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Antioxidant and hepatoprotective effects of *Oxalis corniculata* against carbon tetrachloride (CCl$_4$) induced injuries in rat

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Many human diseases are caused due to oxidative stress involving excessive production of free radicals that can be ameliorated by the antioxidant activities of plant extracts. Present study was designed to characterize the chemical composition of *Oxalis corniculata* methanol extract (OCME) and its various fractions; *O. corniculata* n-hexane (OCHE), *O. corniculata* ethyl acetate (OCEE), *O. corniculata* chloroform (OCCE) and *O. corniculata* aqueous (OCAE); and to determine the antioxidant potential by different *in vitro* assays. OCME was also evaluated for its antioxidant capacity against hepatotoxicity induced with carbon tetrachloride (CCl$_4$: 1 ml/kg b.w., 20% in olive oil, seven doses) in rat. The results showed the presence of flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids in OCME while tannins were absent. Total amount of phenolic and flavonoids was affected by the solvents and the sequence of solvents for phenolic contents was OCME > OCAE > OCCE > OCEE > OCHE while for flavonoids was OCME > OCCE > OCAE > OCEE > OCHE. Free radicals were scavenged by the extract/fraction in a dose response curve in all models. Biochemical parameters of serum; aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase (γ-GT), total bilirubin, cholesterol and triglycerides were significantly increased while total protein and albumin were decreased by CCl$_4$. Treatment of CCl$_4$ significantly decreased the liver contents of reduced glutathione (GSH) and activities of antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), glutathione reductase (GSR) and quinone reductase (QR) whereas elevated the thiobarbituric acid reactive substances (TBARS) contents, and hepatic lesions. All the parameters were brought back to control levels by the supplement of OCME. The results of the present study suggest the antioxidant potential of OCME and its fractions as evidenced by scavenging of free radicals and hepatoprotective capacity.

Key words: *Oxalis corniculata*, carbon tetrachloride (CCl$_4$), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), diphenylpicrylhydrazyl (DPPH), hepatoprotective, lipid peroxidation.

INTRODUCTION

Carbon tetrachloride (CCl$_4$) is an archetype of hepatotoxin used commonly in experimental models to induce oxidative stress in liver (Shyu et al., 2008). CCl$_4$ is also known to be involved in inducing injuries to other organs (Khan and Ahmed, 2009; Khan et al., 2009;

Khan and Zehra, 2011). In its first step of metabolism, CCl$_4$ is converted by cytochrome P450 to a carbon centered radical, the trichloromethyl (•CCl$_3$) that can dismutate to chloroform, cause lipid peroxidation by attacking polyenoic fatty acids (Halliwell and Whiteman, 2004). The trichloromethyl radical can also react with oxygen to form the peroxy trichloromethyl free radical (•CCl$_3$O$_2$), which is more reactive than the 3, 2 trichloromethyl radical with the corresponding health disturbances (Rechnagel et al., 1989; He et al., 2006).

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Multiple processes are involved in the progress of liver diseases. Experimental and clinical results indicate that oxidative stress may be the link, connecting different types of chronic liver injuries (Parola and Robino, 2001). The use of effective antioxidants may be the major therapeutic strategy to reduce the oxidative stress, which leads to progression of liver injuries.

Previous studies have shown that antioxidants like silymarin, vitamins C and E decrease lipid peroxidation and partially ameliorate liver injuries. Over the years, many researchers have reported that plants containing phenolics and flavonoids exhibit a large array of biological activities like hepatoprotection and reversal of fibrosis. These phytochemicals are widely found in fruits, vegetables and herbal plants and are found as reliable hepatoprotective against liver damage, as well as curtailing process in the progression to liver fibrosis (Wang et al., 2008). A number of findings have indicated the scavenging effects of flavonoids and polyphenols *in vivo* and *in vitro* conditions (Khan et al., 2009; Sahreen et al., 2010; Khan and Siddique, 2012).

Oxalis corniculata (Oxalidaceae) is locally used in various ailments. It is rich in niacin, vitamin C and β-carotene (Manandhar, 2002). The juice of the plant is given in jaundice and in stomach troubles (Hussain et al., 2008). The juice of the plant, mixed with butter, is applied to muscular swellings, boils and pimples (Manandhar, 2002). *O. corniculata* is also used as antiseptic, refrigerant, diaphoretic, diuretic and anti diabetic (Hussain et al., 2008). It is used as complementary medicine in wound healing, anemia, dyspepsia, cancer, piles, dementia and convulsions (Taranalli et al., 2004; Madhavachetty et al., 2008). Other alternative uses are; anthelmintic, anti-inflammatory, astringent, depurative, diuretic, emmenagogue, febrifuge, lithontripic, stomachic and styptic. It is also used in the treatment of influenza, fever, urinary tract infections, enteritis, diarrhea, traumatic injuries and sprains (Chopra et al., 1986). It was also reported that *O. corniculata* have hypoglycemic, antihypertensive, antipsychotic, nervous system stimulant and have chronotropic and inotropic effect (Achola et al., 1995; Raghavendra et al., 2006). Chemical characterization of *O. corniculata* showed the presence of glyoxylic acid, oxalic acid, pyruvic acid, vitexin and isovitexin, vitexin-2-O-beta-D- glucopyranoside, neutral lipids, glycolipids; vitamin C; phospholipids; fatty acids, 18:2, 18:3, 16:0; saturated (C10-C14) acids; alpha and beta tocopherols (Raghavendra et al., 2006).

The plant based therapeutics against oxidative stress induced diseases is the research of medicament of these days. Therefore, the present study was conducted to find out the protective effect of *O. corniculata* methanol extract (OCME) against CCl_4-induced oxidative stress and liver injuries in Sprague-Dawley rats. The protective proceedings of OCME are compared with silymarin, which has been used for over 20 years in clinical practice for the treatment of toxic liver diseases. OCME was fractionated with various solvents to determine the chemical composition and *in vitro* antioxidant assays in this study.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, gamma-glutamyl p-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), CCl_4, flavine adenine dinucleotide (FAD), glucose-6-phosphate, Tween-20, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and perchloric acid (PCA) were purchased from Sigma Chemicals Co. USA.

Plant material

At maturity, plants of *O. corniculata* were collected in August, 2009 from the campus of Quaid-i-Azam University, Islamabad Pakistan, identified by Dr. Mir Ajab Khan of Department of Plant Sciences. A voucher specimen was deposited at the Herbarium of Pakistan, Quaid-i-Azam University Islamabad.

