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Hautriwaic acid as one of the hepatoprotective constituent of *Dodonaea viscosa*

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ABSTRACT

It is widely known that hepatitis and its complications such as cirrhosis or hepatocellular carcinoma are one of the major health problems of the world especially since no specific treatment is available. In the present study we investigated the hepatoprotective potential of the methanolic extract of the whole plant of *Dodonaea viscosa* and its ethyl acetate, aqueous, butanol and *n*-hexane fractions against carbon tetrachloride (CCl_4) induced hepatoxicity in rats. Hepatoprotection was assessed in terms of reduction in serum enzymes (ALT, AST, and ALP) that occur after CCl_4 injury, and by histopathology and immunohistochemistry. The methanolic extract reduced the serum enzyme level (ALT, AST, and ALP) down to control levels despite CCl_4 treatment. It also reduced the CCl_4-induced damaged area to 0% as assessed by histopathology. The CD68+ macrophages were also reduced in number around the central vein area by the methanolic extract. These hepatoprotective effects were better than the positive control silymarin. Similar hepatoprotective activities were found with the ethyl acetate, and aqueous fractions of the methanolic extract. The butanol and *n*-hexane fractions showed elevated levels of ALT, AST and ALP as compared to the positive control silymarin. Histopathology showed ~30% damage to the liver cells with the butanol and *n*-hexane fractions which still showed some protective activity compared to the CCl_4 treated control. HPLC fingerprinting suggested that hautriwaic acid present in the methanolic extract and its ethyl acetate, and aqueous fractions may be responsible for this hepatoprotective activity of *Dodonaea viscosa* which was confirmed by *in vivo* experiments.

Introduction

Liver is the first organ to metabolize all foreign compounds and hence it is susceptible to injury that can result in different diseases such as hepatitis, cirrhosis or hepatocellular carcinoma. A major cause of these disorders is exposure to different environmental pollutants and chemicals *e.g.*, paracetamol, carbon tetrachloride, thioacetamide, alcohol, *etc.* Worldwide, hepatitis is an important liver disease with a staggering incidence of 550 million (Alter 2006). It is more common in developing countries. In Pakistan almost 35 million people are believed to be infected with the virus (André 2000). Its complications such as cirrhosis of liver are formidable enemies since there is no specific treatment available for them. Many of the drug-induced liver injury cause morbidity and mortality around the world (Ghabril et al. 2010).

Conventional or synthetic drugs used in the treatment of liver diseases are inadequate and can have serious adverse effects. Many natural products of herbal origin are in use for the treatment of liver aliments. Polyphenolic compounds are widely distributed in plants and known to be excellent antioxidants *in vitro* and have the capacity to scavenge free radicals and protect antioxidant defenses in liver diseases (Sreelatha et al. 2009).

Carbon tetrachloride (CCl_4) is a widely used and well characterized animal model of chemical-induced, oxidative stress-mediated hepatotoxicity (Recknagel and Glende 1973). The symptoms of chronic liver injury in humans are similar to those of CCl_4-induced chronic liver injury (Basu 2003). CCl_4 induces the production of several types of reactive oxygen species (ROS) through cytochrome P450 thereby causing liver injury (Tada et al. 2003). These ROS can bind to polyunsaturated fatty acids, forming different radicals to produce lipid peroxide, which causes membrane damage and changes in enzyme activity (Weber et al. 2003), and consequently

Abbreviations: CCl_4, carbon tetrachloride; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; MeOH, methanolic extract; EtOAc, ethyl acetate; H_2O, aqueous.

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induce hepatic injury, inflammation, necrosis and apoptosis, (Lin et al. 2009).

Dodonaea viscosa, is an evergreen shrub belongs to the family spindaceae, consists of approximately 2000 species and 150 genera. The genus *Dodonaea* consists of 60 species. Previous work on *Dodonaea viscosa* revealed the presence of major secondary metabolites isolated from *Dodonaea viscosa* consist of diterpenoids, triterpenoid saponine organic acids, flavonoids, viscosine (Khan et al. 2013), methylenebissantin (Muhammad et al. 2012a,b) tannins, sterols (Wagner et al. 1987). *Dodonaea viscosa* is widely used in folk medicine for treatment of skin diseases (Pirzada et al. 2010). The crude extracts of *Dodonaea viscosa* have shown antidiabetic, antimalarial, antibacterial (Khurram et al. 2009) and gastroprotective activity (Arun and Asha 2008). Taking into account our interest in the medicinal plants of Pakistan (Riaz et al. 2002) and pharmacological significance of *Dodonaea viscosa*, the present hepatoprotective investigation was undertaken to identify natural compounds from *Dodonaea viscosa* that may be used as potent hepatoprotective agents.

Materials and methods

Plant materials

The whole aerial parts of *Dodonaea viscosa* were collected from the hills of Kurrum agency, Khyber Pakhtoonkhwa province of Pakistan. The identification was done by Dr. Ijaz Khan, a plant taxanomist, Department of Botany, Post Graduate College, Kohat, Khyber Pakhtoonkhwa, Pakistan. A voucher specimen (DVPGCK-098) has been deposited in the herbarium of Department of Botany, Post Graduate College, Kohat.

