Ameliorating role of methanolic leaves extract of *Fraxinus xanthoxyloides* against CCl_4-challanged nephrotoxicity in rats

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Abstract: Roots, bark, stem/twigs, and leaves of *Fraxinus xanthoxyloides* are being used regionally for the cure of malaria, jaundice, internal injuries, pneumonia, pain, rheumatism and also in fracture of bones. Our objective was to assess the methanolic leaves extract of *F. xanthoxyloides* for its antioxidant capability against oxidative stress induced by carbon tetrachloride (CCl_4) in the kidney of Sprague-Dawley rats. Duration of this experiment was 30 days and doses were given on alternative days. Urine of rats was assessed for kidney function and renal tissues for antioxidant enzymes activity, biochemical markers, comet assay and histopathology. Enhanced urinary creatinine, urobilinogen levels and decreased creatinine clearance, protein contents, and albumin levels were observed by CCl_4 administration when matched to controls. CCl_4 injection also decreased the level of reduced glutathione, catalase, super oxide dismutase, peroxidase, glutathione s-transferase, glutathione reductase, and tissue protein while elevated the levels of thiobarbituric acid reactive substances, DNA damages and H_2O_2 in renal tissues of experimental animals. Co-treatment of FXM and silymarin, lead to the restoration of all the above tested parameters of kidney. Through this study we affirmed the ameliorating role of *F. xanthoxyloides* in oxidative stress affiliated disorders of kidney.

Keywords: *Fraxinus xanthoxyloides*, phenolics, antioxidant, kidney, CCl_4, lipid per oxidation.

INTRODUCTION

Carbon tetrachloride (CCl_4) is a colorless, highly pernicious artificial chemical agent belonging to the class of halogenated alkanes (McGregor and Lang, 1996). Contemporary investigations have revealed that CCl_4 is associated with progressive production of reactive oxygen species that lead to the impairment of several organs (Weber *et al*., 2003). Exposure to CCl_4 is a widely used practice for the induction of hepatic steatosis (Shyu *et al*., 2008; Younis *et al*., 2016a), acute renal tubular necrosis (Khan *et al*., 2010a; Khan *et al*., 2010b; Sahreen *et al*., 2015), tissue damage to lungs (Ganie *et al*., 2011; Khan *et al*., 2012), testicle injuries (Premila and Wilfred, 2000) and cardiovascular damage (Khan, 2012; Mohamed, 2010) in laboratory rat models.

CCl_4 leads to the disruption of homeostatic mechanisms by the production of excessive free radicles giving rise to oxidative stress which is main cause of various pathologies (Jacob and Sotoudeh, 2002). Metabolic stimulation of CCl_4 by phase II system (cytochrome P450) to generate free radicals i.e. proxy trichloromethyl ($•OOCCl_3$) and trichloro-4 methyl ($CCl_3•$) are claimed to reinforce protein and lipid degradation by oxidation (Kodai *et al*., 2007). Moreover, these free radicals when react with polyunsaturated fatty acids (PUFA), lead to the formation of lipid alkoxy (R•) and peroxy (ROO•) radicals (Sahreen *et al*., 2011), generating lipid peroxides, which can deteriorate biological membrane integrity ultimately causing tissue damage (Kodai *et al*., 2007). Therefore, CCl_4 is considered best-representative tool for the study of oxidative damage trials.

Free radicals damage the hepatic cell membrane integrity by emancipating enzymes of cytoplasmic matrix like alanine transaminase, lactate dehydrogenase, alkaline phosphatase, and aspartate transaminase in the blood vessels and meliorating thiobarbituric acid reactive substances (TBARS) action with consecutive inflammation in hepatic cells (Singh *et al*., 2008), affecting urinary and serum profile of kidney (Khan and Ahmed, 2009a; Khan *et al*., 2009b; Khan *et al*., 2010a), causing up-regulation of lysosomal enzymes in testis as well as in kidney (Premila and Wilfred, 2000), which result in down-regulation of creatinine kinase (a key enzyme for regeneration of ATP) activity in heart tissues (Mohamed, 2010). Free radical scavenging abilities of antioxidants are mostly evaluated by the activity of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GSR), peroxidase (POD), and glutathione-S-transferase (GST), including TBARS expression's product and oxidative lipid degradation (Srivastava and Shivanandappa, 2010) among variety of tissues.

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Treatment of oxidative stress-induced ailments by naturally occurring antioxidant substances is currently a spot light issue. Regular intake of fresh fruits, leafy green vegetables and *Camellia sinensis* tea as an effective source of natural antioxidants known to be efficacious against neurodegenerative and cardiovascular diseases (Capodice *et al*., 2015; Willcox *et al*., 2004; Zaib and Khan, 2014). Flavonoids are among most important phytochemicals exhibiting antioxidant properties. Plants enriched with flavonoid contents possess strong hepatoprotective, anti-inflammatory, antioxidant, antiviral, antibacterial, antiallergenic, antifungal, cytotoxic and anticancer activities (Bokhari and Khan, 2015; Jan and Khan, 2016; Saeed *et al*., 2012).

