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Anti-fibrotic potential of a *Matthiola arabica* isothiocyanates rich fraction: impact on oxidative stress, inflammatory and fibrosis markers

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The present study is the first one to investigate the glucosinolates (GLS) profile and anti-fibrotic effect of isothiocyanates (ITCs) rich fraction of *Matthiola arabica* (Brassicaceae) using an experimental model of liver fibrosis in rats. Five GLS (ethyl glucosinolate, gluconapin, glucodehydroerucin, glucoerucin and glucoraphanin) were identified by gas liquid chromatography-mass spectrometric (GLC-MS) analysis of their hydrolysis products, produced by the natural autolysis and exogenous myrosinase hydrolysis using one and two units of the enzyme. Spectrophotometric determination of the total intact GLS revealed that content in the fresh sample was 1.8 times higher than in the dry one. ITCs rich fraction was prepared by natural autolysis of the fresh aerial part. Male albino rats were given carbon tetrachloride (CCl_4) (0.5 ml/kg, twice a week) and/or ITCs –rich fraction (30 mg/kg, three times a week) for six weeks. Liver function, different oxidative stress, inflammatory and fibrosis markers were investigated. Treatment of animals with ITCs rich fraction significantly counteracted the changes in liver function induced by CCl_4. Histopathological examination under both light and electron microscope showed the anti-fibrotic effect of ITCs rich fraction. This finding was confirmed with the markedly improved liver fibrosis markers with ITCs rich fraction co-treatment. In elucidation of anti-fibrotic mechanisms of ITCs rich fraction, the significant glutathione depletion and lipid peroxidation caused by CCl_4 intoxication was restored by ITCs rich fraction co-treatment. Besides, ITCs rich fraction showed an anti-inflammatory effect through its ability to counteract the significant increase in nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2) expression, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) levels in liver tissue that caused by CCl_4 intoxication. These findings indicate that ITCs-rich fraction of *M. arabica* possesses a promising anti-fibrotic effect which can be attributed to its antioxidant and anti-inflammatory properties.

1. Introduction

Liver fibrosis is a complex process, where an excessive accumulation of extracellular matrix (ECM) proteins including collagen occurs in most types of chronic liver diseases. Liver fibrosis is considered a dynamic and potentially bidirectional process prior to the establishment of advanced architecture changes to the liver (Pinzani and Rombouts 2004). The hepatic stellate cells (HSCs) are key players in this process (Friedman 2000). Oxidative stress was shown to play an important role in hepatic injury through HSCs activation and stimulation of ECM production (Bataller et al. 2003). Also, reactive oxygen species (ROS) are involved in the activation of transcription factors such as NF-κB that provide signals for the activation of pro-inflammatory genes (Bauerle and Henkel 1994). Indeed, inflammation is an important key element in the initiation and progression of hepatic fibrosis through production and release of several inflammatory mediators by hepatic resident Kupffer cells to initiate local inflammation. Transforming growth factor-beta (TGF-β1) and IL-6 are the two main fibrogenic cytokines. The inhibition of NF-κB had attracted researchers to be a promising target for discovery of new anti-inflammatory drugs due to its ability to stimulate the expression of enzymes whose products lead to the pathogenesis of the inflammation process (Imanifooladi et al. 2010).

Research of natural products has been directed towards the phytochemical products of the Brassicaceae family due to its high content of glycosinolates (GLS). The hydrolysis products of GLS especially ITCs are known for their biological activity particularly their cancer chemopreventive activity (Martinez-Ballesta and Carvajal 2015). The multiple pharmacological effects of dehydroerucin such as antimutagenic (Nakamura et al. 2001), antibacterial (Beevi et al. 2009), apoptotic and direct preventive antioxidant (Valgimigli and Iori 2009) activities caught the attention of scientists. It is present in only five genera of the Brassicaceae family including *Matthiola* (Bennett et al. 2004). Sulforaphane; the ITC hydrolysis product of glucoraphanin, was proven to have indirect antioxidant effect (Boddupalli et al. 2012) beside its ability to inhibit pro-inflammatory signaling molecules and cytokines (Heiss et al. 2001). These antioxidant and anti-inflammatory effects of GLS hydrolysis products are expected to inhibit the activation of HSCs and prevent the progression of hepatic fibrosis. The genus *Matthiola* is represented in Egypt by wild four species; *M. arabica* Boiss. *M. fruticulosa* (L.) Maire, *M. parviflora* (Schousb.) R.Br. and *M. longipetala* (Vent.) D.C (Boulos 1999). Most of them are traditionally used such as *M. fruticulosa* in renal stones and piles (El-Mokasabi 2014) *M. longipetala* as anti-inflammatory (Akrout et al. 2010) and *M. incana* as aphrodisiac, bitter, diuretic, expectorant, stimulant, stomachic and tonic drug for potential antioxidant activity (Rasool et al. 2013) in addition to the reported powerful ability to reduce serum cholesterol in experimental animals (Yaniv et al. 1999). The safety of *M. arabica* showing a weak cytotoxic effect on breast and colon cancer but no effect on liver and lung was evidenced (Moustafa et al. 2014). This explains the absence of any reports about liver toxicity of the plant which is supported

by the traditional Bedouin use of plant flowers and stems as a food in Sinai (Bailey and Danin 1981). It is also used as cattle food in Jordan without any reported animal toxicity (Qasem 2013). The aim of the present study was to explore the glucosinolates profile and the potential anti-fibrotic effect of ITCs-rich fraction of *M. arabica*, usinga a CCl_4 model of liver fibrosis in rats. Also, the possible mechanisms underlying this anti-fibrotic activity were investigated, as were the anti-oxidant and anti-inflammatory activities and its effect on the expression of fibrosis markers.

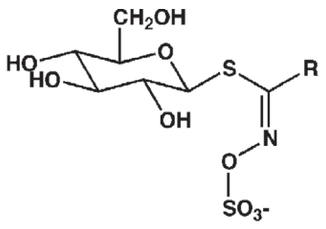
2. Investigations and results

2.1. Identification of glucosinolates hydrolysis products of M. arabica

GLC-MS analysis of glucosinolates hydrolysis products, produced by the natural autolysis and exogenous myrosinase hydrolysis using one and two units of the enzyme (see Experimental) revealed the presence of five glucosinolates. The identification of GLS was based upon the comparison of the mass fragmentation pattern of their hydrolysis products with published data (Al-Gendy and Lockwood 2003; Kjær, et al. 1963; Spencer and Daxenbichler 1980; Blažević and Mastelić 2009) and Wiley & Nist library database as well as reported glucosinolates for other *Matthiola* species (Daxenbichler et al. 1991). Compound names, mass spectral data, relative percentage and the retention time of each compound in the fraction of both ITCs and nitriles are given in Table 1. GLS were identified as ethyl glucosinolate, gluconapin, glucodehydroerucin, glucoerucin and glucoraphanin (Fig. 1). Four ITCs were detected by GLC-MS analysis of GLS hydrolysis products produced by natural autolysis and exogenous myrosinase hydrolysis (1 & 2 units) by different percentages, while two nitriles resulted from natural autolysis and exogenous myrosinase hydrolysis using one unit of enzyme by different percentages. Also a trace amount of 4-(methylthio) butyl ITC was liberated from glucoerucin by exogenous myrosinase hydrolysis using only one unit of the enzyme (see supplementary materials).

2.2. Total intact GLS content of M. arabica

Our spectrophotometric quantitative study was the first one to determine the total content of GLS from *M. arabica*. According to the equation of linear regression relating the concentration to absorbance, A=0.54C+ 0.031 (R^2=0.985), the content of total GLS was calculated. The amount of total intact GLS of *M. arabica* in dry aerial part was 27.5 mg/g dry weight, while it was 48.8 mg/g fresh weight in fresh aerial part.



