturated fatty acids, thereby initiating lipid peroxidation and contributing to liver injury.[43] Hepatocyte injury initiates the activation of Kupffer cells which secrete potent mediators of the early inflammatory response, such as reactive oxygen species (ROS), especially superoxide anions that accounted for the formation of peroxynitrites and hydrogen peroxides (H_2O_2) therefore oxidative stress can be occur.[44] The antioxidants could attenuate this oxidative damage caused by free radicals indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radicals.[45] Antioxidants such as superoxide dismutase (SOD) can scavenge the superoxide anions whereas the glutathion reduced (GSH) is responsible to remove H_2O_2 through the action of glutathione peroxidase.[46] Also, H_2O_2 is consumed by the action of catalase.[47] In this sense, the antioxidant action plays an important role in protecting against CCl_4-induced liver injury.

In the current study, the HCC rats group suffered from severe oxidative stress, achieved by elevation of MDA level and depletion of antioxidant enzymes. This may be due to the

conversion of cellular poly-unsaturated fatty acids to the toxic product MDA which has a cytotoxicity and inhibitory action on cellular protective enzymes.[48] HCC caused by carcinogenic DENA generally reflects instability of liver metabolism associated with free radicals species (ROS) generation, which leads to oxidative stress and alterations in antioxidant defense mechanisms.[49] In rats, DENA is a potent hepatocarcinogen influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation accompanied by hepatocellular necrosis and induces DNA carcinogen adducts, DNA-strand breaks and in turn hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic focal lesions.[50] Increased level of MDA has been reported during DENA-induced hepatocarcinogenesis. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense.[51, 52] Moreover, HCC causes depletion of SOD and GSH contents. Such studies support the current findings, as the current study showed a significant decrease in the activities of antioxidant enzyme in the liver of animals treated with carcinogen. Antioxidants are substances that either directly or indirectly protect cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions.[53] The observed decrease in SOD activity in liver suggests the inactivation of antioxidant enzymes; this is possibly due to increased superoxide radical production or to an inhibition by H_2O_2 as a result of corresponding decrease in the activity of catalase which selectively degrades H_2O_2.[54] The decreased GSH and SOD observed in the HCC group of rats may be due to accumulation of lipid peroxidation that was seen to increase during carcinogenesis.

In this respect, **Naeem and Sohail., (2013)**[55] reported that phytochemical constituents isolated from portulaca oleracea including steroids, vitamins, minerals, fatty acids, alkaloids and saponins played an important role in its antioxidant activity and hepatoprotective effects. **Hao et al., (2009)**[56] mentioned that portulaca oleracea can be used as medicinal plant where it is used for anti-aging thereby increasing the level of SOD and decreasing the level of MDA in the brains of mice treated with D-galactosamine. **Shirwaikar et al., (2003)**[57] reported that decreased levels of uric acid in the portulaca oleracea treated animals may be due to its antioxidant potential. From the above mentioned data, it could be concluded that purslane aqueous extract improved the liver function injured with CCl_4 as compared to silymarin due to its content of phytochemicals. The data also showed increased AFP in HCC rats. Increase of this protein may be due to hepatotoxic agents or hepatocarcinogens that are frequently associated with HCC. Increased glycoprotein AFP levels are considered a good marker for

various malignancies including testicular, bile duct, pancreatic, stomach, colon and hepatic cancer.[58, 59] **In conclusions,** Purslane seeds extract enhanced innate antioxidant activity and ameliorate the DENA-induced liver injury and therefore can be used as a hepatoprotective drug in the future.

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