Fraxinus xanthoxyloides Wall. ex DC belonging to Oleaceae family geographically distributed in hilly regions of Pakistan, Algeria, Morocco, India and Afghanistan. It is known locally as, Afghan ash. In northern areas of Pakistan, indigenous practitioners utilize its root, bark and leaves for the cure of jaundice, pneumonia and malaria (Khan *et al*., 2009). Local communities use decoction of stem bark of *F. xanthoxyloides* to reduce labor pain, pre□mature birth expulsion of infant (Shah and Hussain, 2012) and in traumas (Singh, 2012). Decoction of twigs/stem and wood is used for bone fractures and wound healing in cattle (Mukerji and Manoharachary, 2006; Sharma *et al*., 2006). Antioxidant activities of the extracts from leaves and stem bark of *F. angustifolia* have also been appraised through in vitro studies (Ayouni *et al*., 2016). Aqueous extracts of *F. floribunda* bark have been reported for their in vitro antioxidant potential (Arunika and Palash, 2015). Crude ethanol extract of *F. rhynchophylla* exhibited antioxidant and anti-inflammatory activity on lipopolysaccharide-induced murine Raw 264.7 cells (Hong *et al*., 2012). Water extract of *F. rhynchophylla* leaves was able to fight against the acetaminophen-induced nephrotoxicity in male mice (Jeon and Choi, 2007). We also reported that methanol extract/fractions of *F. xanthoxyloides* leaves have anti-inflammatory, analgesic (Younis *et al*., 2016b), anti-leishmanial (Younis *et al*., 2016c) and hepatoprotective capabilities (Younis *et al*., 2016a).

Based on the earlier studies for the use of Fraxinus species in liver, kidney and anti-inflammatory disorders it was speculated that the traditional use of *F. xanthoxyloides* by the local communities of Pakistan might be attributed through the antioxidant abilities of its phytoconstituents. There are no preceding reports on the nephro-protective activity of any extract/fractions from this plant. So we design the following study to evaluate the methanolic extract of leaves of *F. xanthoxyloides* for its antioxidant capability against CCl_4 stimulated oxidative trauma in renal tissues of rats, by evaluating anti-oxidant enzymes activity, biochemical markers, comet assay and histopathology.

MATERIALS AND METHODS

Plant collection

The leaves of *F. xanthoxyloides* were collected in November, 2016 from Quaid-i-Azam University, Islamabad, Pakistan (QAU). This plant was identified by Dr. Muhammad Zafar, from Department of Plant Sciences, QAU. Sample specimen with voucher number 45679 was submitted to National Herbarium, QAU.

Crude extract preparation

Plant leaves were collected, dried under shadow and grinded in electric grinder to form respectively a fine powder. Dry weight of 1kg powder was soaked in 3 L (95% crude methanol) for duration of 72 hours (h). Filtration was done with Whatman No. 1 filter and methanol was evaporated through rotary evaporator at 40°C under reduced pressure. The above mentioned method was repeated twice. 98.5g of methanolic extract of leaves (FXM) was obtained from 1kg of dry powder after extraction and was stocked in refrigerator at 4°C.

Animal studies

Acute toxicity studies

To find out acute toxicity, the guidelines 425 were followed promoted by the Organization for Economic Cooperation and Development (OECD, 2001). Three male Sprague-Dawley rats (*Rattus norvegicus*) were administered intra-gastrically with 50mg/kg dose of DMSO for 14 days, no mortality was observed and thus DMSO was used as solvent for extract/fractions sample. To check the acute toxicity of the plant, male Sprague-Dawley rats (n = 3) were administered with extract/fractions (50, 250, 500, 1000, 2000, 3000 mg/kg) par oral, whereas saline (10ml/kg body weight (bw) was given to the control group. The animals were examined once daily for 14 days for mortality, behavioral pattern (lethargy, sleep, salivation), changes in physical appearance, injury, pain, and signs of illness. We did not observe any mortality at highest dose of 3000mg/kg, thus 200mg/kg and 400mg/kg bw doses were selected for evaluation of nephro-protective activities (Sakr and Lamfon, 2012).

CCl_4 induced tissue injuries

Nephro-protective effectiveness of FXM on kidney injuries stimulated by CCl_4 and trauma was estimated on Sprague Dawley (150-200g) male rats which were breed in the Primate Facility of QAU, Islamabad. Laboratory animals (48) were divided into 8 groups, in each group 6 rats were placed randomly. Rats were kept in steel cages in 12 h light and dark cycle at QAU, Islamabad's Primate Facility. The protocol of the study (Bch#272) was validated by the ethical committee of QAU, Islamabad. Tap water along with *ad libitum* and rodent chow were given to animals for feeding. Protocol introduced by Shyu *et al*. (2008) was followed but with few modifications.