Shade dried aerial parts of the plant was chopped and grinded mechanically in 1 mm mesh size. 2 kg powder was extracted with methanol (5 L) for 4 days with occasional shaking and the process repeated twice. The extract was concentrated under reduced pressure using rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) at 40°C until a semi-solid sticky mass of OCME was obtained. In order to resolve the compounds contributing to antioxidant capacity of extract, the extract was further subjected to liquid–liquid partition (suspended in 50 ml distilled water) with *O. corniculata* n-hexane fraction (OCHE), *O. corniculata* ethyl acetate fraction (OCEE), *O. corniculata* chloroform fraction (OCCE) while the remaining portion was used as *O. corniculata* aqueous fraction (OCAE). The solvent of the fractions was also removed using a rotary evaporator after partition.

Phytochemical studies

Chemical composition of different extracts for the presence of flavonoids, alkaloids, terpenoids, steroids and saponins were carried out according to Harborne (1973), tannins (Sofowara, 1993) while cardiac glycosides and phlobatannins by Trease and Evans (1989).

Determination of total flavonoid contents

Concentration of flavonoids was measured according to the method of Singleton and Rosi (1996). Different extracts were dissolved in 5% $NaNO_2$, 10% $AlCl_3.6H_2O$ and 1 M NaOH and concentration was measured at 510 nm with a known rutin concentration as a standard (in triplicate). The results were expressed as milligrams of rutin equivalents (RE) per gram of dry weight of various fractions.

Total phenolic contents

The total phenolic content of various fractions was determined using the method of Singleton and Rossi (1996). Methanolic solution of gallic acid (GA) (1 ml; 0.025 to 0.400 mg/ml) with 5 ml

Folin-Ciocalteu reagent (diluted 10-fold) was used for the calibration curve and mixture was incubated for 5 min before the addition of sodium carbonate (4 ml, 0.115 mg/ml). Absorbance was measured at 765 nm. 1 ml of different extracts (0.5 to 5.0 mg/ml) was also mixed with the stated reagents and after 2 h, the absorbance was measured to determine total plant phenolic contents. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

In vitro antioxidant activity

Diphenylpicrylhydrazyl (DPPH) radical scavenging activity

Antioxidant capacity of different samples, GA and ascorbic acid (ASA) was measured with the stable radical DPPH in terms of hydrogen-donating or radical-scavenging activity at 517 nm, according to the described procedure of Gyamfi et al. (1999).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

ABTS assay was carried out by using the protocol of Re et al. (1999) using ABTS and potassium persulfate solution to generate $ABTS^{\cdot+}$ radicals. ASA and GA were used as positive controls and absorbance was determined at 715 nm.

Determination of superoxide radical scavenging activity

Superoxide scavenging for each fraction and for GA and ASA was determined by the nitroblue tetrazolium reduction method (Nishikimi et al., 1972). The tubes were uniformly illuminated with an incandescent visible light for 15 min, and the optical density was measured at 530 nm before and after the illumination.

Hydrogen peroxide scavenging activity

The scavenging capacity for hydrogen peroxide was measured according to the method of Ruch et al. (1989). Hydrogen peroxide scavenging ability of each extract and standards (GA and ASA) was determined at 230 nm against a blank having phosphate buffer.

Phosphomolybdate assay

Phosphomolybdate assay was performed to assess the antioxidant activity of samples and standard compounds; GA and ASA according to the procedure of Umamaheswari and Chatterjee (2008) by using sulphuric acid, sodium phosphate and ammonium molybdate. After incubation at 95°C for 90 min, absorbance of each reaction mixture was measured at 765 nm against a blank.

Chelating power

The iron (II) chelating ability of the extracts was estimated according to the method of Dastmalchi et al. (2008) by using ferrozine. To determine the chelating activity, the absorbance of each assay was recorded at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as control.

Reducing power

The reducing powers of the extracts were determined according to the method of Chung et al. (1998) by using potassium ferricyanide $[K_3Fe(CN)_6]$ and $FeCl_3$ and absorbance was measured at 700 nm in spectrophotometer.

In vivo studies

Animals and treatment

Male Sprague-Dawley rats (190 ± 10 g) were housed in large spacious cages. Feed and water ad libitum were available to the rats. The animals used in this study were treated and cared for their well being in accordance with the guidelines recommended by the Ethical Committee of the Quaid-i-Azam University Islamabad, Pakistan for the care and use of laboratory animals. Animals were divided into seven equal groups with 6 rats in each group. The experimental protocol was approved by the institutional ethics committee.

Group 1 received only raw water and free access to food materials. Group II received olive oil intraperitoneally and dimethyl sulfoxide (DMSO) intragastric for 7 days at a dose of 1 ml/kg body weight. Group III received CCl_4 1 ml/kg body weight (20% in olive oil) intraperitoneally for 7 days. Groups IV and V received CCl_4 and OCME (100 or 200 mg/kg body weight) intragastric for 7 days. Group VI was given OCME (200 mg/kg body weight) intragastric for 7 days. Group VII was treated with CCl_4 and silymarin (50 mg/kg body weight) intragastric for 7 days. After 24 h of the last treatment, all the animals were weighted, sacrificed; collected their blood, weighted and perfuse liver in ice-cold saline solution. Half of liver tissues were treated with liquid nitrogen for further enzymatic analysis while the other portion was processed for histology.

Serum analysis for liver marker enzymes

Serum samples were assayed for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (γ-GT) by using standard diagnostic kits standard AMP diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

Serum analysis for biochemical studies

Total bilirubin, cholesterol, triglycerides, total protein and albumin were estimated by using standard diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

Hepatic biochemical studies

Hepatic tissue was homogenized in 10 volume of 100 mM KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and used for various biochemical studies. Protein concentration of the supernatant of liver tissue was determined by the method of Lowry et al. (1951) using crystalline BSA as standard.

Catalase (CAT) assay

CAT activities were determined by the method of Chance and Maehly (1955) by using H_2O_2 as substrate. Changes in absorbance

of the reaction solution at 240 nm were determined for 1 min. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Peroxidase (POD) assay

Activities of POD were determined by the method of Chance and Maehly (1955) with guaiacol and H_2O_2 as substrates. Changes in absorbance of the reaction solution at 470 nm were determined for 1 min. One unit of POD activity was defined as an absorbance change of 0.01 as units/min.

Superoxide dismutase (SOD) assay

SOD activity of thyroid was estimated by the method of Kakkar et al. (1984). Reaction mixture of this method contained phenazine methosulphate and sodium pyrophosphate buffer. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed in units/mg protein.

Glutathione-S-transferase (GST) assay

GST activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of GSH and CDNB. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nM CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3 \, M^{-1} cm^{-1}$.

Glutathione peroxidase (GSH-Px) assay

GSH-Px activity was assayed by the method of Mohandas et al. (1984). The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nM NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3 \, M^{-1} cm^{-1}$.