R	Glucosinolate
CH_3 — CH_2 —	Ethyl glucosinolate
CH_2 ═ CH — CH_2 — CH_2 —	Gluconapin
CH_3 — S — CH_2 ═ CH — CH_2 — CH_2 —	Glucodehydroerucin
CH_3 — S — CH_2 — CH_2 — CH_2 — CH_2 —	Glucoerucin
CH_3 — SO — CH_2 — CH_2 — CH_2 — CH_2 —	Glucoraphanin

Fig. 1: Glucosinolates identified in *Matthiola arabica* plant

2.3. Hepatotoxicity indices

Screening for the hepatoprotective dose of ITCs rich fraction in a model of CCl_4-induced acute hepatotoxicity showed that the highest dose of 30 mg/kg was the most protective one**.** This was evident through the significant reduction of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels by 61and 51.5%, respectively as compared to the CCl_4-intoxicated group (Mohammed et al. 2015). Also, histopathological examination using H& E stain of liver sections confirmed these findings. Injection of CCl_4 (0.5 mL/kg) twice a week for six consecutive weeks (see Experimental) significantly elevated all hepatotoxicity indices confirming the induction of liver hypertrophy (Mantawy et al. 2011) with a marked decrease in albumin level. The co-treatment of animals with the fraction rich in ITCs in alternating days with CCl_4 significantly counteracted or even prevented the changes in all serum parameters as shown in Table 2. It is worth to mention that the animals treated with ITCs rich fraction alone did not show

Table 1: Glucosinolates hydrolysis products obtained by the natural autolysis and the exogenous myrosinase enzymatic hydrolysis of the aerial part of *M. arabica*

Parent compound Hydrolysis product	Rt (min)	Major fragments (% relative intensity)	Relative% of hydrolysis products		
			NA	EH 1unit	EH 2units
Ethyl glucosinolate					
Ethyl isothiocyanate	7.91	59, 72 and 87 (M^+)	7.1	10.7	7.8
Gluconapin					
3-Butenyl isothiocyanate	11.99	41(9.4%),55(22.6%),72(100%),85(11.3%), and 113 (M^+; 64.2%)	2.1	0.38	1.4
Glucodehydroerucin					
5-(Methylthio)-4-pentenenitrile	19.81	41(49%), 55(28.3%), 57(26.4%), 59(13.2%), 87 (100%) and 127 ($M^{+\cdot}$ 51%)	0.22	0.81	-
4-(Methylthio)-3-butenyl isothiocyanate	37.97	41(22.6%), 57(20.7%), 72 (54.7%), 87(100%), 137 (37.7%) and 159 (M^+; 24.5%)	5.9	15.6	7.6
Glucoerucin					
5-(Methylthio)pentanenitrile	20.42	41(27%), 61(100%), 82(42.3%), 93 (9.6%), 114 (7.7%) and 129 (M^+; 57.6%)	1.3	0.24	-
4-(Methylthio) butyl isothiocyanates (Erucin)	38.65*	41, 61,72, 82, 115 and 161 (M^+)	-	Traces	-
Glucoraphanin					
4-(Methylsulfinyl) butyl isothiocynate (sulforaphane)	38.92	41 (36%), 55 (66%), 72(100%), 85(36%), 115(43.3%), 160 (66%), 177 (M^+; 2%) and 178 (M^{+1}; 7.5%)	0.74	9	2.7

All retention time related to autolysis except * related to the external enzymatic hydrolysis using 1 unit of enzyme**.**
NA, natural autolysis; EH, exogenous enzymatic hydrolysis

Table 2: Effects of ITCs rich fraction (30 mg/kg given orally three times per week for 6 weeks) on hepatotoxicity indices in rats subjected to CCl_4-induced liver fibrosis

Group	Liver index	ALT (U/L)	AST (U/L)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Albumin (g/dl)
Control	2.56±0.17 [b]	23.20±7.09[b]	88.50±8.86[b]	94.92±9.23[b]	44.32±9.77[b]	5.77±0.83[b]
ITCs (30 mg/kg)	2.72±0.15 [b]	25.72±1.70 [b]	89.40±6.91[b]	76.94±11.47[a,b]	41.62±8.51[b]	5.32±0.94[b]
CCl_4	3.77±0.13 [a]	54.23±8.66[a]	168.00±5.70[a]	202.40±10.83[a]	157.00±10.36[a]	2.47±0.56[a]
CCl_4+ITCs (30 mg/kg)	3.34±0.15[a,b]	39.38±7.47[a,b]	151.60±4.44[a,b]	115.10±9.39[a,b]	84.76±9.02[a, b]	5.60±0.93[b]

Data presented as mean±SD (n= 6).
a or b: Significantly different from the control or the CCl_4-intoxicated group respectively at P< 0.05 using ANOVA followed by Tukey–Kramer as a post-hoc test.
AST:aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; TG:triglycerides.

any significant changes in all liver function indices except a significant reduction in serum total cholesterol (TC) level by 19% as compared to the control group.

2.4. Histopathological examination

Liver sections stained with H&E and obtained from both control and ITCs rich fraction alone-treated groups showed a normal hepatic architecture where the hepatocytes showed a well-preserved cytoplasm and a well-defined nucleus (Fig. 2A and B), respectively. On the contrary, CCl_4 induced hepatocellular degeneration with formation of fibrous connective tissue originating from the portal area and in hepatic parenchyma which divided into lobules (Fig. 2C). Liver sections obtained from the rats co-treated with ITCs rich fraction beside CCl_4 revealed that the drug was able to reduce fibrosis, but with remaining centrolobular necrosis and ballooning degeneration in a diffused manner all over the hepatocytes in the parenchyma (Fig. 2D).

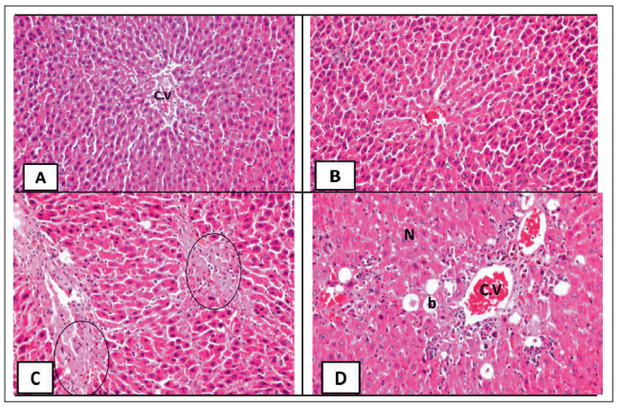


Fig. 2: Representative photomicrographs of liver sections stained by H &E.
A and B:liver sections taken from the control group and that treated with the ITCs fraction alone, respectively showing normal hepatic architecture, hepatocyte structure and central vein (CV) (×20). C: Liver sections taken from CCl_4-intoxicated rats showing heavy cells infiltration and fibrosis (circles) in the portal tract and central vein and peripheral darkly-stained and pyknotic nuclei of hepatocytes (×40). D: Liver sections taken from rats from intoxicated with CCl_4 and concomitantly treated with the ITCs fraction showing the apparent normal looking of central vein (CV) and hepatocytes that regain their eosinophilic cytoplasm containing central rounded and vesicular nuclei with moderate necrosis(N) and ballooning (b) in absence of fibrosis (×40).