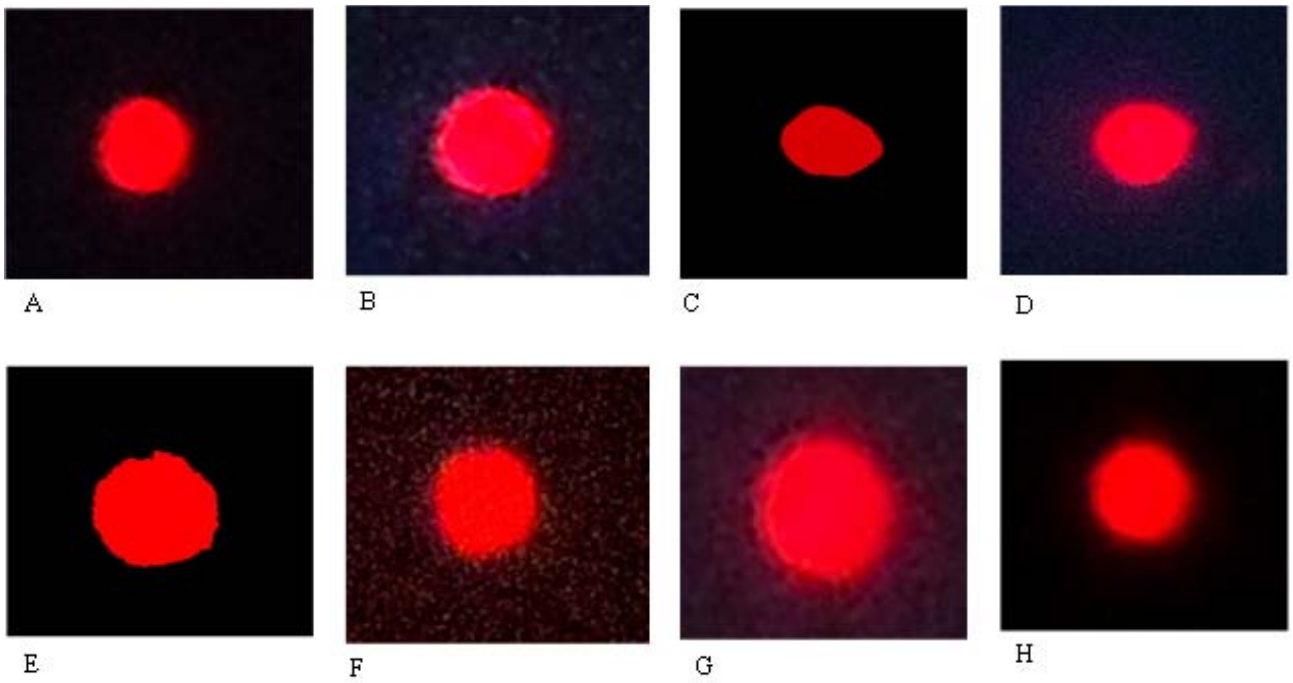

Fig. 1: Comet studies of renal tissues. Ethidium bromide stain; 40× (A) Untreated control (B) vehicle treated (DMSO +Olive oil) (C) CCl_4+Olive oil treated (D) CCl_4+Silymarin (100 mg/kg) treated (E) CCl_4+FXM (200 mg/kg) treated (F) CCl_4+FXM (400 mg/kg) treated (G) FXM (200 mg/kg) treated (H) FXM (400 mg/kg) treated rats. FXM; *F. xanthoxyloides* leaves methanol extract.

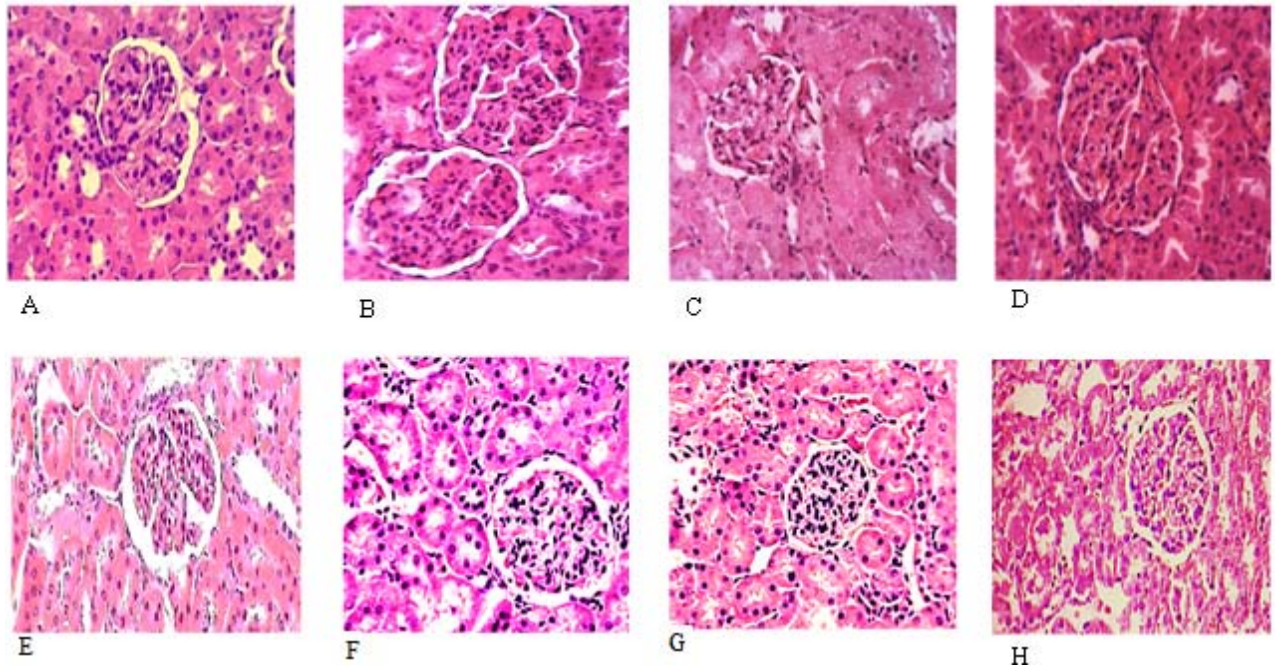

Fig. 2: Histopathological studies of kidney. Hematoxylin and eosin stain; 40× (A) Untreated control (B) vehicle treated (DMSO +Olive oil) hepatic tissues (C) CCl_4+Olive oil (D) CCl_4+Silymarin (100 mg/kg) (E) CCl_4+FXM (200 mg/kg) (F) CCl_4+FXM (400mg/kg) (G) FXM (200 mg/kg) (H) FXM (400 mg/kg). FXM; *F. xanthoxyloides* leaves methanol extract.

Group I was control group (untreated rats) and Group II was vehicle control (10% DMSO in olive oil; 1 ml/kg bw). Intraperitoneally CCl_4 (30% CCl_4 in olive oil; 1ml/kg bw) was given to Group III for 1 month (15 dosages) on alternative days. Group IV, V and VI rats were treated with silymarin (100mg/kg bw) and FXM (200, 400 mg/kg bw par oral) respectively for 30 days along with CCl_4 (15 dosages). Only FXM (200, 400 mg/kg bw par oral) was given to Group VII-VIII for 1 month (15 dosages). After 30 days, normal feed without any treatment was given to the rats for 24 h, urine was collected and preserved at -70ºC for further analysis. Animals were anaesthetized with ether than euthanized, renal tissues were removed and placed in chilled saline solution. A section of each kidney was stored in liquid nitrogen, to conduct biochemical investigations. For histopathology and comet assay small section of each renal tissue was preserved in 10% phosphate buffered formalin.