Glutathione reductase (GSR) assay

This assay was conducted according to the protocol of Carlberg and Mannervik (1975). The reaction solution contained EDTA, oxidized glutathione, and NADPH. After mixing, the absorbance was measured at 340 nm. GST activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3 \, M^{-1} cm^{-1}$.

Reduced glutathione (GSH) assay

GSH was estimated by the method of Jollow et al. (1974) using DTNB. The yellow color developed was read immediately at 412 nm on a SmartSpecTM plus Spectrophotometer. It was expressed as µM GSH/g tissue.

Estimation of lipid peroxidation [thiobarbituric acid reactive substances (TBARS)] assay

The assay for lipid peroxidation was carried out following the method of Iqbal et al. (1996) by using TBA as the substrate. The amount of TBARS formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer against a reagent blank. The results were expressed as nM TBARS/min/mg tissue at 37°C using molar

extinction coefficient of $1.56 \times 10^5 \, M^{-1} cm^{-1}$.

Histopathalogical studies

For microscopic evaluation, hepatic tissues were fixed in a fixative (absolute alcohol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 µm and subsequently stained with hematoxylin/eosin (Fischer et al., 2006). Sections were studied under light microscope (DIALUX 20 EB) at 20X magnifications. Slides of all the treated groups were studied and photographed.

Statistical analysis

Parametric data, expressed as mean and standard deviation (SD), were analyzed through one way analysis of variance (ANOVA), followed by the *post hoc* Fisher least significant difference (LSD) for comparison of various treatments using the SPSS 13.0. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Plant composition studies

The aerial part of *O. corniculata* was extracted with methanol and fractionated with various solvent of increasing polarity such as n-hexane, ethyl acetate, choloroform and water. The results obtained in this study indicated the presence of flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids in OCME and OCAE fraction. Cardiac glycosides and phlobatannins were absent in OCCE. Alkaloids and cardiac glycosides were not present in OCEE while alkaloids, cardiac glycosides, phlobatannins and steroids were absent in OCHE. Presence of tannins was not confirmed in OCME and in other fractions; OCAE, OCCE, OCEE and OCHE (Table 1).

According to the data shown in Table 2, the significant difference in the amount of the total phenolic or total flavonoid was observed among these extracts, respectively ($P < 0.05$). The richest amount of total flavonoid was found in the OCME (6.92 mg RE/g extract), followed by OCCE (5.32 mg RE/g extract), OCAE (4.68 mg RE/g extract), OCEE (5.95 mg RE/g extract) and OCHE (2.52 mg RE/g extract). Similarly, the highest phenolic content (7.76 mg GAE/g extract) was observed in OCME, while OCHE had the lowest content (4.41 mg GAE/g extract). The amounts of total phenolics were affected by the extraction solvents with the following order: OCME > OCAE > OCCE > OCEE > OCHE ($P < 0.05$ among the extracts).

In vitro antioxidant studies

Scavenging activity methanol extract and various fractions on DPPH radical

It is shown in Figure 1A that the tested samples dose-

Table 1. Composition of various fractions of *O. corniculata*.

Chemical	Methanol extract	Chloroform fraction	Ethyl acetate fraction	n-hexane fraction	Aqueous fraction
Flavonoids	++	++	++	+	++
Alkaloids	+	+	-	-	+
Terpenoids	++	+	++	+	++
Saponins	++	+	+	+	++
Tannins	-	-	-	-	-
Cardiac glycosides	+	-	-	-	++
Phlobatannins	++	-	+	-	++
Steroids	++	++	+	-	+

++, + and -, strong presence, presence and absence respectively.

Table 2. Total phenolic and flavonoid contents in *O. corniculata*.

Plant extract	Total phenolics (mg GAE/g extract)	Total flavonoids (mg RE/g extract)
Methanol extract	7.76 ± 0.36^a	6.92 ± 0.52^a
Ethyl acetate fraction	4.76 ± 0.14^c	2.86 ± 0.35^d
Chloroform fraction	6.06 ± 0.27^b	5.32 ± 0.41^b
n-Hexane fraction	4.41 ± 0.17^c	2.52 ± 0.23^d
Aqueous fraction	6.78 ± 0.34^{ab}	4.68 ± 0.26^{bc}

n, 03 ±SD; [a to d], Means with different superscript letters indicate significance at $P < 0.05$.

**Table 3.** Scavenging activity of OCME and its various fractions.

Plant extract	IC_{50} (µg/ml)					
	Scavenging of DPPH radicals	Scavenging of ABTS radicals	Scavenging of hydrogen peroxide	Scavenging of superoxides	Phosphomolybdate assay	Iron chelating ability
Methanol extract	68.5 ± 0.96^b	269.2 ± 4.2^c	26.7 ± 1.5^b	29.7 ± 2.4^g	213.2 ± 2.7^a	52.8 ± 2.4^e
Ethyl acetate fraction	34.4 ± 1.17^d	340.5 ± 4.4^b	11.6 ± 0.9^c	173.0 ± 3.0^a	211.1 ± 2.9^a	201.7 ± 3.5^a
Chloroform fraction	52.4 ± 1.03^c	112.9 ± 3.8^e	9.6 ± 0.8^c	$34.7 \pm 1.4f$	49.0 ± 3.3^c	100.9 ± 2.6^c
n-Hexane fraction	32.0 ± 1.42^e	268.9 ± 2.6^c	10.5 ± 0.7^c	115.4 ± 2.0^b	108.6 ± 1.6^b	150.7 ± 3.2^b
Aqueous fraction	78.5 ± 1.65^a	$>500^a$	26.0 ± 1.4^b	105.7 ± 2.4^c	105.3 ± 2.3^b	57.8 ± 2.2^d
Gallic acid	7.6 ± 0.88^f	170.1 ± 3.8^d	32.0 ± 3.3^a	52.5 ± 2.7^d	34.5 ± 2.5^d	-
Ascorbic acid	1.1 ± 0.14^g	55.7 ± 1.0^f	7.9 ± 0.2^d	32.7 ± 1.8^e	31.0 ± 2.3^d	-
EDTA	-	-	-	-	-	51.2 ± 2.1^e

-, Not determined; Mean ± SD (n = 03); [a to d], Means with different superscript letters indicate significance at $P < 0.05$.

dependently exhibited a DPPH• -scavenging ability at all the investigated concentrations. OCHE exhibited maximum scavenging ability (IC_{50}, 32.0 ± 1.42), while OCAE was found the least effective (IC_{50}, 78.5 ± 1.65) for DPPH radical scavenging. IC_{50} values indicated significant differences among all the extracts ($P < 0.05$), where the IC_{50} values can be ranked as OCAE > OCME > OCCE > OCEE > OCHE (Table 3). It is also observed that none of the extract was as effective as the positive controls ($P < 0.05$, GA, ASA).