Furthermore, electron microscopic examination of sections taken from the CCl_4 group confirmed the light microscopic results through the presence of pyknotic and darkly stained nuclei in some hepatocytes together with the fragmentation of parts of cytoplasm (Fig. 3C).These results provided further evidence for the occurrence of fibrosis that led to loss of normal hepatic architecture and mitochondrial dysfunction. In addition, a well observed flat

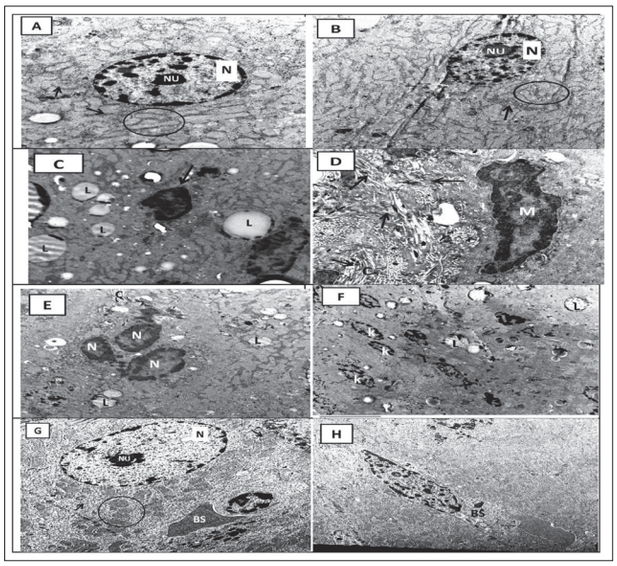


Fig. 3: Representative electron micrograph ultrathin sections of the liver:
A and B:liver sections taken from the control group and that treated with the ITCs rich fraction alone, respectively showing hepatocytes containing euchromatic nucleus (N) with prominent nucleolus (Nu), mitochondria (circle) and rough endoplasmic reticulum (black arrows) (×8000). C, D, E and F:Liver sections taken from the CCl_4-intoxicated rats; C: showing a pyknotic darkly-stained nucleus (arrow) and a lipid droplet (L) in the hepatocyte cytoplasm(×8000), D: showing CT septa (arrows) between hepatocytes containing collagen fibers (C) and myofibroblast (M) (×15000), E: showing neutrophils (N), collagen fibers (C) and lipid droplets (×8000) and F: showing multiple kupffer cells (k) with multiple lipid droplets(L) (×3000).G andH:Liver sections taken from the CCl_4-intoxicated rats and concomitantly treated with the ITCs rich fraction; G: showing a central rounded euchromatic nucleus (N) with prominent nucleolus (Nu), rough endoplasmic reticulum between multiple mitochondria (circle) and Blood sinusoid (BS) (×6000) and H: showing a well-defined blood sinusoid (BS) (×6000).

connective tissue (CT) cell beside collagen fibers assumed to be most probably a myofibroblast (Fig. 3D). Also, multiple neutrophils and Kuppfer cells were observed (Fig. 3E and F, respectively), reflecting mild to moderate inflammation that could be associated with perisinusoidal fibrosis (Mantawy et al. 2011). On the other hand, co-treatment of animals with ITCs fraction nearly preserved the normal liver ultra-structures as shown in Fig. 3G and H.

2.5. Liver fibrosis markers

Hepatic fibrosis is characterized by both qualitative and quantitative changes of hepatic ECM which can be shown by Masson's trichrome stain. In sections stained with Masson's trichrome, the connective tissue septa containing collagen fibers were not demarcated around the classical hepatic lobules in liver sections of both control and ITCs only-treated groups (Fig. 4A and B, respectively).

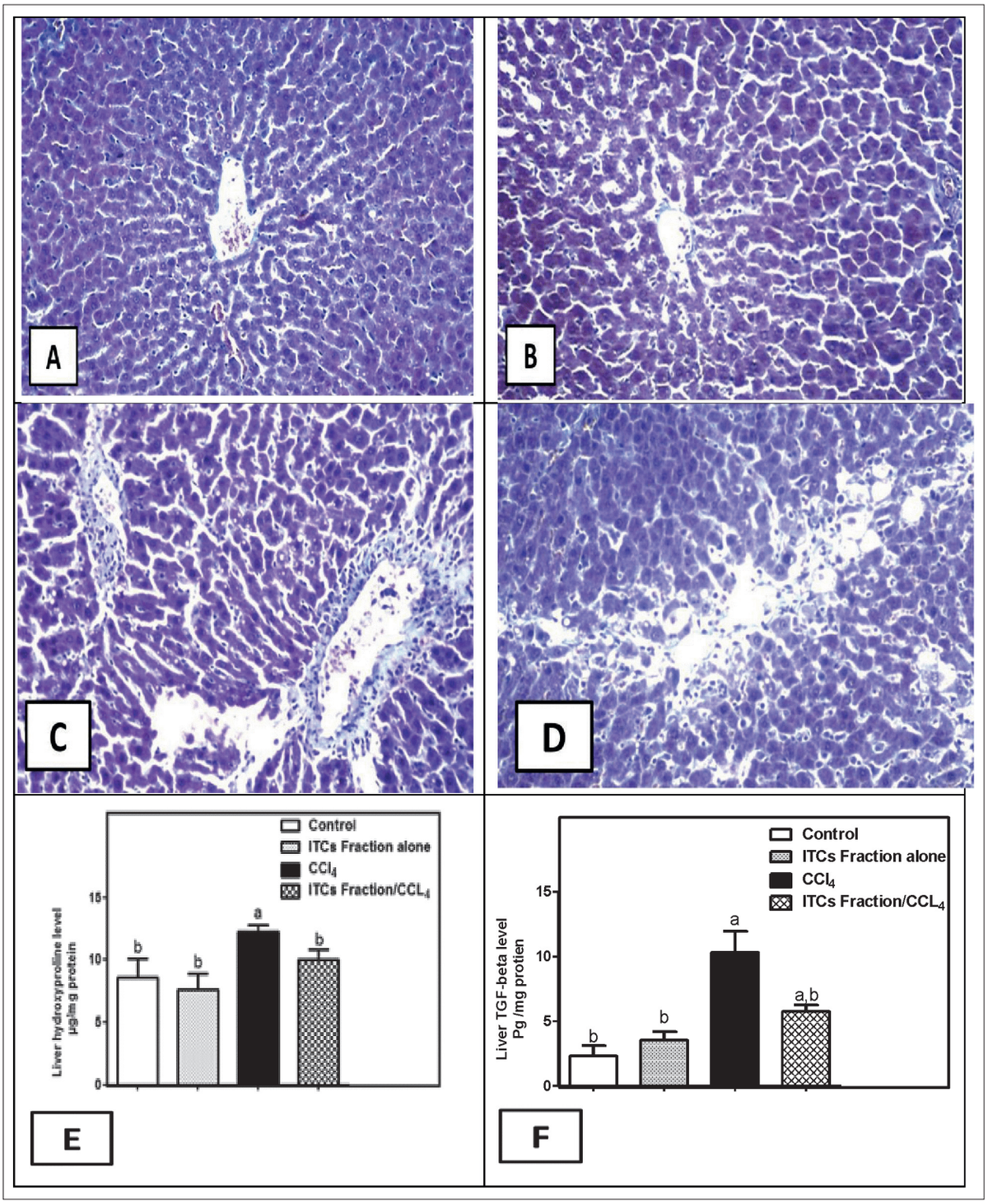


Fig. 4: Representative photomicrographs of liver sections stained by Masson's trichrome.
A and B: Liver sections obtained from the control rats and those treated with the ITCs rich fraction alone, respectivelyshowing complete absence of collagen fibers, stained blue, between hepatic lobules (×20). C: Liver sections obtained from the CCl_4-intoxicated rats showing excessive collagen fibers deposition and pseudolobules formation (bridging fibrosis) (×40). D: Liver sections taken from the CCl_4-intoxicated ratsand concomitantly treated with the ITCs rich fraction showing complete absence of pseudolobules (×40). E: Liver hydroxyproline content expressed as µg/mg protein. F: Liver TGF-β1 content expressed as pg/mg protein. Values are given as mean ± SD. a or b: Statistically significant from the control orCCl_4 group, respectively at $P< 0.05$ using one-way analysis of variance (ANOVA) followed by Tukey–Kramer as a post-hoc test.
TGF-β1: transforming growth factor-beta 1.

