Chemical composition and protective effect of *Juniperus sabina* L. essential oil against CCl₄ induced hepatotoxicity

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The hepatoprotective activity of the total extract of *Juniperus sabina* L. against CCl₄ induced toxicity in experimental animals was previously reported and indicated promising results. Essential oil of *J. Sabina* was prepared by hydrodistillation method. Components of the oil were identified by comparison of GC-MS and retention indexes with reported data. The hepatoprotective effect of the essential oil against CCl₄ induced toxicity was studied using male Wistar rats and silymarin at 10 mg/kg p.o as standard drug. The protective effect was evaluated via serum biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT), total bilirubin and non-protein sulfhydryl groups (NP-SH), malonaldehyde (MDA) and total protein (TP). Histopathological study was applied on the liver tissues using Mayer’s hematoxylin stain, Periodic Acid Schiff – Hematoxylin (PAS-H) and Masson trichrome technique on light microscope. Electron microscope images were also obtained for more detailed study.

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1. Introduction

Essential oils are highly concentrated mixtures of saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters, ethers, ketones, oxides, phenols and terpenes (Schiller and Schiller, 1994; Wildwood, 1996). Essential oils are of great importance in therapeutic and cosmetic uses (Evans, 2000). Essential oils obtained from different parts or exudates of the plants are the main therapeutic agents in aromatherapy (Dunning, 2013). Few studies were conducted on the chemical composition and biological activity of *Juniperus sabina* L. essential oil. Essential oils obtained from fruits and leaves of *J. sabina* were proved to possess inhibitory activity against protein glycation and oxidative stress (Asgary et al., 2013). Essential oil from berries and branches of *J. sabina* composed mainly of sabinen and α-pinene showed antioxidant (Emami et al., 2009) and weak antimicrobial activities (Asili et al., 2010). Other studies were concerned with the effect of the geographical source on the oil chemical composition (Adams et al., 2006). Study of *J. phoenicea* and *J. procera* for the search of hepatoprotective secondary metabolites resulted in the identification of hinokiflavone, 4-epi-abietol, sugiol as the most active components (Alqasoumi and Abdel-Kader, 2012; Alqasoumi et al., 2013).

We previously reported on the hepatoprotective effect of *J. sabina* total extract of the aerial part against CCl₁₄ induced toxicity in rats (Abdel-Kader et al., 2016). In the current study we investigated the chemical composition and hepatoprotective effect of the essential oil obtained from the green branches of *J. sabina*. 

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2. Materials and methods

2.1. Plant materials

Aerial parts of Juniperus sabina L. (Cupressaceae) were described earlier (Abdel-Kader, 2016).

2.2. Preparation of the oil

The dried aerial parts of J. sabina (0.5 kg) were subjected to hydrodistillation for 8 h using a Clevenger apparatus with 5 L rounded-bottomed flask. The condensate was extracted with ether. The ether extract was dehydrated over anhydrous sodium sulfate and evaporated to obtain the essential oil. The yield of the oil was 0.5% w/w.

2.3. GC/MS analysis

The GC/MS analyses was carried out on Gas chromatography Mass spectrometer SHIMAZU model 2010 plus equipped with flam ionization detector (FID). Mass spectrometer model MS-2010-Ultra equipped with electron multiplier detector and Quadruple system analyzer. Auto injector model Aoc-20i. GC, injector and detector temperature were set at 220–290 °C respectively. Column temperature was programmed from 60 to 220 °C at a rate of 4 °C/min, lower temperature held for 10 mins, 220 °C to 290 °C at rate of 5 °C/min. Carrier gas: Helium at a flow rate of 1.5 mL/min. Column: Rtx 5MS, crossbond 5% diphenyl, 95% dimethyl polysiloxane. Sample volume 1.0 mL and split ratio was set at 40:1. The mass analyzer was scanned from m/z 35–450 at a scan rate of (3.46) s⁻¹.

The peak identity was confirmed by comparing their mass spectra against commercial (Wiley GC/MS Library, MassFinder 3 Library) (Zaghloul et al., 1989; McLafferty and Stauffer, 1989).

2.4. GC analysis

GC spectrum obtained under same conditions as the above mentioned conditions was used for identification of peaks by comparison of their relative retention index (RRI) to a series of n-alkanes. The quantitative estimation of each compound was carried out based on computerized peak area measurement (Table 1).