Scavenging activity of ABTS and various fractions extracts on superoxide radical

Characterization of scavenging activity of OCME and its different fractions against ABTS radical revealed that all the tested samples exerted scavenging effects in a concentration dependent manner (Figure 1B). As shown in Table 3, the IC_{50} values was found to be 269.2 ± 4.2, 340.5 ± 4.4, 112.9 ± 3.8, 268.9 ± 2.6, > 500, 170.1 ± 3.8, 55.7 ± 1.0 µg/ml for OCME, OCEE, OCCE, OCHE,
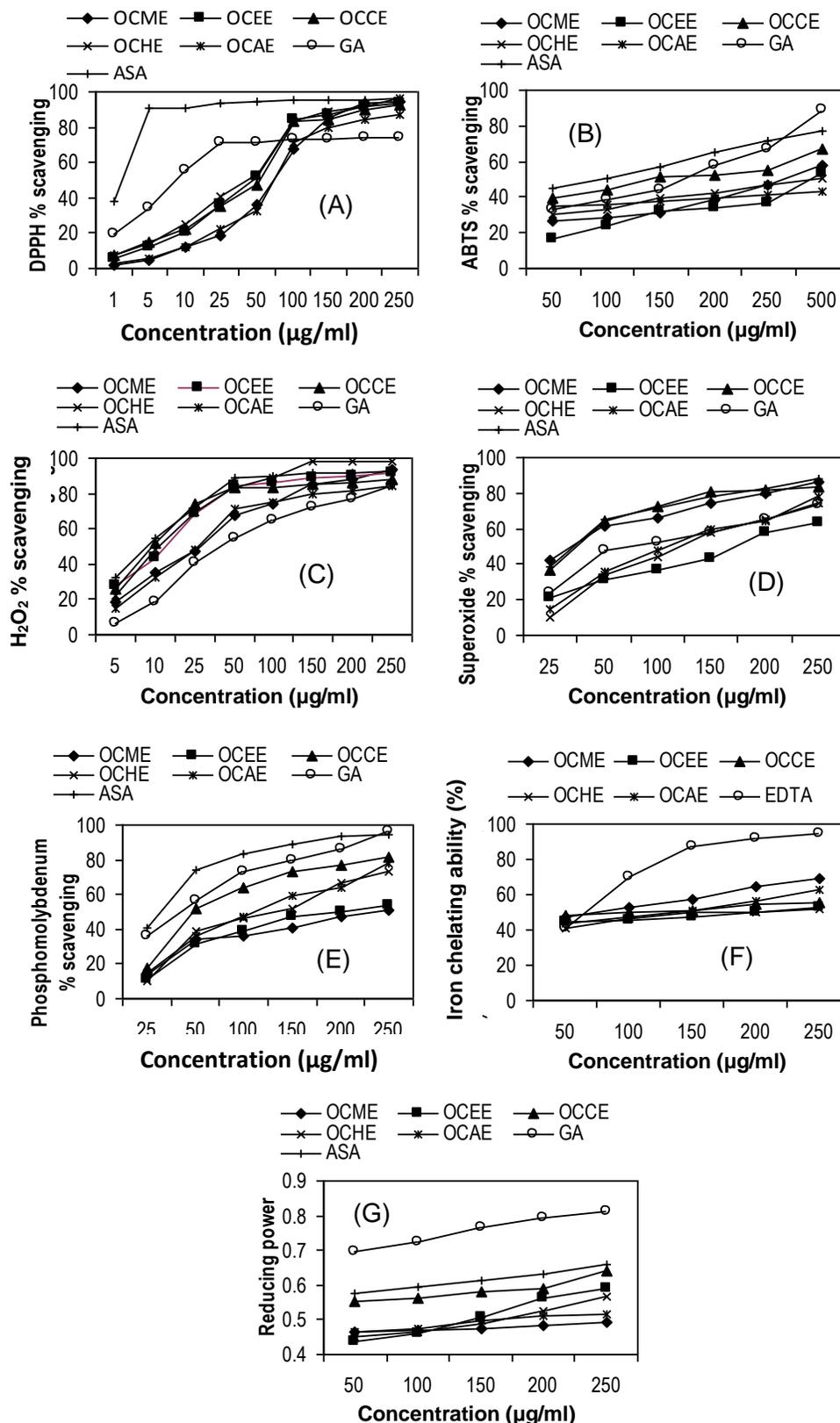
Figure 1. Antioxidant effects of *O. corniculata* in different *in vitro* assays. OCME, *O. corniculata* methanol extract; OCEE, *O. corniculata* ethyl acetate fraction; OCCE, *O. corniculata* chloroform fraction; OCHE, *O. corniculata* n-hexane fraction; OCAE, *O. corniculata* aqueous fraction; GA, gallic acid; ASA, ascorbic acid; EDTA, ethylene diamine tetracetate.

OCAE, GA and ASA, respectively. The order of IC_{50} values for different samples was OCAE > OCEE > OCME > OCHE > GA > OCCE > ASA where the OCME and OCHE were statistically similar to each other (P > 0.05).

Scavenging activity of OCME and its various fractions on hydroxyl radical

Scavenging of hydroxyl radical with the different test samples showed a dose response curve (Figure 1C). All the test samples showed strong scavenging effects against hydroxyl radical with the IC_{50} values, 26.7 ± 1.5, 11.6 ± 0.9, 9.6 ± 0.8, 10.5 ± 0.7 and 26.0±1.4 for OCME, OCEE, OCCE, OCHE and OCAE, respectively. Evidently, the order of scavenging activity for hydroxyl radical was OCME > OCAE > OCEE > OCHE > OCCE where the IC_{50} value of OCME and OCAE did not reach the level of statistical significance (P > 0.05). However, the least and the maximum IC_{50} value for the positive controls ASA (7.9 ± 0.2) and GA (32.0 ± 3.3) were obtained in this study.

Scavenging activity of OCME and its various fractions on superoxide radical

Figure 1D shows the dose response curve for superoxide radical scavenging ability for OCME and different fractions. All the test samples exerted strong scavenging activity against the superoxide radicals with IC_{50} values of 29.7 ± 2.4, 173.0 ± 3.0, 34.7 ± 1.4, 115.4 ± 2.0, 105.7 ± 2.4 for OCME, OCEE, OCCE, OCHE and OCAE, respectively. The IC_{50} values obtained for standard compounds GA and ASA were 52.5 ± 2.7 and 32.7 ± 1.8, respectively. All the test samples and positive control were statistically different from each other (P < 0.05). However, OCME exhibited significantly (P < 0.05) less IC_{50} value as compared to the GA and ASA.