In contrast, the collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-intoxicated group and extended from the central vein to the portal tract resulting in the formation of pseudolobules (Fig. 4C). Interestingly, ITCs rich fraction, when administered along with CCl_4, prevented the abnormal collagen deposition (Fig. 4D)**.** Biochemical measuring of hydroxyproline, the main diagnostic compound in collagen as an important reflection of the degree of hepatic fibrosis (Hayasaka and Saisho 1998), supported these results in which chronic administration of CCl_4 resulted in a significant increase in the liver content of hydroxyproline by 42 % as compared to the control group. However, ITCs rich fraction when administered along with CCl_4 prevented this

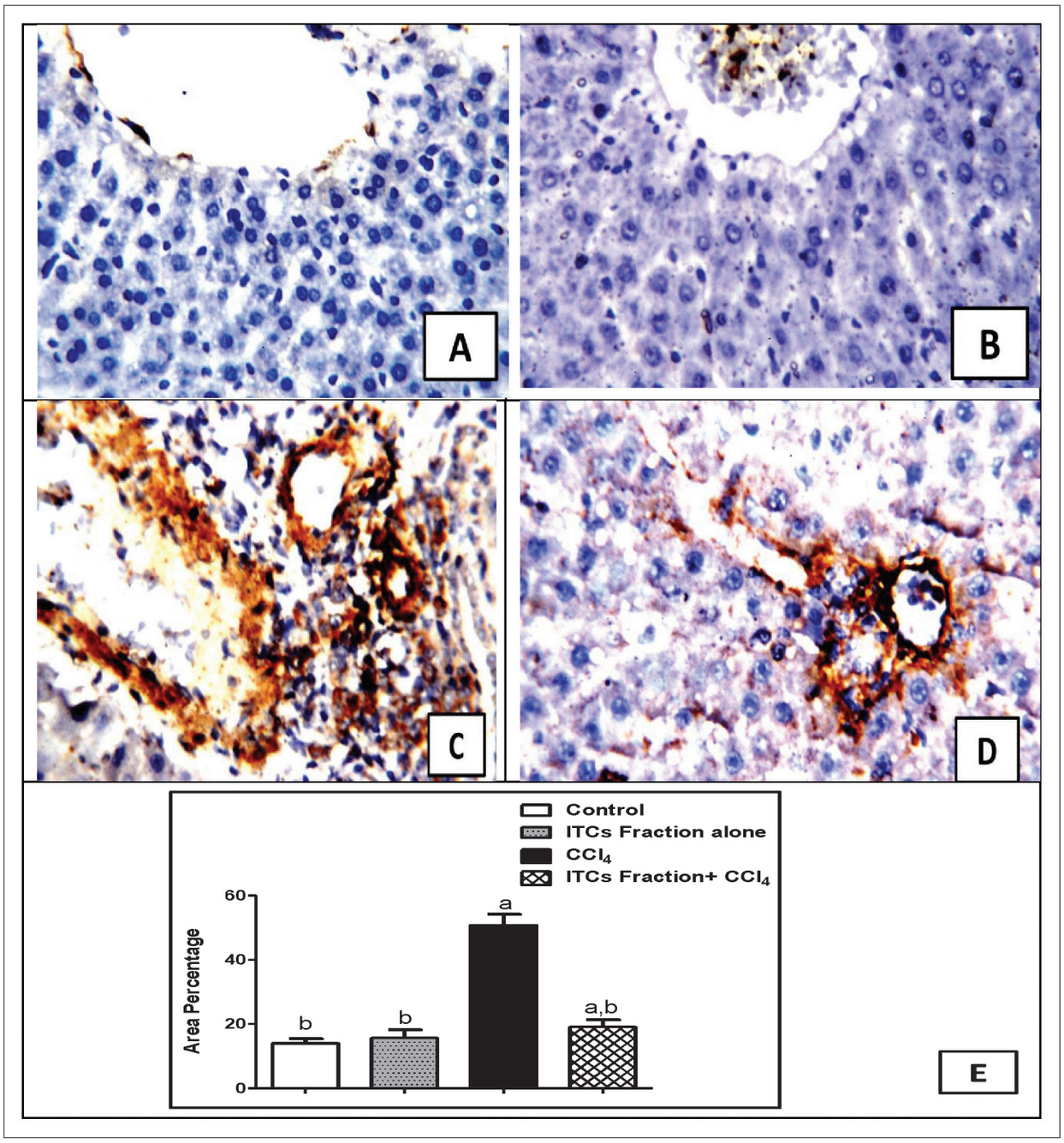


Fig. 5: Expression of α-SMA antigen by immunohistochemical staining (×40).
A and B:Sections obtained from the control rats and those treated with the ITCs fraction alone, respectivelyshowing a minimal α-SMA expression detected in the myofibroblastsand the vascular endothelial cells. C: A liver section obtained from the CCl_4-intoxicated rats showing extensive α-SMA (brown color) expression in portal tracts and sinusoids. D: The liver section taken from the CCl_4-intoxicated rats and concomitantly treated with the ITCs rich fraction showing limited α-SMA expression. E: Quantification of α-SMA staining represents area percentage was averaged across 6 fields for each rat section. Values are given as mean ± SD. a or b: Statistically significant from the control or CCl_4group, respectively at $P < 0.05$ using one-way analysis of variance (ANOVA) followed by Tukey–Kramer as a post-hoc test.
α-SMA: alpha-smooth muscle actin.

abnormal collagen deposition and induced a significant decrease in hydroxyproline content by 18% as compared to the CCl_4-intoxicated group (Fig. 4E).
Furthermore, immunohistochemical detection of alpha-smooth muscle actin (α-SMA) as fibrosis marker and as a marker of activated HSCs revealed minimal staining in the blood vessels of the control and fraction rich in ITCs alone-treated groups (Fig. 5A and 5B, respectively). In contrast, HSCs strongly positive due to over- expression of α-SMA and the significant elevation of TGF-β1 were observed in liver samples of CCl_4-intoxicated group reaching approximately 4 folds as compared to the control group (Fig. 5C and 4F, respectively). Co-treatment of animals with ITCs rich fraction markedly attenuated these elevated expressions by 62 and 44%, respectively compared to the CCl_4 group (Fig. 5D and 4F, respectively). These findings confirmed the anti-fibrotic effect of the whole fraction of ITCs.