Biochemical investigation of urine

Albumin, creatinine, creatinine clearance and total protein was estimated by using standard diagnostic kits (MediScreen kit France).

Anti-oxidant enzymes assessment

150 mg of kidney tissue was taken in a tube then 1.5ml phosphate buffer (100mM) in 1mM EDTA with pH 7.4 was added. Samples were homogenized and centrifuged at 12000 × g at 4°C for the period of 30 min and supernatant was obtained which was used later to perform the antioxidant enzyme assays which are as follows.

Catalase (CAT) activity

For CAT activity, we followed Chance and Maehly (1955) method with few amendments. Reaction mixture was consisted of 35µl supernatant, 625µl of phosphate buffer (50mM, pH 5.0) and 100µl of 5.9mM H_2O_2. Absorbance was measured at 240 nm with first reading at 0 minute and second after 1 min. One unit of CAT activity was identical to 0.01 as units/min.

Peroxidase (POD) activity

To assess POD activity methodology of Chance and Maehly (1955) was followed. Reaction mixture was consisted of 625µl of phosphate buffer (50mM, pH 5.0), 75µl of H_2O_2 (40mM), 25µl of guaiacol (20mM) and 25 µl supernatant. After 1 min, change in absorbance was assessed at 470 nm wavelength. One unit of POD activity level was identical to 0.01units/min.

Superoxide dismutase (SOD) activity

Protocol of Habig *et al*. (1974) was followed for SOD activity. Tissue homogenate was centrifuged for 10min at 1500×g and then again at 10,000×g for duration of 15 min, then supernatant was collected. PMS (50µl, 186µM), sodium pyrophosphate buffer (600µl, 0.052mM) was mixed with 150µl of supernatant. Reaction was induced by adding NADH (100µl, 780µM) and glacial acetic acid (500µl) was added after 1min to stop the reaction. The amount of chromogen formed was evaluated by determination of color change at wavelength of 560nm and findings were evaluated in units/mg protein.

Glutathione-S-transferase (GST) activity

Protocol of Younis *et al*. (2016a) was supervened for the evaluation of GST activity. Principle of assay was the generation of conjugate [1-chloro-2,4-dinitrobenzene (CDNB)]. Phosphate buffer (0.1M, 720µl, pH 6.5), reduced glutathione (100µl, 1mM), CDNB (12.5µl, 1mM) and 150µl of enzyme solution were mixed in an eppendorf's tube. Change in absorbance was measured at 350 nm. Activity of the enzyme was estimated as nM CDNB conjugate made/min/mg protein.

Glutathione reductase (GSR) activity

GSR activity was estimated by the method of Carlberg and Mannervik (1975). Basic mechanism of GSR activity depends upon the transformation of oxidized glutathione to reduce glutathione by consuming NADPH. 25 µl of 1mM oxidized glutathione, 50µl of EDTA (0.5mM) and 825µl of phosphate buffer (pH 7.6, 0.1M) was mixed with 50µl of renal tissue supernatant. Then NADPH (50µl, 0.1mM) was added to start the reaction. Absorbance was noted after 20 min at 340 nm wavelength. At 6.22×10^3/M/cm coefficient of molar excitation, the activity of GSR was determined as NADPH oxidized/min/mg protein.

Estimation of biochemical markers

Reduced glutathione (GSH) estimation

GSH was measured by following the protocol of Jollow *et al*. (1974). Firstly tissue homogenate (500µl) was precipitated with 500µl of sulfosalicylic acid (4%). Then test samples were placed for 1 h at 4ºC and in next step these samples were centrifuged at 1200×g for 20 min at 4ºC. 33µl of resulting supernatant was then mixed with potassium phosphate buffer (900µl, 0.1M, pH 7.4) and DTNB (66µl, 100mM). Basic principle of this assay is the reaction of GSH and DTNB to generate 5′-thio-2-nitrobenzoic acid which is detected as yellow color. This color change was assessed by measuring the absorbance at 412 nm. Enzyme activity was measured as µM GSH/g tissue.

Lipid peroxidation assay (TBARS)

TBARS were measured by following the methodology of Iqbal *et al*. (1996). Reaction mixture was consisted of phosphate buffer (290µl, pH 7.4, 0.1M), ascorbic acid (100µl, 100mM), homogenized sample (100µl) and $FeCl_3$ (10µl, 100 mM) with total volume 1000µl. Shaking water bath was used for incubation at 37ºC for 1 h. 500 µl of 10% TCA was added to above reaction mixture to stop the reaction. After that, TBA (500µl, 0.67%) was mixed in the same reaction mixture and the tubes were placed in

water bath (100°C) for 20 min. Then the tubes were kept in crushed ice-bath and centrifuged at 2500×g for 10 min. TBARS formed in respective samples were measured by taking the absorbance at 535 nm wavelength against blank and were represented as nM TBARS/min/mg tissue at temperature 37ºC employing the coefficient of molar extinction 1.56×10^5 $M^{-1}cm^{-1}$.

Protein assessment

The total soluble tissue protein was assessed by following the protocol of Lowry *et al*. (1951). 80mg renal tissue was homogenized in the potassium phosphate buffer and centrifuged for 20 min at 10,000 rpm at 4ºC and supernatant was collected. After that, 0.1ml supernatant was mixed with 1ml alkaline solution and incubated for 10 min. Then Folin-Ciocalteu phenol reagent (1:1) was added in each sample and thoroughly mixed by vortexing. Incubated for 30 min and change in absorbance was noted at 590 nm. Amount of protein present in renal tissue was estimated by using bovine serum albumin standard curve.