2.5. Animals

Male Wistar albino rats (160–180 g) of similar age (8–10 weeks) and Swiss albino mice of either sex (25–30 g), provided by the Experimental Animal Care Center, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, KSA, were used. The animals were kept under controlled temperature (22 ± 2 °C), humidity (55%) and light/dark conditions (12/12 h). The animals were provided with Purina chow and free access to drinking water ad libitum (Alqasoumi et al., 2009). The experimental and procedures were approved by the Ethical Committee at Prince Sattam Bin Abdulaziz University.

2.6. LD₅₀ determination

Acute oral toxicity of the essential oil was evaluated in in Swiss albino mice. Thirty animals were equally divided into five groups (n = 6) as per sex. Group I was kept as control, treated with 1% Tween 80. Other groups received the oil as suspension in Tween 80 at doses of 0.8, 1.6, 3.2 and 6.4 mL/kg by intraperitoneal injection. Animals were observed for symptoms of toxicity for 24 h and at the end of the experiment. The number of mortality in each group were counted. LD₅₀ were calculated using Karber’s method (1931).

2.7. Hepatoprotective activity

Rats were divided into four groups and four subgroups five animals each. Group I received 1% Tween 80 in normal saline and was kept as a control. Groups II–IX received 1.25 mL of CCl₄ in liquid paraffin (1:1) per 1 Kg body weight intraperitoneally. Group II received only CCl₄ treatment. Group III was treated with 10 mg/kg p.o. (20.7 μmole/kg) of silymarin (Sigma-Aldrich, St. Louis, MO, USA) (Abdel-Kader et al., 2016). Groups IV was divided into four sub groups IVa- IVd treated with 50, 100, 150 and 200 mg/kg of J. sabina essential oil. Treatment started 5 days prior to CCl₄ administration and continued till the end of the experiment. After 48 h, following CCl₄ administration the animals were sacrificed under ether anesthesia. Blood samples were obtained by heart puncture and the serum was separated for biochemical parameters measurements. The livers were immediately removed and representative pieces were immersed in10% formalin for fixation necessary for histopathological study.

2.7.1. Determination of enzyme levels

Serum glutamate oxaloacetate transaminase Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT), (ALP) and total bilirubin were determined following the reported methods.
(Edwards and Bouchier, 1991). The enzyme activities were measured by Reflotron® diagnostic strips (Roche, Basel, Switzerland) and Reflotron® Plus instrument (Roche).

2.7.2. Determination of tissue parameters

Non-protein sulfhydryl groups (NP-SH) were measured following Sedlak and Lindsay method (1968). Livers were cooled in ice bath. Weight of 200 mg of liver tissues was homogenized in 8 mL of 0.02 M ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 mL of the homogenate were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid (TCA). To precipitate protein, the tubes were shaken for 10–15 min at intervals and centrifuged at 3000 rpm for 15 min. Two

For the measurement of MDA level, aliquots from liver homogenate were incubated with shaking at 37 °C for 3 h, mixed with 1 mL of 10% aqueous TCA and centrifuged at 800 rpm for 10 min. From the supernatants, 1 mL from each were mixed with 1 mL aqueous solution of 0.67% 2-thiobarbituric acid and heated for 10 min on boiling water bath. Mixtures were cooled, mixed with 1 mL distilled water and the absorbance values were measured at 535 nm. The content of MDA (Table 3) (nmol/g wet tissue) were estimated from the calibration curve of MDA solution (Utley et al., 1967).

For the determination of the TP, portions of the homogenate were mixed with 0.7 mL Lowry’s solution and kept at room temperature for 20 min in dark then 0.1 mL of diluted Folin’s reagent were added. Mixtures were kept for 30 min at room temperature away from light. The absorbance values then measured at 750 nm (Table 3) (Lowry et al., 1951).

2.8. Statistical Analysis

Analysis of variance (ANOVA) test was used to judge whether the difference between groups is significant or not. Non paired samples such as control and CCl4-treated group were compared for significance using Dunnette's test (Woolson and Clarke, 2002). All the reported values are presented as mean ± S.E.

2.9. Histopathology

The livers samples were dehydrated, cleared and infiltrated by immersion in increasing concentrations of ethanol (70–100%), xylene (3 times, 1 h each) followed by paraffin wax (4 times, 1 h each). The tissues were oriented by hot forceps in moulds and then stained with methyl blue, dehydrated, cleared and mounted sections in DPX (Hamad and Ahmed, 2016).

Deparaffinized liver sections were stained with Weigert's iron hematoxylin for 2 min, differentiated in acid alcohol 2 dips, rinsed with tap water 2 dips, and blued in running tap water for 10 min, dehydrated, cleared, and a coverslip mounted with DPX (Product 03600, Loba Chemie Pvt. Ltd., Mumbai, India) (Hamad and Ahmed, 2018).