2.6. Oxidative stress markers

Chronic administration of CCl_4 resulted in a significant depletion of liver GSH by 65% accompanied with a significant increase in

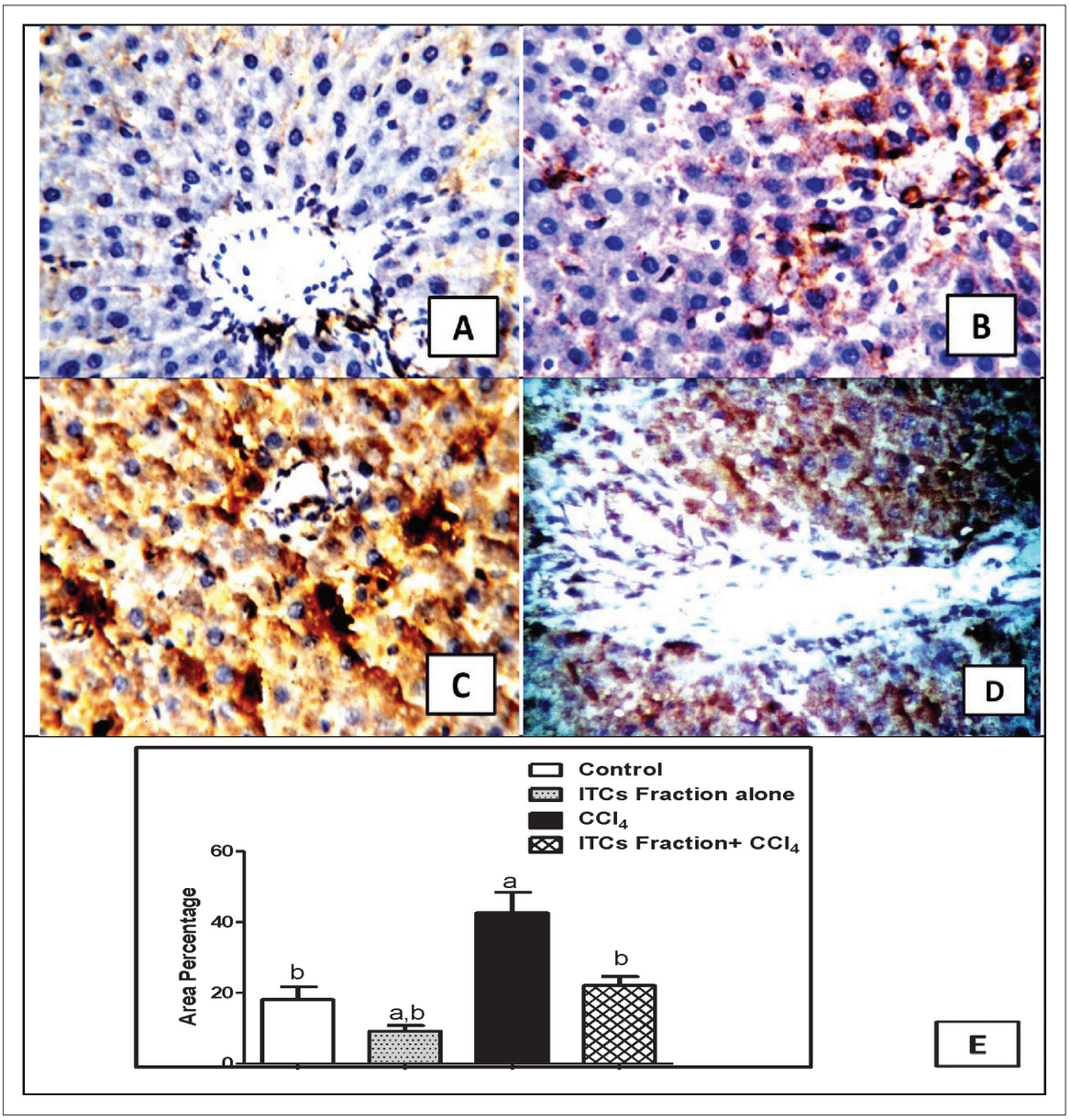


Fig. 6: Expression of COX-2 by immunohistochemical staining (×40).
A and B: Liver sections of the control rat and those treated with ITCs rich fraction alone showing a minimal COX-2 (brown color) expression. C: A liver section obtained from the CCl_4-intoxicated rats showing extensive COX-2 (brown color) expression in tissue. D:Aliver section taken from the CCl_4-intoxicated rats and concomitantly treated with the ITCs rich fractions showing a limited COX-2 (brown color) expression. E: Quantification of COX-2 staining represents area percentage was averaged across 6 fields for each rat section. Values are given as mean±SD. a or b: Statistically significant from the control or the CCl_4group, respectively at $P < 0.05$ using one-way analysis of variance (ANOVA) followed by Tukey–Kramer as a post-hoc test.
COX-2: cyclooxygenase-2

MDA by 19% as compared to the control group. In the present study, GSH and lipid peroxide levels were almost normalized by ITCs rich co-treatment as shown in Table 3.

2.7. Inflammation markers

Assessment of different inflammation markers is important to detect initiation and the progression of liver fibrosis in both animals and humans (Saile and Ramadori, 2007). Intoxication with CCl_4 induced a two fold increase in the liver COX-2 expression (Fig. 6C) together with a significant elevation in tissue levels of both TNF-α and IL-6 by 31 and 40%, respectively as compared to the control group (Fig. 7A and B, respectively). These results reflect an amplified inflammatory response during the chronic liver injury. The consequence of the high levels of COX-2 is the production of high concentrations of nitric oxide and eicosanoids leading to cellular inflammation and necrosis (Hu 2003). The sustained liver inflammation provoked by long-term administration of CCl_4 is believed to be mediated by the NF-κB pathway (Son et al. 2007) which was increased reaching 437.5% in CCl_4-intoxicaed animals as compared to the control group (Fig. 8C and E). In the present study, co-treatment with ITCs rich significantly reduced the expression of NF-κB by 69% when compared to CCl_4-intoxicated group (Fig. 8D) and hence, inhibited the down-stream inflammatory cascade as shown by a decreased the expression of COX-2 (Fig. 6D) as well as the tissue levels of TNF-α and IL-6 to almost that of the control value. Treatment of animals with ITCs rich alone did not induce any significant change in all studied inflammatory markers except a slightly significant increase in IL-6 value as compared to the control group (Fig. 7A and B, respectively).

Table 3: Effects of ITCs rich fraction (30 mg/kg given orally three times per week for 6 weeks) on oxidative stress markers in rats subjected to CCl_4-induced liver fibrosis

Groups	GSH (µmol/g wet tissue)	MDA (nmol/g wet tissue)
Control	0.46±0.10[b]	10.66±0.70[b]
ITC (30 mg/kg)	0.37±0.06[b]	10.02±0.62[b]
CCl_4	0.16±0.05[a]	12.70±0.75[a]
CCl_4+ITC (30 mg/kg)	0.43±0.12[b]	9.86±0.92[b]

Data presented as mean±SD (n= 6).
a or b: Significantly different from the control or the CCl_4-intoxicated group, respectively at P< 0.05, using ANOVA followed by Tukey–Kramer as a post-hoc test.
GSH: Reduced glutathion; MDA: Malondialdehyde.

3. Discussion

The present work is the first study to prepare the ITCs-rich fraction, the hydrolysis product of glucsinolates of *M. arabica*, and to explore its potential anti-fibrotic effect in a carbon tetrachloride model of liver fibrosis in rats. Also, possible mechanisms underlying this anti-fibrotic activity were investigated as were anti-oxidant and anti-inflammatory activities.

Ethyl isothiocyanate is the only hydrolysis product of ethylglucosinolate (Table 1). Gluconapin was identified by its 3-butenyl isothiocyanate. Glucodehydroerucin showed two hydrolysis products; 5-(methylthio)-4-pentenenitrile and 4-(methylthio)-3-butenyl isothiocyanate. Similarly, 5-(methylthio) pentanenitrile and 4-(methylthio) butyl isothiocyanates were liberated from glucoerucin. Glucoraphanin was detected by its highly biologically active 4-(methylsulfinyl) butyl isothiocynate known as sulforaphane. Glucodehydroerucin and glucoerucin were previously reported in different *Matthiola* species (Daxenbichler et al. 1991).

Quantitative determination of the total intact GLS indicated that it was 1.8 fold higher in the fresh sample than in the dry one. To the best of our knowledge there is no previously published data for the total GLS content of *M. arabica.* The total GLS content in the seeds of different *Matthiola* species except *M. arabica* expressed as amount of glucose liberated was previously reported (Daxenbichler et al. 1991).