Scavenging activity of OCME and its various fractions on phosphomolybdate assay

The data obtained indicated the marked scavenging potency of OCME and its various fractions for phosphomolybdate assay in a dose-dependent way (Figure 1E). The IC_{50} values obtained were 213.2 ± 2.7, 211.1 ± 2.9, 49.0 ± 3.3, 108.6 ± 1.6, 105.3 ± 2.3, 34.5 ± 2.5, 31.0 ± 2.3 for OCME, OCEE, OCCE, OCHE, OCAE, GA and ASA, respectively. The IC_{50} values of OCME, OCEE and OCHE, OCAE did not reach the level of significance with each other (P < 0.05). However, inferior IC_{50} values were obtained for all the test samples with respect to the positive controls GA and ASA.

Scavenging activity of OCME and its various fractions on Fe^{2+} chelating potency

As shown in Figure 1F, all the samples displayed different magnitudes of Fe^{2+} chelating potency in a dose-dependent manner. From the estimated IC_{50} values in Table 3, it can be seen that the most effective Fe^{2+} chelating extract was OCME (52.8 ± 2.4) and then followed by OCAE (57.8 ± 2.2), OCCE (100.9 ± 2.6), OCHE (150.7 ± 3.2) and OCEE (201.7 ± 3.5). However, the lowest Fe^{2+} chelating IC_{50} value was obtained for positive control EDTA (51.2 ± 2.1). The sequence of IC_{50} value for Fe^{2+} chelating activity for different samples was EDTA > OCME > OCAE > OCCE > OCHE > OCEE (P < 0.05).

Scavenging activity of OCME and its various fractions on reducing power

As shown in Figure 1G, OCME and its different fractions exhibited varied degrees of reducing power. Among all the extracts, at 250 µg/ml OCCE exhibited the strongest reducing power with absorbance values of (0.641 ± 0.004) followed by OCEE (0.595 ± 0.014), OCHE (0.565 ± 0.013) and OCAE (0.515 ± 0.006). At a concentration 250 µg/ml, the absorbance recorded for positive controls GA and ASA was (0.809 ± 0.018) and (0.655 ± 0.007), respectively (Table 4).

Effect of OCME on liver marker enzymes

Ameliorative effects of OCME on liver marker enzymes such as AST, ALT, ALP, LDH and γ-GT in serum are shown in Table 5. The treatment of CCl_4 to rats significantly elevated the serum level of AST, ALT, ALP, LDH and γ-GT as compared to the control group. Administration of both CCl_4 and OCME (100 and 200 mg/kg) alleviated the toxicity of CCl_4 and the changed serum level of AST, ALT, ALP, LDH and γ-GT, reverted towards the control group with significance difference from the CCl_4 group. Similarly, treatment of CCl_4 alleviated the toxicity of CCl_4 and serum level of AST, ALT, ALP, LDH and γ-GT was observed significantly less than that of CCl_4 only. However, treatment of OCME alone did not statistically change the level of AST, ALT, ALP, LDH and γ-GT in serum compared with controls.

OCME on serum biochemical profile

In the CCl_4 treated group the serum level of total bilirubin, cholesterol, triglycerides were significantly increased while total protein and albumin were decreased compared with the control group (Table 6). The groups treated with both CCl_4 and OCME (100 and 200 mg/kg) showed a significant change in the level of serum total bilirubin, cholesterol, triglycerides, total protein and albumin compared with the CCl_4 group. Higher dose of OCME (200 mg/kg) produce more appreciable change in the level of serum biochemical profile with respect to the

Table 4. Reducing activity of OCME and its various fractions.

Plant extract	Reducing power absorbance at 700 nm (250 µg/ml)
Methanol extract	0.498 ± 0.020^e
Ethyl acetate fraction	0.595 ± 0.014^c
Chloroform fraction	0.641 ± 0.004^b
n-Hexane fraction	0.565 ± 0.013^d
Aqueous fraction	0.515 ± 0.006^e
Gallic acid	0.809 ± 0.018^a
Ascorbic acid	0.655 ± 0.007^b

n, $03 \pm$ SD; [a to d], Means with different superscript letters indicate significance at $P < 0.05$.

Table 5. Protective effect of OCME on liver marker enzymes.

Group	Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)	γ-GT (U/L)
I	Control	42.2 ± 3.7^A	135.3 ± 9.7^A	227.8 ± 12.4^A	57.5 ± 11.9^A	36.6 ± 7.7^A
II	DMSO + olive oil	40.9 ± 4.2^A	132.7 ± 5.4^A	226.12 ± 10.3^A	55.3 ± 7.9^A	38.9 ± 5.7^A
III	CCl$_4$ (1 ml/kg)	$78.8 \pm 6.^a$	298.5 ± 9.6^a	331.4 ± 15.3^a	101.3 ± 12.8^a	176.6 ± 7.0^a
IV	CCl$_4$ + OCME (100 mg/kg)	68.8 ± 2.7^{Aa}	217.2 ± 6.3^{Aa}	293.4 ± 11.7^{Aa}	69.2 ± 6.8^{Aa}	85.9 ± 8.5^{Aa}
V	CCl$_4$ + OCME (200 mg/kg)	48.5 ± 2.5^A	145.7 ± 7.1^A	238.2 ± 12.8^A	56.1 ± 4.8^A	42.4 ± 8.0^A
VI	OCME (200 mg/kg)	43.2 ± 3.9^A	128.3 ± 7.2^A	231.5 ± 8.08^A	54.7 ± 9.6^A	32.1 ± 7.5^A
VII	CCl$_4$ + silymarin (100 mg/kg)	44.2 ± 2.6^A	147.8 ± 6.5^A	240.3 ± 9.84^A	57.4 ± 8.1^A	46.7 ± 7.3^A

Mean $\pm$ SD (n=6 number). [a], Significance at $P < 0.01$ from control group; [A], significance at $P < 0.01$ from CCl$_4$ group; OCME, *Oxalis corniculata* methanol extract.

Table 6. Protective effect of OCME on serum biochemical profile.