Hydrogen peroxide (H_2O_2) assay

To evaluate the quantity of H_2O_2, protocol introduced by Pick and Keisari (1981) was followed. Reaction solution was consisted of tissue homogenate (100µl), phenol red (100µl, 0.28nM), 8.5 units horse radish peroxidase, 250µl of dextrose (5.5nM) and 500 µl phosphate buffer (0.05M, pH 7.0), followed by incubation for 60 min at 37ºC. To stop the reaction, NaOH (100µl, 10N) was pipetted and centrifuged (800 × g) for 5 min. The absorbance was measured against blank at 610 nm. Amount of H_2O_2 produced was measured as nM H_2O_2/min/mg tissue by utilizing H_2O_2 oxidized phenol red standard curve.

DNA damages

Comet assay

We have followed the methodology of Dhawan *et al*. (2009) to check the DNA damages, induced by CCl_4 through comet assay. Autoclaved slides were infused in 1 % normal melting point agarose and were solidified. A tiny section of renal tissue was placed in chilled 1ml lysis solution then homogenized and mixed with low melting point agarose (75µl). The resulting mixture was then coated on the pre-coated slides, then cover slips were placed over them. The gel was then solidify by placing the slide on ice-cubes for nearly 8-10 min. Then, cover slip of slide was gently detached and agarose was added one more time and permitted to solidify by placing on ice cubes. Slide with three coatings of low melting agarose gel was dipped in the lysis solution for nearly 10 min and then employed in the refrigerator for about 2 h. Gel electrophoresis was performed proceeded by slide staining with 1 % ethidium bromide and viewed under fluorescent microscope. For the evaluation of intensity of DNA damage, we use CASP 1.2.3.b software for the analysis of image and 50–100 cells were analyzed from each of the samples.

DNA fragmentation assay

For DNA fragmentation assay, method of Wu *et al*. (2006) was adopted. 0.1ml tissue homogenate in Tris triton EDTA was centrifuged at 200 × g for 10 min at 48°C and labelled as B. Supernatant was collected in separate tube and labelled as S. Again centrifugation was done at 20,000 ×g at 48°C for 10 min. As the result undamaged chromatin was obtained and labelled as C. To all the tubes i.e. B, S and C 1ml of 25% TCA was added and placed in incubator at 48°C for overnight. Centrifugation was done at 18000×g at 48°C, to get precipitated DNA. Then 160µl of 5% TCA was added and samples were warmed for 15 min at 90°C, then 320µl of diphenylamine solution was added. Contents in each tube were mixed by vortex and incubated for 4 h at 37°C. Absorbance was taken at 600 nm. Percent fragmentation of DNA was calculated as:

$$\text{DNA fragmentation (\%)} = \left[\frac{C \times 100}{C + B}\right]$$

Histopathology

Histopathological evaluation was performed for renal tissues. Fixation of samples was done in fixative solution which was consisted of 20% forlmaldehyde, 70% absolute alcohol and 10% glacial acetic acid, after that samples were embedded in paraffin. Sections (3□4 µm) of embedded-tissues were prepared, stained with hematoxylin/eosin and visualized under the light microscope (Nikon Eclipse E100LED MVR, Tokyo, Japan) at 40X.

STATISTICAL ANALYSIS

Data values are shown as Mean ±SD. For this study, data was assessed by using Graph Pad prism 4.0 and Statistix 8.1 software. For the comparison of different treatments with control group, Dunnet test with one way analysis variance was followed at: *=$p<0.05$, $^{\#}$=$p<0.01$ and †=$p<0.001$. Turkey's HSD test was used for the multiple comparisons among treatments. Alphabets in superscript demonstrate significance between treatments on ($p<0.01$), not sharing common letters.

RESULTS

Consequence of FXM on urine profile of rat

Table 1 demonstrates a remarkable rise in the levels of urobilinogen and urinary creatinine however urinary albumin, creatinine clearance and level of urinary proteins was notably lower down by CCl_4 treatment ($p<0.05$) in comparison to control. Co-administration of FXM showed defensive potential against CCl_4 toxicity by restoring these levels. In rats treated with FXM alone, no toxic effects were observed as the values were observed to be near to control. Data showed that FXM had pronounced pharmacological effects on urine profile in comparison to CCl_4 induced toxicity.

Consequence of FXM on antioxidant enzymes of kidney

Treatment of rats with CCl_4 led to the synthesis of free radicals that inhibited the action of antioxidant enzymes. Table 2 clearly reveals substantial ($p<0.001$) decline in the levels of antiradical enzymes of kidney; CAT, SOD, POD, GSR and GST when matched to control. Administration of FXM along with silymarin significantly decreased the CCl_4 toxicity and the antioxidant enzymes level of kidney was restored in the direction of control group. Several correlations of different treatments represent that lower dose of FXM (200mg/kg) as compared to higher dose was less efficient when matched with control group. Higher dose of FXM (400mg/kg) and silymarin restored the antioxidant enzymes activity. Nonetheless, administration of FXM (200 and 400 mg/kg) un-accompanied, did not modify the level of indicated parameters when matched to the control.

Consequence of FXM on renal biochemical markers

The defensive effects of FXM on renal biochemical markers against CCl_4 stimulated toxicity are represented in table 3. Administration of CCl_4 led to the rise in level of H_2O_2, DNA injuries, and TBARS whereas decrease ($p<0.001$) in protein and GSH level in renal tissues when matched to control group. It was also observed that co-administration of FXM reduced the load of H_2O_2, DNA injuries and TBARS which was observed in CCl_4 treated group. Significant rise in tissue protein and GSH level was observed when subjected to co-treatment of FXM and silymarin. In case of protein and TBARS both doses of FXM were effective while in others, effect of FXM was dose dependent. Multiple comparison among various treatments represent the restoring capability of FXM (400mg/kg) along with silymarin on protein, H_2O_2, TBARS and GSH concentration in the direction of control and these were statistically identical to one another. Administration of both the doses of FXM only, did not modify ($p>0.05$) the levels of renal biochemical parameters when matched to the control animals.