Depending on the significant reduction of the elevated ALT and AST serum levels attributed to CCl_4 toxicity which was confirmed by histopathological routine examination of liver sections, the hepatoprotective dose of ITCs-rich fraction of 30 mg/kg was selected and used in hepatic fibrosis model (Mohammed et al. 2015).

Regarding the hepatic fibrosis model, ITCs-rich fraction, the hydrolysis product of glucsinolates of *M. arabica*, was able to affect different fibrosis markers particularly α-SMA and TGF-β1 which play a central role in in liver fibrosis, contributing to the influx as well as the activation of inflammatory cells and HSCs (Goodman 2003). This anti-fibrotic activity was preceded through different mechanisms including the anti-oxidant and anti-inflammatory processes.

Isothiocyanates rich fraction was able to restore the exhausted intracellular antioxidants such as GSH by CCl_4 which is very important in maintaining the normal redox state of cells (Masella, et al. 2005) beside its ability to decrease lipid peroxides, degradation products of the attacked lipids by CCl_4 radicals (Mantawy et al. 2011).These findings were supported and confirmed by previously published data which proved the antioxidant effect of erucin ITC, a component of our ITCs-rich fraction, due to its ability to normalize GSH and MDA (Arora et al. 2014). Also research has

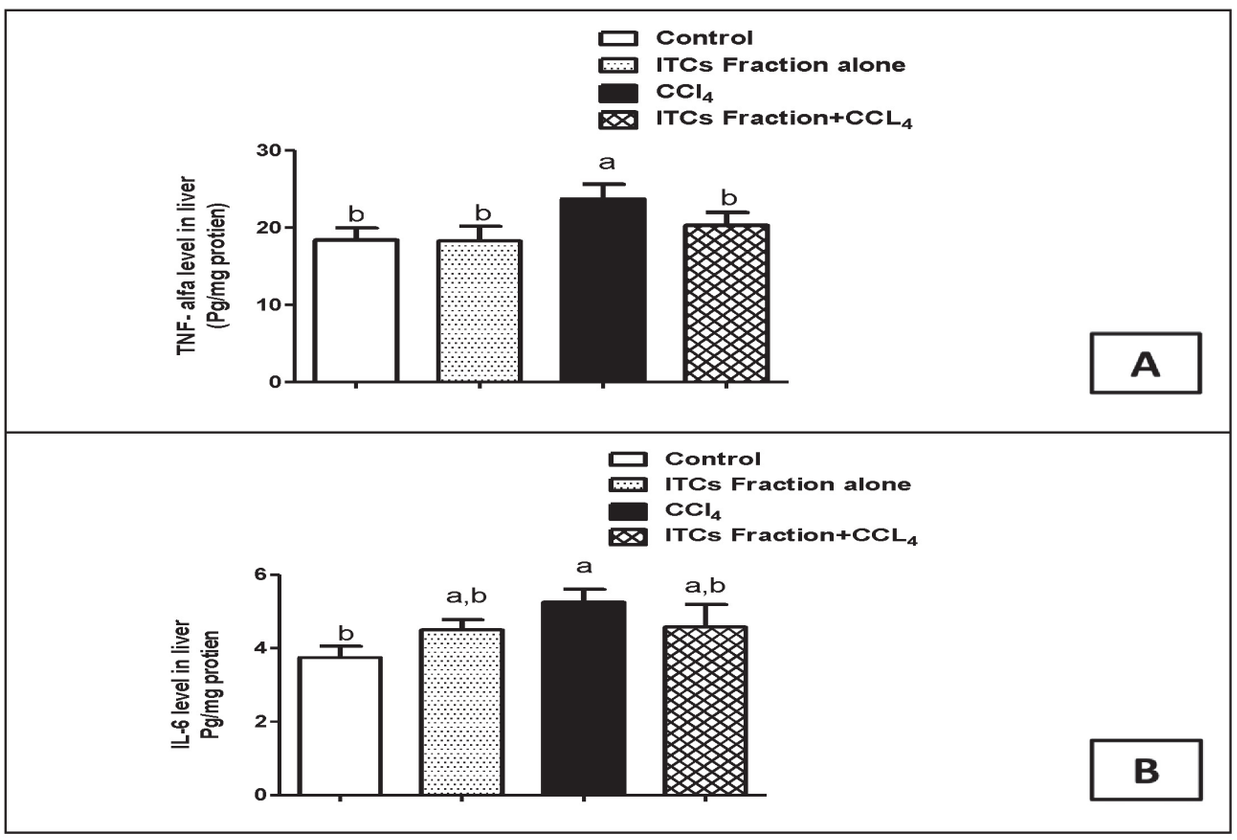


Fig. 7: Effects of the ITCs rich fraction on (A) TNF-α and (B) IL-6 levels in liver tissue in rats subjected to CCl_4 induced liver fibrosis. Values are given as mean± SD. a or b: Statistically significant from the control or CCl_4 group, respectively at P < 0.05 using one-way analysis of variance (ANOVA) followed by Tukey–Kramer as a post-hoc test.TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin 6