Group	Treatment	Total bilirubin (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Total protein (g/dl)	Albumin (g/dl)
I	Control	10.1 ± 1.5^B	56.3 ± 5.1^B	26.7 ± 5.1^B	52.9 ± 3.5^B	32.8 ± 3.1^B
II	DMSO+ Olive oil	10.4 ± 1.7^B	55.7 ± 4.4^B	23.3 ± 2.4^B	52.0 ± 5.2^B	30.0 ± 5.2^B
III	CCl$_4$ (1 ml/kg)	20.6 ± 1.4^b	71.9 ± 5.0^b	51.0 ± 5.8^b	41.0 ± 3.6^b	21.0 ± 2.6^b
IV	CCl$_4$ + OCME (100 mg/kg)	16.3 ± 1.9^{Bb}	66.2 ± 5.9^{Ab}	43.2 ± 5.9^{Bb}	44.8 ± 2.8^{Ab}	26.8 ± 3.8^{Ab}
V	CCl$_4$ + OCME (200 mg/kg)	13.4 ± 1.0^{Ba}	59.4 ± 7.6^B	29.4 ± 7.1^B	49.3 ± 3.8^B	29.1 ± 3.0^B
VI	OCME (200 mg/kg)	9.3 ± 1.5^B	57.6 ± 4.6^B	27.2 ± 1.6^B	51.4 ± 2.2^B	31.4 ± 3.2^B
VII	CCl$_4$ + silymarin (100 mg/kg)	11.3 ± 1.2^B	58.1 ± 2.0^B	28.1 ± 2.5^B	48.2 ± 3.8^B	30.5 ± 2.8^B

Mean $\pm$SD (n=6 number). [a and b], Significance at $P < 0.05$ and $P < 0.01$ from control group; [A and B], significance at $P < 0.05$ and $P < 0.01$ from CCl$_4$ group; OCME, *Oxalis corniculata* methanol extract.

lower dose of OCME (100 mg/kg). Treatment of silymarin (50 mg/kg) in combination with CCl$_4$ significantly restored the level of serum total bilirubin, cholesterol, triglycerides, total protein and albumin as against the CCl$_4$ group. Ameliorating effects of OCME at higher dose (200 mg/kg) were determined similar to silymarin for the stated parameters. However, treatment of OCME (200 mg/kg) alone did not cause any significant change in the level of total bilirubin, cholesterol, triglycerides, total protein and albumin compared with the control group.

Effect of OCME on hepatic antioxidant enzymes

In the CCl$_4$ treated group, the activity level of antioxidant enzymes, CAT, POD and SOD in hepatic samples (Table 7) showed a significant (P < 0.01) decrease as compared with the control group. The groups treated with both CCl$_4$ and OCME (100 and 200 mg/kg b.w.) showed a significant (P < 0.01) increase in the activity level of CAT, POD and SOD compared with the CCl$_4$ group. However, the activity levels of these antioxidant enzymes were

Table 7. Effect of OCME on antioxidant enzymes of liver in rat.

Group	Treatment	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GSH-Px (nM/mg protein)	GSR (nM/min/mg protein)	GST (nmol/min/mg protein)	QR (µM/mg protein)
I	Control	25.4 ± 1.2^A	13.5 ± 0.89^A	5.8 ± 0.06^A	63.5 ± 5.5^A	117.2 ± 4.5^A	14.5 ± 1.2^A	25.1 ± 0.2^A
II	DMSO + olive oil	24.3 ± 2.3^A	12.9 ± 0.45^A	5.4 ± 0.78^A	62.4 ± 4.2^A	114.5 ± 4.7^A	15.4 ± 1.0^A	24.2 ± 0.3^A
III	CCl$_4$ (1 ml/kg)	12.8 ± 1.8^b	7.3 ± 0.57^b	3.1 ± 0.23^b	35.6 ± 3.6^b	220.3 ± 5.7^b	7.8 ± 1.3^b	10.6 ± 1.0^b
IV	CCl$_4$ + OCME (100 mg/kg)	16.8 ± 2.0^{Ab}	9.3 ± 0.67^{Ab}	4.7 ± 0.2^{Ab}	43.0 ± 5.3^{Ab}	176.9 ± 8.2^{Ab}	10.9 ± 1.0^{Ab}	17.2 ± 2.4^{Ab}
V	CCl$_4$ + OCME (200 mg/kg)	22.8 ± 1.6^A	11.4 ± 0.34^{Aa}	5.5 ± 0.32^A	54.2 ± 3.4^{Aa}	126.5 ± 3.9^{Aa}	12.4 ± 2.1^A	20.3 ± 1.5^{Aa}
VI	OCME (200 mg/kg)	26.4 ± 2.9^A	13.0 ± 0.56^A	6.0 ± 0.12^A	60.2 ± 4.5^A	115.3 ± 4.9^A	15.2 ± 2.1^A	25.2 ± 1.5^A
VII	CCl$_4$ + silymarin (100 mg/kg)	21.3 ± 1.0^{Aa}	12.6 ± 0.76^A	4.9 ± 0.12^{Aa}	52.2 ± 3.6^{Aa}	134.8 ± 5.7^{Aa}	12.8 ± 1.2^A	21.2 ± 0.9^{Aa}

Mean ±SD (n=6 number). ^{a} and ^{b}, Significance at $P < 0.01$ from control group; ^{A}, significance at $P < 0.01$ from CCl$_4$ group; OCME, *Oxalis corniculata* methanol extract.

more pronounced at the higher dose (200 mg/kg b.w.) and activity level of CAT and SOD was statistically similar to the control group while POD activity was significantly ($P < 0.05$) less as compared with the control group. Group treated with CCl$_4$ and silymarin showed statistical increase ($P < 0.01$) in CAT, POD and SOD activity level with respect to the CCl$_4$ group whereas CAT and SOD showed significantly ($P < 0.05$) lower activity compared with the control group. The OCME treated group (200 mg/kg b.w.) did not exhibit antioxidant activity of CAT, POD and SOD that was different from the control group.

As shown in Table 7 the activity levels of GSH-Px, GST, GSR and quinone reductase (QR) were significantly ($P < 0.01$) lowered in CCl$_4$ treated group in hepatic samples compared with controls. The groups treated with both CCl$_4$ and different dosages of OCME (100 and 200 mg/kg b.w.) had significantly ($P < 0.01$) elevated the activity levels of GSH-Px, GST, GSR and QR in liver samples compared with the group given CCl$_4$ only. However, the increase in the activity level was more pronounced at higher dose of OCME (200 mg/kg b.w.). Activity level of GSH-Px, GSR and QR at higher dose of OCME (200 mg/kg b.w.) was

obtained statistically lower ($P < 0.05$) as against the control group. Treatment of silymarin (50 mg/kg b.w.) effectively ameliorated the toxicity of CCl$_4$ and activity level of GSH-Px, GST, GSR and QR activity was restored towards the control group. However, activity level of GSH-Px, GSR and QR was found statistically lower ($P < 0.05$) compared with the control group. OCME (200 mg/kg b.w.) administration alone did not significantly change the activity levels of GSH-Px, GST, GSR and QR in hepatic samples when compared with those of the control group.