Consequence of FXM on comet parameters

Table 4 and fig.1 displayed the defensive impacts of FXM on comet parameters after CCl_4 induced toxicity in renal cells of rats. In our study, DNA injuries induced by the treatment of CCl_4 in renal cells and % DNA in tail and head, tail length and moment, head length, comet length were considerably ($p<0.001$) altered. Nonetheless, % DNA in the head was considerably ($p<0.001$) reduced as compared to control group. Administration of FXM lessened the harmful effects of CCl_4 and bring them near the control. Multiple comparison between different treatments clearly revealed that higher dose of FXM (400mg/kg) expressively ($p<0.01$) re-established most of the parameters of comet along with its shielding affects in contrast to the silymarin treated group. Administration of FXM only did not modify ($p>0.05$) the status of comet specifications in comparison to control.

FXM and consequences of CCl_4 on renal histopathology

Alterations in renal histopathology are abridged in fig. 2. Normal histology of renal tissue was observed in control and DMSO + Olive oil treated group (fig. 2A and B). Both groups displayed normal glomeruli with normal distal and convoluted tubules and Bowman's capsule. Unembellished histopathological aberrations were noticed in the CCl_4 + Olive oil administered group. Malpighian body exhibited anomalous display with impaired corticular segments and accumulation of necrotic cells. There was also cramming of blood vessels, stretching of tubules, inflammatory cell's penetration in corticular and medullary sections, and narrowing of Bowman's capsule space (fig. 2C). Silymarin co-administration diminished the renal injuries with alleviated glomerular degeneration and inflammatory cells infiltration and normal architecture near to control group (fig. 2D). Treatment with CCl_4 and 200mg/kg of FXM presented renal injuries (fig. 2E). Nonetheless, the greater dose of FXM displayed the shielding capability almost close to control (fig. 2F). The administration of FXM alone have no potential to stimulate histopathological changes in renal tissues (fig. 2G and H).

DISCUSSION

Nature has supplied us with plentiful depository in the form of medicinal herbs and plants from hundreds of years and this approach has resulted in isolation of significant number of contemporary drugs from natural products, mainly which has plant origin (Afsar *et al*., 2016). In our previous investigation, HPLC-DAD examination of FXM affirmed the presence of two flavonoides, rutin and caffiec acid (Younis *et al*., 2016a). Three isolated pure compounds; nummularic acid and rutin from ethyl acetate fraction and plectranthoic acid from chloroform fraction of FXM, were characterized by spectroscopic and NMR analysis (data not shown). Two of them belongs to triterpenoid class of phytochemicals i.e. plectranthoic acid and nummularic acid. Plectranthoic acid utilization during in vitro studies has revealed its activity as 5'AMP-activated kinase (AMPK) that is being recognized as metabolic hub for the cure of cancer and type-2 diabetes (Akhtar *et al*., 2015). Furthermore, another analysis (GC-MS) of FXM affirmed the existance of variety of compounds belonging to 15 major classes among them; 26.61% were terpenoids (3), 16.47% lactam (4), 15.81% esters (3), 8.37% phenols (3), 6.91% steroid (2), 5.02% alcohols (3), 4.49% ketones (3), 3.89% aldehyde (1), 3.01% fatty acid glycerol (2), 2.64% nitrile (1), 2.31% lactones (2), 2.25% silyl-ether (1), 1.31% alkene (1), and 0.89% alkyne (1) (Younis *et al*., 2016b).

FXM and its prepared fractions displayed anti-inflammatory activity during *in vivo* and *in vitro* studies by reducing the number of inflammatory mediators such as nitric oxide. GC-MS analysis of FXM marked the

existence of 2-linoleoyl glycerol, squalene, and 2-palmitoyl glycerol, compounds having anti-inflammatory properties, which prevented the activation of monocytes, neutrophils and macrophages (Younis *et al*., 2016b). Kidney can be influenced by the discharge of cytokines and CCl_4 metabolites that cause oxidative damage and procreate inflammatory cascade (Sahreen *et al*., 2015). Anti-oxidant potential of FXM in these studies might be linked to the role of caffeic acid, rutin, and other anti-inflammatory compounds or antioxidant compounds in defending the body system against the lethal effects of reactive oxygen species or free radicals.

Preceeding studies have declared that urine analysis provide clues about the functional situation of kidneys. It could also be mentioned that damaged kidneys show ailed urine profiles. In normal conditions urobilinogen is not the component of urine but its presence in higher amount in urine may be the result of CCl_4-induced renal toxicity in rats. Along with this the augmented urinary creatinine and decreased proteins of urine, albumin, creatinine clearance, are the markers for sevsre oxidative damage to the kidneys (Khan *et al*., 2010b). The present study dipicted that FXM showed the ameliorating effects by making urinary profiles normal.