All values represent mean ± SEM.*p < 0.05; **p < 0.01; ***p < 0.001; ANOVA, followed by Dunnett’s multiple comparison test. A As compared with Control group. B As compared with CCl4 only group.
2.10. Electron microscopy

Tissue specimens of about 2–3 mm thick were fixed in 4% glutaraldehyde (Product 16210, Electron Microscopy Sciences (EMS), Hatfield, PA, USA) for 2 h followed by 1% osmium tetroxide (Product 19100, EMS) for 1 h. Then, dehydrated using 70% ethanol for 10 min, 100% ethanol for 10 min, and 100% propylene oxide (Product 8.07027.1001, Merck KGAa, Darmstadt, Germany) for 15 min × 2. Samples were subjected to infiltration using mixture of EMbed 812 one-step single mix formula composed of 20 mL of EMbed 812 (Product 14900, EMS), 16 mL of Dodecenyl Succinic Anhydride (DDSA) (Product 13710, EMS), 8 mL of Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride (NMA) (Product 19000, EMS) and 0.66–0.88 mL of 2,4,6-Tris(dimethylaminomethyl) phenol (DMP-30) (Product 13600, EMS). Tissues were drained of most of the propylene oxide. Then tissues were soaked in 1:1 solution of propylene oxide: embedding medium for 1 h at room temperature followed by 2:1 embedding medium to propylene oxide at RT overnight. Finally, the mixture was replaced with 100% embedding medium for 2 h at RT. Specimen rotator (Product 15920D, Thermo Fisher Scientific, Carlsbad, CA, USA) was used during fixation, dehydration and infiltration. Embedding was finalized by transferring tissues in EMS embedding capsules (Product 69910-05, EMS) then filled with the embedding medium. Capsules were then incubated at 60 °C in oven for 24 h to make blocks. Ultramicrotome (Product PT-PC #75,840, RMC Boeckeler Instruments, Inc., Tucson, AZ, USA) was used to obtain ultra thin section of 100–200 nm after cooling to room temperature. Sections were loaded on grid (Product G200-Cu, EMS) and stained manually in 1% uranyl acetate (Product 93-2840, STREML CHEMICALS, Newburyport, MA, USA) for 15 min away from light, rinsed with normal saline for 6 times followed by 0.5% lead citrate (Product 17810, EMS) beside several pellets of sodium hydride then rinsed in distilled water. Tissues were then dried and examined under transmission electron microscope (TEM) (Product FEI TECNAI 12, Thermo Fisher Scientific, Hillsboro, Oregon, USA) (Luft, 1961, Woods and Stirling, 2013).

3. Results and discussion

The facts that total extract of J. sabina showed marked hepatoprotective activity (Abdelkader, 2016) and that essential oils are highly concentrated mixtures of low molecular weight diverse secondary metabolites (Schiller and Schiller, 1994; Wildwood, 1996) initiate the study of the plant essential oil chemical composition and hepatoprotective effect.

Combination of GC-MS and GC analyses of the essential oil of J. sabina enable the identification of 36 components representing 97.07% of the oil components. Hydrocarbons represent the major components of the oil as their percentage was 77.14. Sabineol represents 55.820% of the oil components followed by α-pinenene (5.210%). Total alcohols represent 9.01% of the oil with the major alcohol citronellol 1.040%. Acetate esters represents 3.84% out of them linalyl acetate represents 2.21%. Caryophyllene oxide represents 1.48% while the total oxides representing 3.07% of the oil. Ketones represent the least component in the oil with total percentage of 2.03 of which pinocarvone represents 0.83% of the oil. Monoterpenes derivatives were the major components of the oil representing 76.24% while sesquiterpenes derivatives represents 19.76%. Only one fatty acid; dodecanoic acid was detected in the oil (Table 1).

The induction of hepatotoxicity using CCl₄ cause severe disturbances of calcium homeostasis leading to necrotic cell death (Weber et al., 2003). Significant increase of transaminases (AST and ALT) and alkaline phosphatase (ALP) levels was due to hepatocytes damage (Zafar and Ali, 1998). Severe jaundice was diagnosed via elevated levels of serum bilirubin (Table 2) (Lin et al., 1997).

The standard drug silymarin at a dose of 10 mg/kg (20.7 μmol/kg) provides protective effect mediated via; scavenging the free radicals, increase the intracellular concentration of GSH, enhancement of the cellular membrane permeability, stimulation of protein synthesis leading to regeneration of liver cells (Saller et al., 2007; Dehmolow et al., 1996). These effects resulted in normalization of the biochemical and tissue parameters (Tables 2 and 3).