Effect of OCME on lipid peroxidation and glutathione

Treatment of CCl$_4$ to rats significantly ($P < 0.01$) elevated the TBARS level an indicator of lipid peroxidation while the contents of GSH, an endogenous antioxidant were significantly ($P < 0.01$) decreased in hepatic samples compared with the control group (Table 8). The groups administered both with CCl$_4$ and OCME (100 and 200 mg/kg b.w.) had significantly ($P < 0.01$) decreased TBARS while increased GSH levels (P

< 0.01) in liver samples compared with the group given CCl$_4$ only. Higher dosage of OCME (200 mg/kg b.w.) was found to be more potent in the reversal of TBARS and GSH level in liver. Treatment of silymarin (50 mg/kg b.w.) in combination with CCl$_4$ was able to restore the TBARS and GSH level towards the control group. OCME administration alone did not statistically change the TBARS and GSH levels in the hepatic samples when compared with the controls.
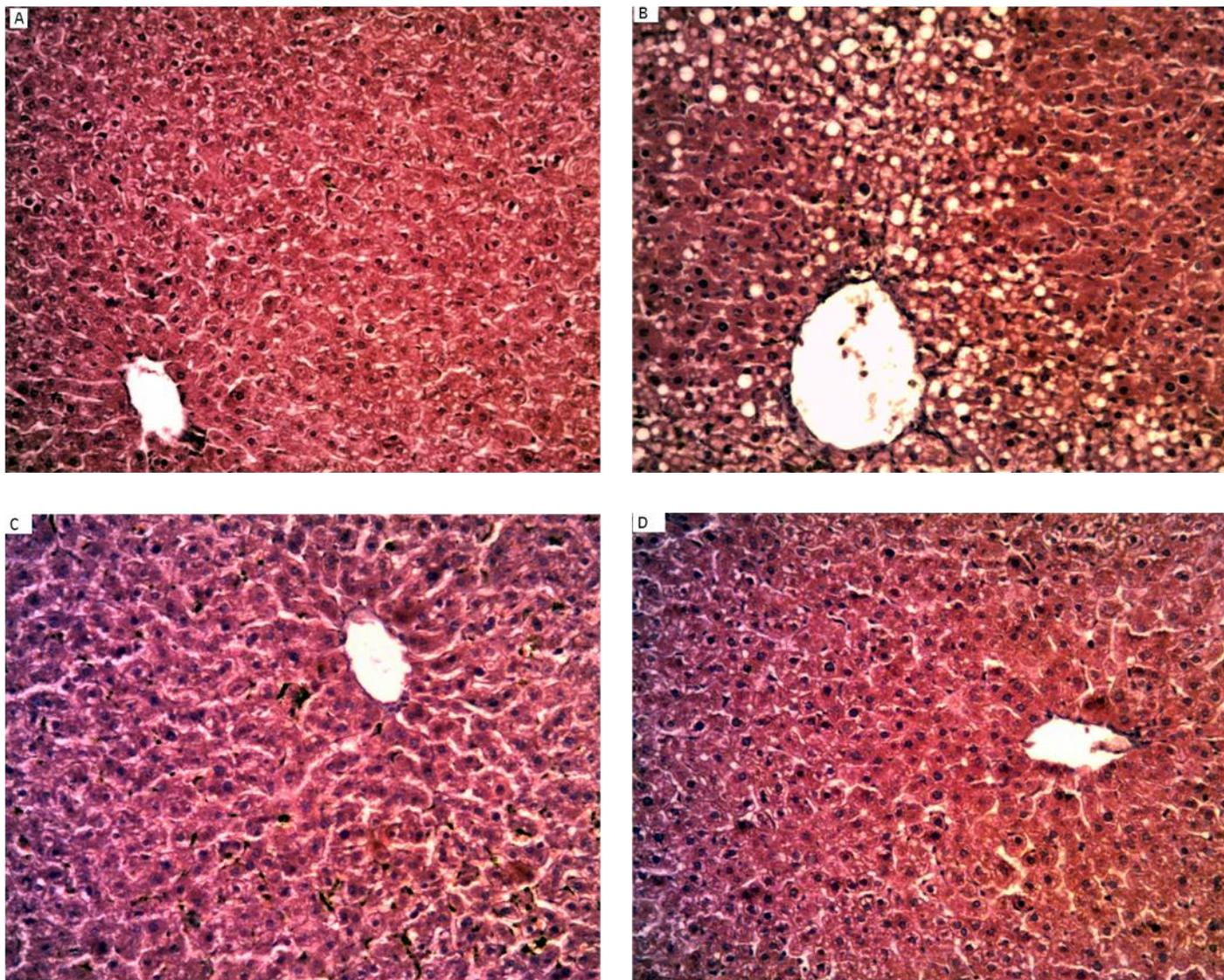
Effect of OCME on histopathology of liver

Hematoxylin and eosin stained section (Figure 2) indicated that administration of CCl$_4$ markedly increasedthe fatty changes with white and yellow areas, cellular hypertrophy, and necrotic foci, degeneration of the lobular architecture and the formation of septa and congested blood vessels with disturbed epithelium. Co-administration of various doses of OCME attenuated the hepatic injuries with very less or no fatty changes, dilation of blood vessel, uniform morphology of hepatocytes near to control group was found. Co-treatment with silymarin showed near to normal

Table 8. Effect of OCME on lipid peroxidation and glutathione in hepatic of rat.

Group	Treatment	Lipid peroxidation TBARS (nM/min/mg protein)	GSH (µM/g tissue)
I	Control	19.1 ± 1.2[A]	1.69 ± 0.002[A]
II	DMSO + olive oil	18.4 ± 2.1[A]	1.73 ± 0.004[A]
III	CCl$_4$ (1 ml/kg)	39.8 ± 1.6[b]	0.51 ± 0.006[b]
IV	CCl$_4$ + OCME (100 mg/kg)	30.7 ± 2.0[Ab]	0.96 ± 0.006[Ab]
V	CCl$_4$ + OCME (200 mg/kg)	22.2 ± 1.5[A]	1.54 ± 0.003[A]
VI	OCME (200 mg/kg) alone	19.4 ± 1.3[A]	1.71 ± 0.001[A]
VII	CCl$_4$ + silymarin (50 mg/kg)	22.8 ± 1.3[A]	1.44 ± 0.001[Aa]

Mean ±SD (n = 6 number). [a] and [b], Significance at $P < 0.05$ and $P < 0.01$ from control group; [A], significance at $P<0.01$ from CCl$_4$ group; OCME, *Oxalis corniculata* methanol extract.

Figure 2. H & E stain (20x). Effects of OCME on histopathology of liver in rat. (A) Control group; CL centrilobule; HP, hepatocytes. (B) CCl$_4$ (1 ml/kg b.w., 20% in olive oil) group; ICL, injured centrilobule; MC, macrosteatosis. (C) CCl$_4$ + OCME (200 mg/kg b.w.) group; CL, centrilobule; MIS, microsteatosis. (D) CCl$_4$ + silymarin (50 mg/kg b.w.) group; CL, centrilobule; HP, hepatocytes..

morphology. No abnormal changes was found in the morphology of control group was found.