In this experimental work, concentration of antioxidant enzymes i.e. CAT, POD, SOD, GST and GSR in tissues of kidney was markdly decreased revealing the fact that the physiological stress was stimulated via administration of CCl_4. It is widely affirmed that the correlative activity of antioxidant enzymes is actually a pre-requirement for scavenging the harmful free radicals. The long-lasting and overwhelming production of free radicals reduce the activity of these enzymes. The superoxide radicals created during intoxication or in the process of normal metabolism are transformed by enzyme SOD into H_2O_2 that is consequently degenerated by GPx and CAT thus wiping out the deletrious effects of •OH, to the body tissues. During this study, treatment of CCl_4 when given to rats, lead to the increase in concentration of H_2O_2 in renal tissues. The serious renal injuries examined during these investigations specify a clue that OH radicals created during the transformation process of H_2O_2 can detach hydrogen from PUFA in the biological membranes. Furthermore, the concessed action of secondry antioxidant enzymes decline in status by the production of free radicals. Our results are inaccordance to other studies where CCl_4 stimulated toxication lead to the augmented concentrations of H_2O_2 and reduced action of antioxidant enzymes in tissues of kidney (Al-Yahya *et al*., 2013; Sahreen *et al*., 2015). Co-treatment of FXM mitigates the oxidative stress by scavenging the free radicals that lead to the recovery of antioxidant enzymes activity. Thus, these results are in agreement to various other studies where the treatment with antioxidant producing agents have mitigated the harmful consequences of CCl_4 via restoring the activity of these antioxidant enzymes (Sahreen *et al*., 2015).

We also observed that the toxicity caused by CCl_4 lead to a remarkable rise in TBARS while decrease in total protein and GSH in samples of renal tissues when correlated to non-treated group. Administration of CCl_4, stimulated the oxidative stress in renal tissues via creation of free radicals which are further involved in oxidative lipid peroxidation (Sajid *et al*., 2016). Evaluation of lipid peroxides in our samples is a useful to validate the existance of renal anomalies. Recovery of TBARS and tissue protein via co-treatment of FXM in our study revealed the defensive potential in contrast to CCl_4 stimulated renal injuries in rat. Results of this study have been validated by observing lesser damages in histological studies.

In the same study degree of DNA injury was being appraised in CCl_4 intoxicated Sprague Dawley rat's renal cells and in FXM treated groups by means of comet assay (fig. 1). In our results, various criterion displayed a dose-dependent amelirating effect with CCl_4 co-treatment as compared to control. Remarkable change was assessed in tail moment, % DNA in tail and comet tail length in renal cells of CCl_4 administered group presenting a high degree of DNA damage. Co-treatment of FXM restored the levels to normal. Comet tail length is a dipiction of DNA fragmentation in each and every cell assessed by comet assay. In our findings, increased tail length of comet indicate marked DNA damage in CCl_4 treated kidney cells. Comparable consequences of CCl_4 induced genotoxicity in hepatic cells were also evaluated by Beddowes *et al*. (2003). According to this study at remarkably greater concentration, CCl_4 induced an physiological stress by the decline in antioxidant defense system and reduced glutathione that finally lead to genotoxicity. Single strand breaks in DNA were estimated by the comet assay.

In renal tissues, CCl_4 intoxication mediates the lipid peroxidation in lipid molecuels which lead to cellular damages as shown in histopthological studies (fig. 2). In current study, renal tissues of CCl_4 administered animals indicated the disruption of glomeruli, fibrosis of interstitium, edema of distal and proximal tubules, degeneration of glomerular cells, and necrosis in epithelium.These serious anomalies were not observed in rats that were co-treated with FXM, indicating the defensive effects of this plant against CCl_4 induced toxicity. These results were in coherence with (Sahreen *et al*., 2015) who observed the histopathological progressions in rat kidney tissues treated with CCl_4 and these histopathological anomalies were reduced after administration of *Carissa opaca* fruit extract.

Table 1: Effects of FXM on urine profile *viz*; creatinine clearance, albumin, creatinine, urinary proteins and urobilinogen

Treatment	Creatinine (mg/dl)	Creatinine clearance (ml/min)	Albumin (mg/dl)	Urobilinogen (mg/dl)	Urinary protein (mg/dl)
Control	2.08±0.11^{b}	1.52±0.07^{ab}	9.75±0.24^{a}	4.42±1.31^{de}	30.69±2.10^{a}
DMSO+Olive oil	2.00+0.12^{b}	1.55±0.03^{a}	9.69±1.71^{a}	4.45±0.8^{*cd}	30.65±2.45^{a}
CCl_4+Olive oil	4.67±1.39^{*a}	0.85±0.02^{*d}	4.06±1.11^{*d}	12.14±1.34$^{*\dagger a}$	15.79±1.22$^{* c}$
CCl_4+Silymarin (100)	2.77±0.46^{b}	1.40±0.07^{*c}	8.70±1.15^{*c}	4.39±0.68^{*e}	30.68±2.09^{a}
CCl_4+FXM (200)	2.60±0.55^{b}	1.50±0.06^{b}	9.10±0.55^{*b}	5.13±1.89$^{*\dagger b}$	29.84±0.87^{*b}
CCl_4+FXM (400)	2.57±0.51^{b}	1.53±0.09^{ab}	9.68±1.29^{a}	4.42±1.99^{de}	30.66±1.43^{a}
FXM (200)	2.14±0.20^{b}	1.55±0.08^{a}	9.72±1.21^{a}	4.44±1.43^{cd}	30.68±1.37^{a}
FXM (400)	2.01±0.87^{b}	1.51±0.03^{ab}	9.77±0.90^{a}	4.46±1.24^{*c}	30.65±1.78^{a}