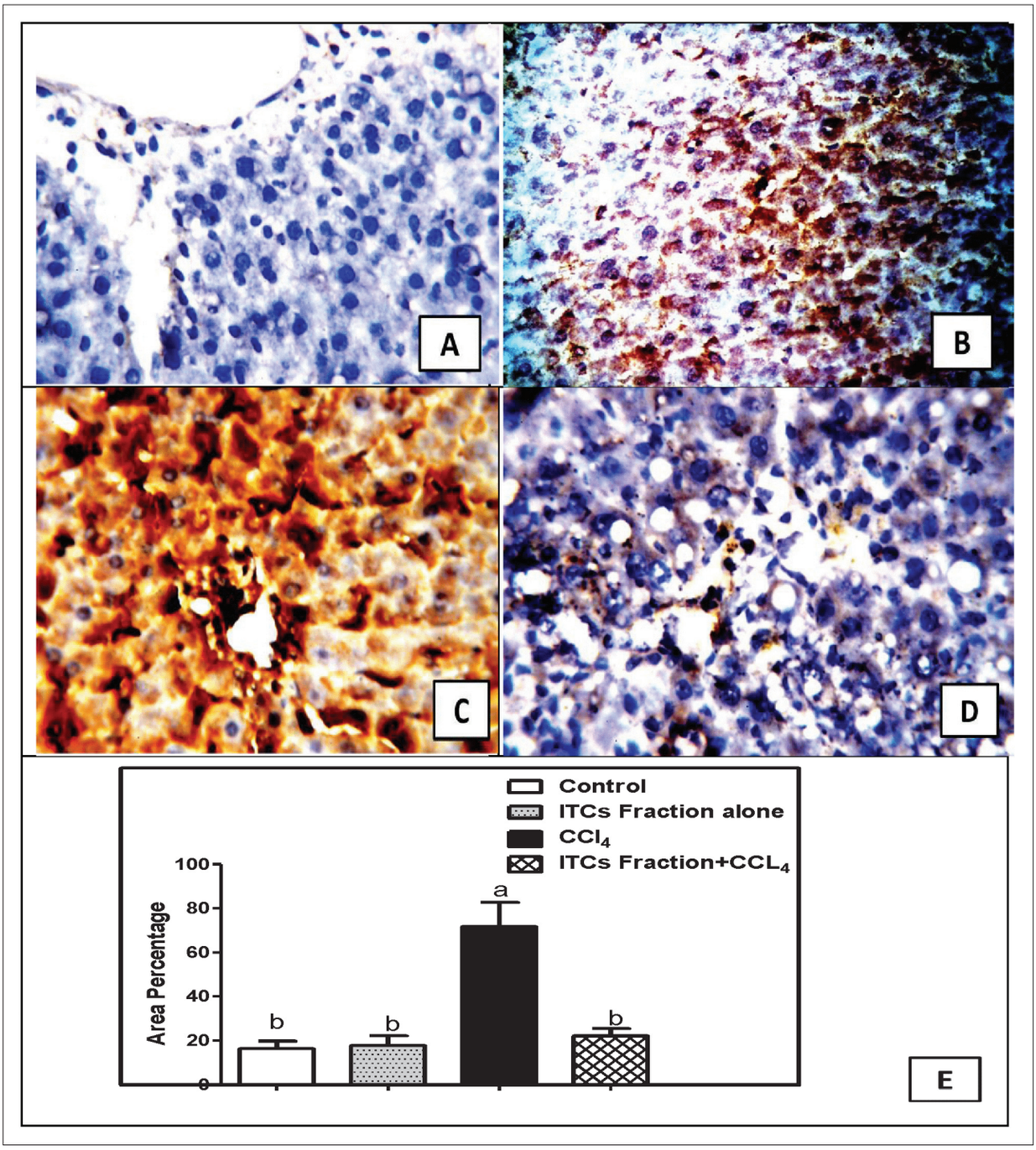


Fig. 8: Expression of NF-κBbyimmunohistochemical staining (magnification ×40).
A and B: Liver sections ofcontrol rat and those treated with the ITCs rich fraction alone showing a minimal NF-κB (brown color) expression. C: A liver section obtained from the CCl_4-intoxicated rats showing extensive NF-κB (brown color) expression in tissue. D:Aliver section taken from the CCl_4-intoxicated rats and concomitantly treated with the ITCs rich fraction showing a limited NF-κB (brown color) expression. E: Quantification of NF-κB staining represents the area percentage was averaged across 6 fields for each rat section. Values are given as mean±SD. a or b: Statistically significant from the control or the CCl_4 group, respectively at $P < 0.05$ using one-way analysis of variance (ANOVA) followed by Tukey–Kramer as a post-hoc test.
NF-κB nuclear factor-kappa B.

been focused on the preventive antioxidant activity of glucodehydroerucin *in vivo* as well as *in vitro* due to its ability to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical quickly. Interestingly, dehydroerucin, the major hydrolysis product in our ITCs-rich fraction, proved to be about three times more reactive than its parent GLS and ten times more potent than common phenolic antioxidants such as 2,6-di-tert-butyl-4-methoxyphenol (BHA) and 2,6-di-tert-butyl-4-methylphenol (BHT) (Papi et al. 2008).

In addition to the antioxidant effect, the ITCs rich fraction provides promising anti-inflammatory effects. These results coincided with those of previous studies that reported the anti-inflammatory effect of sulforaphane, the ITC hydrolysis product of glucoraphanin, where it proved to reduce NF-kB, nitric oxide and prostaglandin E2 (Ritz et al. 2007). Also, it was shown to inhibit the production of some cytokines such as IL-17 and TNF-α *in vitro* and markedly decrease the production of IL-6 *in vivo* (Kong et al. 2010).

In conclusion, ethyl glucosinolate, gluconapin, glucodehydroerucin, glucoerucin and glucoraphanin were detected for the first time in *M. arabica* growing in the Egyptian deserts. The present study is the first one to prepare fraction rich in isothiocyantes; hydrolysis product of GLS, from *M. arabica* as well as to quantify the total GLS content of this plant. Also, it is the first to provide the evidence for the potent hepatoprotective and anti-fibrotic effects of the whole ITCs-rich fraction against CCl_4 induced hepatotoxicity and fibrosis. These promising effects of ITCs-rich fraction could be through attenuating oxidative stress as well as decreasing the expression of NF-κB and subsequent pro-inflammatory cytokines production *in vivo*.

4. Experimental

4.1. Chemicals

The following materials were purchased from Sigma Chemical co. (St. Louis, Mo, USA) and other chemicals used were of the highest grade commercially available. Myrosinase enzyme, L-ascorbic acid, chloroform, sinigrin standard, palladium chloride II, sodium carboxy methylcellulose (SCMS), hydrochloric acid, CCl_4, reduced glutathione (GSH), Ellman's reagent [5,5-dithio-bis(2-nitrobenzoic acid); DTNB], hydroxyproline, chloramine-T, p-dimethylaminobenzaldehyde, bovine serum albumin /(BSA), thiobarbituric acid (TBA), N-butanol, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and trichloroacetic acid (TCA).

4.2. Plant material

Matthiola arabica Boiss. (Brassicaceae), common name komkom, aerial parts were collected from Wadi-Gebal, Saint Catherin governmental protectorate, south Sinai, Egypt in April 2015. It was identified by Dr. Atia Eisa, lecturer of plant taxonomy, Faculty of Science, Damanhour University, Egypt. A voucher sample (Ma-Ap-2015) was kept in the Medicinal and Aromatic Plants Department, Desert Research Center, Egypt.

4.3. Preparation of fraction rich in isothiocyanates

4.3.1. Method A: Exogenous myrosinase enzymatic hydrolysis

Two samples of fresh aerial part rich in leaves (10 g) were homogenized with distilled water (200 mL), myrosinase enzyme (1 and 2 units, Sigma-Aldrich) and 2–5 mg l-ascorbic acid and left to hydrolyse for 2 h. The mixtures were shaken with chloroform (50 mL) for 30 min and centrifuged for 5 min at 3500 rpm. Anhydrous sodium sulphate was used to dry the separated organic layer which then concentrated under nitrogen to about 0.5 mL and kept (in a tightly closed vial) at – 80 °C until analysis (Al-Gendy and Lockwood 2003).

4.3.2. Method B: Natural autolysis

Fresh aerial parts rich in leaves (10 g) were homogenised with distilled water (200 mL) and left for natural autolysis overnight (17 h) at room temperature, followed by extraction of autolysate as described above (Al-Gendy and Lockwood 2003).

4.4. Identification of the hydrolysis products using gas liquid chromatography –mass spectrometry (GLC–MS)

Two microlitre aliquots of the concentrated fraction prepared by previously described methods were subjected to GLC–MS analysis using Agilent GC-MS system chromatograph attached to a Finnigan MAT SSQ 7000 mass spectrometer operated at 70 eV. The column used was a HP- 5MS fused silica capillary column (30 m × 0.25 mm ×0.25 µm; Agilent, Santa Clara, CA). Helium was used as carrier gas in flow rate of 4 ml/min. The injector temperature was 250 °C and the temperature program was 40 °C initially for 2 min, followed by an increase of 4 °C /min to 280 °C.

4.5. Spectrophotometric quantification of the total intact GLS of M. arabica

The spectrophotometric quantification of total glucosinolates was carried out by an external standard method according to a previous publication (Hu et al. 2010) with some modifications. Sample of aerial parts of *M. arabica* was heated at 100 °C for 2 h and another fresh sample was homogenized directly without heating and used in the extraction of GLS. Triplicate of 0.100±0.001 g ground materials were extracted three times with 45 mL deionised water at 50 °C. Each mixture was sonicated and centrifuged for 15 min at 5000 rpm. Each extract was collected into a volumetric flask of a 50 mL and the deionised water was used for volume adjustment to 50 mL. All samples were filtered through a 0.45 mm aqueous filter membrane.

The standard procedures for the measurement of GLS were carried out according to reported conditions (Thies 1982).The reaction was carried out by mixing 2 mL of standard solution or extract, 4 mL of 0.1% (w/w) SCMC in deionised water and 2 mL of 0.004N palladium chloride in 2N HCl. Then the reactions were left at 25 °C for 1 h in test tubes. The mixture absorbance was measured at 520 nm against solvent using 1 cm light-path cuvettes. A blank containing all ingredients except palladium chloride was carried out for each sample. A calibration curve for sinigrin was created from serial dilutions ranged from 0.05 to 0.25 mg/mL. The concentrations of total intact GLS in *M. arabica* extracts were measured according to the equation generated by linear regression.