DISCUSSION

In recent years, plant extracts have been widely used as natural antioxidants because of the presence of polyphenolics (Nuengchamnong et al., 2009). Flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids have been determined in OCME. In the present investigation, highest quantity of total phenolics and flavonoid components was found in OCME. The presence of these phenolic and flavonoid compounds, contribute diverse biological activities such as anti-carcinogenic, anti-inflammatory, and anti-atherosclerotic. These activities might be related to their antioxidant activity (Nuengchamnong et al., 2009). The polar solvents such as methanol and water are the best solvent in extracting the flavonoid from *O. corniculata*, indicating that most of the flavonoid exist in a conjugated form through their hydroxyl groups with glycosides, lead to the increasing polarity and solubility in methanol and water (Mohsen and Ammar, 2009).

The antioxidant ability of OCME and its fractions was measured through the DPPH and ABTS radical scavenging potential. The present results indicated that OCME and OCAE have high IC_{50} values as compared to the non polar solvents (Table 3). However, reverse order was obtained for the iron chelating ability. The antioxidant activity of phenolic is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers and some of the pharmacological effects might be due to these valuable compounds. In addition, they have a metal chelating potential (Rice-Evans et al., 1995). Chelating agents may act as secondary antioxidants because of their ability to reduce the redox potential to stabilize the oxidized form of the metal ions. Therefore it is of importance to screen the iron (II) chelating ability of extracts. Accordingly it is suggested that the iron (II) chelating properties of these fractions may be attributed to the nature of endogenous chelating agents like phenolics and flavonoids. Some phenolic compounds have properly oriented functional groups, which can chelate metal ions to protect against oxidative damage.

It is evident from these results that the chemical constituents found in OCME and its fractions are potent scavengers of free radicals such as $O^{\bullet-}$ and hydrogen peroxide, at very low concentrations (Sahreen et al., 2010). Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell. Among different reactive oxygen substance (ROS), $O_2^{\bullet-}$ is one of the precursors of $HO^{\bullet}$ or singlet oxygen, and also can produce other kinds of oxidizing agents, where it indirectly initiates lipid peroxidation and magnifies cellular damage. Antioxidants

presents in the OCME and other fractions are able to scavenge the $O_2^{\bullet-}$ and H_2O_2 to encounter the deleterious chain reactions.

The reducing capacity is a significant reflection of the antioxidant activity in assessing potential antioxidants (Sahreen et al., 2010). In this assay system, the presence of antioxidants causes the reduction of the $Fe^{3+}/K_3Fe(CN)_6$ complex to the ferrous form (Fe^{2+}), and consequently, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. It is suggested that there is a direct correlation between antioxidant activities and reducing power of components of some plants (Sahreen et al., 2010). The results obtained in this study indicate that OCME and its different fractions have a remarkable potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

In this study, elevated levels of liver marker enzymes such as AST, ALT, ALP, LDH and γ-GT in serum are obtained with CCl_4 treatment indicating the pathophysiology of liver. Generally, CCl_4 is metabolized by the liver in to highly reactive metabolites which either directly or indirectly cause lipid peroxidation of the hepatocytes. Different cytosolic liver marker enzymes would then leaked out from these swollen and necrotic hepatocytes in to blood circulation and evidently elevated levels are obtained that is associated with the massive centrilobular necrosis, ballooning, degeneration and cellular infiltration of the liver (Singh et al., 2008). Treatment of rats by both CCl_4 and the OCME/silymarin reversed the increase in liver marker enzymes towards the control group and hepatic lesions are also minimized (Figure 2).

It is well known that the liver play a pivotal role in the regulation of various chemicals. Administration of CCl_4 causes hepathopathy as indicated by elevation in serum level of total bilirubin, total cholesterol and triglycerides whereas decreases total protein and albumin in rat. These pathological changes signify the potential damage in hepatic induced with CCl_4 treatment. Treatment of rat with CCl_4 and with either OCME or silymarin ameliorated the toxic effects of CCl_4 and restored the level of above biochemicals towards the control group in accordance with other findings (Lin et al., 2008).

Treatment of rats with CCl_4 decreases the level of endogenous glutathione due to its increased utilization in hepatocytes in scavenging of CCl_4 metabolites. GSH is catalyzed by GST for conjugation to different xenobiotics and their metabolites and converting them in to more soluble compounds. GSH is oxidized to GSSH by GSH-Px and then GSSH is reverted back to GSH by GSR. On account of excessive oxidative stress induced by CCl_4 lead to decline in GSH contents and in the activity of GSH-Px, GSR and GST of liver (Hayes et al., 2005).

Activity level of CAT, POD, SOD and QR are reduced in CCl_4 group. Among them CAT is actively engaged in

the catalytic breakdown of H_2O_2 into H_2O and oxygen. Superoxides are highly reactive and the precursor of singlet oxygen and hydroxyl radicals, are dismutated by SOD to H_2O_2 and oxygen (Reiter et al., 2000). Administration of OCME/silymarin along with CCl_4 scavenge the toxic radicals of CCl_4 thereby maintained the GSH level towards the control, increased the capacity or synthesis that confers enhanced protection against oxidative injuries, consequently increase the hepatic level of CAT, POD, SOD, GSH-Px, GST, GSR and QR (Benson et al., 1980).

Measurement of TBARS serves as an indirect indicator of lipid peroxidation of polyunsaturated fatty acids of hepatocyte membrane. Elevation in TBARS level by CCl_4 treatment in this study exhibits the liver damage involving series of chain reactions (Ohkawa et al., 1979). Treatment of OCME/silymarin alleviated the toxicity of CCl_4 and restored the level of TBARS near to control group in conformity to other studies.

It was apparent from the results that administration of CCl_4 induces extensive fatty change with white and yellow areas due to lipid peroxidation, congestion in blood vessels, cellular hypertrophy, and necrotic foci, destruction of the lobular architecture, the formation of septa and congested blood vessels with disturbed epithelium and nuclear degeneration in some areas occur which was significantly recovered by the various concentrations of OCME. The study revealed that the OCME was comprised of polyphenol and terpenoids which show significant protective effect against hepatotoxicity induced by CCl_4. Similar histological observation was found by various investigators (Lin et al., 2008; Khan et al., 2011) while evaluating the protective effect of medicinal plant against CCl_4 and other drugs induced hepatotoxicity in rats.

Conclusion

OCME and various fractions possess efficient scavenging potential as evident in different *in vitro* antioxidant assays. Present findings indicate the protective potential of OCME against CCl_4 induced oxidative stress, elevated the GSH and activity level of various enzyme defenses, decrease lipid peroxidation, restore serum level of liver marker enzymes and hepatic lesions possibly through polyphenolic and other active constituents and accredit its local use in liver disorders.

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