Extract preparation from Dodonaea viscose

The shade-dried plant (20 kg) material was ground into powder and extracted at room temperature with MeOH to yield the methanolic extract (2 kg). After removal of the solvent, the extract was suspended in H_2O, and extracted with *n*-hexane, Ethyl acetate (EtOAc), and butanol to yield hexane (620 g), EtOAc (507 g), and butanol (750 g) fractions. The extract and fractions were kept at 4 °C for use in hepatoprotective activity. Hautriwaic acid was isolated and characterized as described earlier (Salinas-Sánchez et al. 2012).

Animals

Male wistar rats weighing 180–200 g were housed in individual cages kept at 22–26 °C under 12-h light/dark cycles, with free access to standard laboratory chow and tap water *ad libitum*. All animals received humane care and all protocols involving the animals were in compliance with the guidelines approved by the institutional ethics committee of ICCBS, Karachi University.

CCl_4-induced liver injury

The animals were randomly divided into eight groups of six rats each as described below: Group 1 (normal control) were injected with vehicle only (1 ml/kg body weight olive oil); Group 2 (hepatitis model) were injected with intraperitoneal injection of CCl_4 (1 ml/kg) with 1:1 olive oil; Group 3 (positive control) were injected with intraperitoneal injection of CCl_4 (1 ml/kg) with 1:1 olive oil and also received silymarin 200 mg/kg (oral), per day 3 days before treatment and 2 days after treatment; Groups 4–8 were injected with intraperitoneal injection of CCl_4 (1 ml/kg) with 1:1 olive oil but also received methanolic extract, aqueous, ethyl acetate, butanol, *n*-hexane fractions and hautriwaic acid respectively, at a dose of 100 mg/kg body weight per day for 3 days before treatment and 2 days after treatment.

Blood biochemistry

Wistar rats were sacrificed 48 hr after CCl_4 administration under sodium pentothal anesthesia. Blood was collected by cardiac puncture. Serums were obtained for determination of liver damage by measuring the serum level of alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) using a dry chemistry analyzer (Roche Diagnostics, (Mannheim, Germany).

Histopathological examination of the liver

Liver tissues were rapidly removed and fixed in 10% neutral buffered formalin, dehydrated through a graded series of alcohol, embedded in paraffin, and cut into 6 μm thick sections. The liver tissues were stained with Hematoxylin–Eosin (H&E). The tissues were then examined under bright field microscope at different magnification using a Nikon *90i* microscope. Histopathological analysis of the liver under different conditions was carried out as follows. First the necrotic area was measured by in 30 different sections of the liver using the NIS-elements software from Nikon, Japan. Then the damaged area was expressed as percentage of the whole area of the section.

Immunohistochemistry

For immunohistochemistry, the liver sections were deparaffinized in xylene, rehydrated in graded alcohol, washed in water for 10–15 min. Each section was incubated with a blocking solution in phosphate buffered solution (PBS, Roti-immunoblock, Carl Roth, Karlsruhe, Germany) for 30 min at room temperature. Then, the sections were incubated with monoclonal CD68 (clone ED1, abcam) primary antibody (1:100) for 1 hour at 37 °C. After thoroughly washing with PBS, the sections were incubated with Texas Red-conjugated goat anti-mouse IgG (1:100) for 45 min. After washing extensively with PBS the nuclei were stained with DAPI (1:10,000 dilution of a 1 mg/ml stock solution) for 1 min, washed with PBS and mounted in mowiol 4-88 mounting media. Fluorescent images were acquired and analyzed using a Nikon *90i* multichannel fluorescence microscope and a Nikon DXM 1200 C camera with NIS-Elements image analysis software AR 3.0 (Nikon, Japan). Image processing was performed with Adobe Photoshop software. CD68+ macrophage cells were counted from number of the DAPI stained nucleus that showed the CD68 antibody staining around it using the NIS-elements software. The CD68+ macrophages were counted around the central vein region in a round area of 104,025 mm^2.

HPLC analysis

HPLC analysis of MeOH extract of *Dodonaea viscosa* and its various fractions were performed on Agilent 1200 Series, Rapid Resolution LC (RRLC) system, comprising Agilent binary pump SL with degasser, high performance autosampler SL with thermostat, thermostatted column compartment (TCC) and diode-array detector SL (DAD SL). Data acquisition and integration was controlled by Agilent Technologies ChemStation software. An Agilent Zorbax Eclipse XDB-C8 column (3.0 mm × 30 mm I.D., 1.8 μm) was used. The HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (USA). Water was purified using a Millipore® Milli-Q Plus system (Bedford, USA). The mobile phase was a binary gradient system composed of eluent A (95% water containing 0.01% HCCOH + 5% acetonitrile) and eluent B (0.01% formic acid in ACN) properly filtered and degassed for 15 min in ultra-sonic bath before use. The gradient program was: 10–60% B from 0 to 35 min, 60–35%