The safety of the oil was accessed via LD₅₀ determination following Karber’s method (1931). The LD₅₀ of the oil was 3.4325 mg/kg. For the hepatoprotective effect four doses were used 50, 100, 150 and 200 mg/kg. The essential oil showed dose dependent hepatoprotective effect. The effect was comparable with that of silymarin at the highest dose used at 200 mg/kg. The protective effect was accessed via the measurement of biochemical and tissue parameters as well as histopathological study. The effect of J. sabina essential oil at 200 mg/kg on reducing the levels of aspartate amino transferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) and total bilirubin (36.64, 43.39, 30.44, 25.10, 53.11 % respectively) was less than the effect observed in the group of animals treated with silymarin (53.15, 65.53, 56.73, 31.01 and 64.27 % respectively) was less than the effect observed in the group of animals treated with silymarin (53.15, 65.53, 56.73, 31.01 and 64.27 % respectively) was less than the effect observed in the group of animals treated with silymarin (53.15, 65.53, 56.73, 31.01 and 64.27 % respectively) was less than the effect observed in the group of animals treated with silymarin (53.15, 65.53, 56.73, 31.01 and 64.27 % respectively).

Histopathological study was conducted using Mayer’s haematoxylin stain, Masson trichrome technique and Periodic Acid Schiff – Hematoxylin (PAS-H) on light microscope (Fig. 1). Masson trichrome stain collagen with blue colour and give an indication about fibrosis due to liver injury. Using PAS-H confirms the presence of glycogen in the liver cells (Krishna, 2013). Tissue specimens were also examined by electron microscopy (Fig. 2). Liver cells of the CCl₄ treated group showed degenerative liver tissue, necrosis with complete occlusion of the blood vessels (b-i), large amount of collagen fibers with the blue colour (b-ii), and absence of PAS positivity (b-iii). Electron microscope pictures (Fig. 2) showed restored cellular and nuclear membranes with decreased vacuolization and presence of fat droplets (c-i), regaining many normal nuclei without clumps (c-ii), normal cytoplasm with normal Golgi apparatus and very few small fat droplets are present in the cytoplasm (c-iii).

Liver cells of CCl₄ and 150 mg/kg J. sabina essential oil treated group (Fig. 1) showed partial healing of central veins (d-i), moderate amount of collagen fibers (d-ii), and moderate amount of PAS.
positive materials (d-iii) indicating partial healing. Treatment with CCl₄ and 200 mg/kg *J. sabina* essential oil resulted in complete recovery of liver cells (e). Electron microscopy for liver specimen of animals treated with CCl₄ and 150 mg/kg *J. sabina* essential oil (Fig. 2) showed partial recovery indicated by some degeneration, necroses (d-i), regaining intact nuclear membrane but still some uneven distribution of chromatin material in the nucleus (d-ii), partial degenerated pattern of Golgi apparatus, weak cytoplasmic production and very few fat materials but no fat droplets (d-iii). Liver cells of CCl₄ and 200 mg/kg *J. sabina* essential oil treated group showed almost normal appearance of nucleus, functioning cytoplasm and fat materials (e-i), very high improvement and healing activity by regaining intact nuclear membrane and even distribution of chromatin material in the nucleus (d-ii). The cytoplasm shows normal pattern of Golgi apparatus and very few fat materials with complete absence of fat droplets.

4. Conclusion

The yield of the essential oil from the aerial parts of *J. sabina* was 0.5% w/w. GC-MS study of the oil enable the identification of 36 components representing 97.07% of the oil components. Sabinene was the major components of the oil representing 55.820% followed by α-pinene (5.210%). The LD₅₀ of the oil found to be 3.4325 mg/kg. The essential oil showed dose dependent hepatoprotective effect. The effect was comparable with that of silymarin at the highest dose used 200 mg/ml. The effect of the oil on
reducing the level of MDA toward the normal level and restoring NP-SH groups was superior to the effect observed with silymarin treatment. Light microscope pictures showed complete recovery of cells in the group treated with CCl4 and 200 mg/kg J. sabina essential oil. Similarly, electron microscope study revealed very high level of protection for the same group which received CCl4 and 200 mg/kg J. sabina essential oil. The fact that the LD50 is fifteen fold higher than the effective dose gives an indication about the safety of the essential oil for use.

**Declaration of Competing Interest**

The authors declare that; there is no conflict of interest.

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