4.6. In vivo study

4.6.1. Animals

The study was conducted according to the ethical guidelines (Faculty of Pharmacy, Ain Shams University, Egypt). Male albino rats (150–220 g) were obtained from Nile Co. for Pharmaceutical and Chemical industries (Egypt). Rats were housed in an air-conditioned atmosphere of 25 °C with alternatively 12 h light and dark system. Before experimentation, animals were acclimated for 2 weeks and kept on a standard diet pellets (El-Nasr, Abu Zaabal, Egypt) including not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture beside water ad libitum.

4.6.2. Experimental design

Part 1: Screening for the hepatoprotective dose of ITCs rich in a model of acute hepatotoxicity induced by CCl_4

ITCs-rich fraction was prepared by natural autolysis where the aqueous autolysate was given to animals at the required doses.

Thirty animals were randomized into five different groups and treated for one week as follows:

Group 1 received 1 mL/kg, interpretonially (i.p) corn oil on the seventh day (control group).

Group 2 was given CCl_4 at a dose of 1 mL/kg, i.p. (prepared as 1:1 mixture with corn oil) on the seventh day.

Groups 3, 4 and 5 were given ITCs-rich fraction (autolysate) at three different doses of 7.5, 15 and 30 mg/kg, per intragastric gavage (ig) for six consecutive days followed by CCl_4 at a single dose of 1 mL/kg, i.p. (prepared as 1:1 mixture with corn oil) on the seventh day.

At day eight, the rats were anesthetized and blood samples were collected from the retro-orbital plexus and allowed to clot, centrifuged at 1000 rpm for 10 min for serum separation to be used for the assessment of liver functions. Then, rats were sacrificed and specimens from the three major lobes of each liver were fixed in 10% formalin for histopathological examination.

Part 2: Studying the potential anti-fibrotic effect of the selected dose of ITCs rich fraction in chronic CCl_4 intoxication model of liver fibrosis

Forty animals were randomized into four different treatment groups and treated for six weeks as follows:

Group 1 served as control group and received vehicles

Group 2 served as ITCs rich fraction treated alone group and received dose of 30 mg/kg, ig, ITCs rich fraction, three times per week.

Group 3 served as CCl_4 intoxicated (fibrosis) group and received CCl_4 at dose of 0.5 mL/kg, i.p (prepared as 1:1 mixture with corn oil), twice per week.

Group 4 received ITCs rich fraction at a dose of 30 mg/kg, ig, three times per week, alternatively with CCl_4 at a dose of 0.5 mL/kg, i.p. (prepared as 1:1 mixture with corn oil), twice per week.

After 6 weeks, rats were anesthetized and blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 1000 rpm for 10 min and used for the assessment of liver functions. Rats were sacrificed and liver tissues were dissected, washed with ice-cold saline, homogenized in saline and used for the assessment of liver fibrosis markers (hydroxyproline content and TGF-β1), oxidative stress markers (reduced glutathione (GSH) and malondialdehyde (MDA) as well as inflammatory makers (TNF-α and IL-6). Additional specimens from the three major lobes of each liver were fixed in appropriate buffer for light and electron microscopical examination as well as immunohistochemical detection of the inflammatory markers (NF-κB (p65) and COX2) and the fibrosis marker; alpha-smooth muscle actin (α-SMA),

4.7. Assessment of the liver function

Hepatotoxicity was determined by the assessment of liver function parameters such as serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) that were determined according to previous reports (Reitman and Frankel 1957). Besides, serum levels of total cholesterol (TC), triglycerides (TG) and albumin were estimated using the available commercial kits (Spectrum diagnostics, Cairo, Egypt). The formula of (liver weight/body weight) ×100 was used for liver index calculation.

4.8. Assessment of liver fibrosis markers

Hydroxyproline was estimated in the liver as a parameter of liver fibrosis using a reported method (Woessner 1961). Assessment of TGF-β1 tissue level was performed using a commercial ELISA kit (R&D Lab.), according to the manufacturer's instructions. The quantities of rat TGF-β1 was expressed as pg/mg protein. Protein was determined as described previously (Bradford 1976) using BSA as a standard. Beside hydroxyproline assay and TGF-β1, liver fibrosis was further assessed using Masson's trichrome staining for collagen fiber detection as well as immunohistochemical technique to determine α-SMA tissue expression.

4.9. Assessment of oxidative stress markers

To determine GSH, fresh homogenate was used for spectrophotometric method (Ellman, 1959). The results were expressed as µM of GSH/g of wet tissue. As described previously (Uchiyama and Mihara, 1978), lipid peroxidation was estimated by measuring the level of thiobarbituric acid reactive substances (TBARS) expressed as MDA. The results were recorded as nmol of MDA/g of wet tissue using 1,1,3,3-tetraethoxypropane as a standard.

4.10. Assessment of inflammation markers

The TNF-α and IL-6 levels in liver homogenate were determined using a commercial ELISA kit (R&D Lab.), according to the manufacturer's instructions. The quantities of rat TNF-α and IL-6 were expressed as pg/mg protein. Also, NF-κB (p65) and COX-2 tissue expression was determined by immunohistochemistry technique.

4.11. Histopathological examination

4.11.1. Light microscopy

The liver specimens were fixed in 10% formalin for 24 h for histopathological examination using hematoxylin & eosin (H&E) stain and Masson's trichrome stain for demonstration of collagen fibers as described previously (Banchroft et al. 1996).

4.11.2. Electron microscopic examination

The liver tissue samples were immediately fixed in 2.5% phosphate buffered glutaraldehyde (pH 7.4) at 4 °C for 24 h and then fixed in 1% osmium tetraoxide for 1 h, dehydrated by ethanol in ascending manner, immersed in propylene oxide and finally embedded in epoxy resin mixture. Semithin sections (1 µm thickness) were cut, stained with toluidine blue and examined by light microscopy. Ultrathin sections (80–90 nm thickness) were stained with uranyl acetate and lead citrate (Bozzola and Russell 1998), examined and photographed with JEOL 1010 transmission electron microscopy at Regional Center for Mycology and Biotechnology (RCMB), Al Azhar University.

4.12. Immunohistochemical detection of α-SMA, NF-κB (p65) and COX-2

Paraffin embedded tissue sections of 3 µm thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were blocked with 5% BSA in tris buffered saline (TBS) for 2 h then immunostained with one of the following primary antibodies; mouse monoclonal to rat α-SMA (Santacruz Biotech, Cat No. sc-53142), rabbit polyclonal IgG to rat NF-κB p65 (Santacruz Biotech, Cat No. sc-372), or rabbit polyclonal anti rat COX-2 antibody (Thermo Fisher Scientific, Cat No. RB-9072-R7) at a concentration of 1 µg/mL containing 5 % BSA in TBS and incubated overnight at 4 °C. After washing the slides with TBS, incubation of the sections with goat anti-rabbit secondary antibody was established and followed by the washing with TBS and incubation again for 5–10 min in a solution of 0.02 % diaminobenzidine containing 0.01 % of H_2O_2. Counter staining was performed using hematoxylin and the slides were visualized under a light microscope. The quantification of α-SMA, NF-κB p65 and COX-2 staining was performed using Image J image analysis software which was represented as the area percentage of the average of 6 fields for each section.

4.13. Statistical analysis

The data which are presented as mean±S.D was statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post-hoc test. The criterion for significance was determined using 0.05 level of probability. Instat software package (version 3) was used for data statistical analyses and Graphs were sketched using GraphPad Prism software version 5 (ISI® software, USA).

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