Table 2: Consequence of FXM on antioxidant enzymes of kidney in rat

Treatment	CAT (U/l)	POD (U/l)	SOD (mg/dl)	GST (mg/dl)	GSR (mg/dl)
Control	3.65±0.03^{a}	8.34±0.21^{a}	2.19±0.07^{ab}	139.57±8^{ab}	190.87±9.98^{abc}
DMSO+Olive oil	3.59±0.06^{a}	8.86±0.1^{*a}	2.22±0.06^{ab}	135.9±6.06^{ab}	201.82±10.31^{a}
CCl_4+Olive oil	1.1±0.04$^{\dagger c}$	3.05±0.02$^{\dagger c}$	0.82±0.01$^{\dagger c}$	76.41±4.07$^{\dagger d}$	88.38±4.25$^{\dagger d}$
CCl_4+Silymarin (100)	3.50±0.46^{a}	7.45±0.04$^{\dagger b}$	2.24±0.02^{a}	132.39±7.12^{b}	179.02±4.68^{c}
CCl_4+FXM (200)	2.75±0.12$^{\dagger b}$	6.9±0.38$^{\dagger b}$	2.11±0.05^{*b}	97.13±3.67$^{\dagger c}$	176.29±6.43^{*c}
CCl_4+FXM (400)	3.54±0.05^{a}	7.26±0.08$^{\dagger b}$	2.27±0.05^{a}	131.6±3.79^{b}	180.84±9.96^{bc}
FXM (200)	3.48±0.13^{a}	8.36±0.52^{a}	2.17±0.04^{ab}	139±7.03^{ab}	202.5±7.76^{a}
FXM (400)	3.7±0.03^{a}	8.66±0.31^{a}	2.23±0.02^{a}	145.4±5.86^{a}	198.21±7.42^{ab}

Table 3: Consequence of FXM on biochemical parameters of kidney in rat

Treatment	Protein	GSH (U/l)	TBARS (U/l)	H_2O_2 (mg/dl)	DNA
Control	3.01±0.03^{a}	19.63±0.88^{ab}	2.02±0.01^{b}	1.32±0.03^{c}	32.72±2.11^{c}
DMSO+Olive oil	2.97±0.08^{a}	22.08±1.71$^{\dagger a}$	2.05±0.02^{b}	1.31±0.11^{c}	31.64±2.17^{c}
CCl_4+Olive oil	0.81±0.03$^{\dagger b}$	9.3±0.45$^{\dagger d}$	6.04±0.26$^{\dagger a}$	7.16±0.15$^{\dagger a}$	62.26±3.65$^{\dagger a}$
CCl_4+Silymarin (100)	3.00±0.04^{a}	16.59±1.12$^{\dagger c}$	2.04±0.02^{b}	1.42±0.02^{c}	35.38±1.45^{c}
CCl_4+FXM (200)	2.93±0.06^{a}	11.66±1.05$^{\#d}$	2.1±0.06^{b}	1.90±0.48$^{\dagger b}$	48.6±2.83$^{\dagger b}$
CCl_4+FXM (400)	2.96±0.04^{a}	17.73±1.48^{bc}	2.13±0.04^{b}	1.47±0.01^{c}	36.11±2.17^{c}
FXM (200)	2.96±0.07^{a}	18.69±1.27^{bc}	2.05±0.02^{b}	1.4±0.07^{c}	33.46±1.84^{c}
FXM (400)	2.92±0.02^{*a}	19.27±1.27^{b}	2.05±0.03^{b}	1.30±0.04^{c}	31.41±1.36^{c}

Table 4: Consequence of FXM on comet parameters of kidney cells in rat

Treatment	Comet Length	Head Length	Tail Length	DNA head	DNA Tail	Tail Moment
Control	31.04±1.16^{d}	27.88±1.08^{cd}	3.16±0.99^{d}	97.15±0.65^{a}	2.85±0.65^{d}	0.52±0.02^{c}
DMSO +Olive oil	32.16±1.29^{e}	26.11±0.70^{c}	6.05±1.93^{e}	96.89±1.00^{a}	3.105±1.00^{d}	0.53±0.00^{c}
CCl_4+Olive oil	63.38±1.69$^{\dagger ea}$	40.68±3.15$^{\dagger a}$	22.7±2.55$^{\dagger a}$	69.84±1.28$^{\dagger d}$	30.16±1.29$^{\dagger a}$	1.84±0.07$^{\dagger a}$
CCl_4+Silymarin (100)	34.12±1.05$^{\dagger c}$	29.21±0.73^{*c}	9.91±1.00$^{\dagger c}$	90.48±0.98$^{\dagger b}$	9.52±0.98$^{\dagger c}$	0.59±0.01$^{\#bc}$
CCl_4+FXM (200)	48.08±1.25$^{\dagger b}$	32.62±1.05$^{\dagger b}$	15.46±1.71$^{\dagger b}$	84.86±1.10$^{\dagger c}$	15.14±1.10$^{\dagger b}$	0.68±0.02$^{\dagger b}$
CCl_4+FXM (400)	41.48±2.00$^{\dagger c}$	25.86±0.79^{cd}	15.62±2.34$^{\dagger c}$	89.82±1.25$^{\dagger b}$	10.18±1.25$^{\dagger c}$	0.61±0.03$^{\#bc}$
FXM (200)	34.77±1.21^{de}	27.39±0.88^{e}	7.38±1.62^{d}	94.57±2.08^{a}	5.42±2.08^{d}	0.54±0.01^{c}
FXM (400)	35.82±0.72^{d}	27.18±1.30^{de}	8.64±1.56^{d}	95.04±1.22^{a}	4.96±1.22^{d}	0.53±0.01^{c}

FXM; *F. xanthoxyloides* methanol extract. Mean ± SD (n = 6). For the Dunnet comparison of treatments with control one way analysis of variance was followed at: * = $p<0.05$, $^{\#}$ = $p<0.01$ and † = $p<0.001$. Tukeys' HSD test was used for the multiple comparisons among treatments. Alphabets in superscript demonstrate significance between treatments on ($p<0.01$), not sharing common letters.

CONCLUSION

In conclusion our study dipicted that *F. xanthoxyloides* has the capability to mitigate the CCl_4 provoked injuries in kidney and has resulted in restoration of the urine markers, level of anti-oxidant enzyme activity, DNA damages and histological changes. The ameliorating effects of FXM might possibly be correlated with its antioxidant and anti-inflammatory properties.

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