Hepatoprotective Effects of *Avicennia marina* (Forssk.) Vierh.

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Abstract

The xenobiotics-induced liver injury is a major clinical complication. Hence, finding hepatoprotective agents could have clinical value. Herbal medicines are a major source of biologically active chemicals which could be applied as hepatoprotective agents. The current study was designed to assess the hepatoprotective properties of *Avicennia marina* (AM) extract and its different fractions. *In vivo*, the hepatoprotective effect of AM total extract against CCl₄-induced acute liver injury was evaluated in rats, and a series of histopathological, biochemical, and oxidative stress parameters were monitored. *In vitro*, the protective effect of AM extract fractions (Petroleum ether, Chloroform, Ethyl acetate, and Ethanol) was evaluated on human liver hepatoma cells (HepG2). Severe elevation in serum level of liver injury biomarkers, along with liver tissue histopathological changes, lipid peroxidation, and liver tissue glutathione depletion were detected in CCl₄-treated rats. On the other hand, CCl₄-induced toxicity was evident *in vitro* by significant cell death. It was found that AM extract provided significant protection against CCl₄ toxicity *in vivo* by decreasing serum biomarkers of liver injury and tissue markers of oxidative stress. *In vitro*, the protective effect of AM extract fractions (Chloroform, Ethyl acetate, and Ethanol) was evident as these fractions significantly decreased CCl₄ cytotoxicity. As AM extract exhibited significant suppression of oxidative stress markers, its antioxidant effect could play a significant role in its hepatoprotective properties.

Keywords: Hepatoprotection, Natural product, Oxidative Stress.

1. Introduction

The liver is prone to xenobiotics-induced injury due to its central role in the metabolism of foreign compounds (1,2). Liver injury is a significant clinical problem induced by different xenobiotics including drugs and environmental pollutants (2-7). The xenobiotics-induced liver injury could lead to patient death if not appropriately managed. Hence, finding new pharmacological interventions to protect liver has clinical value. Many hepatoprotective chemicals have been studied to find a way to protect the liver from toxic insults (6, 8-15). Recently, interest in the discovery of natural hepatoprotective agents has increased. Principal candidates in this discovery process are medicinal plants (16-18). Due to the presence of a wide range of biologically active chemicals, different herbal medicine could be applied as the
source of hepatoprotective molecules.

Carbon tetrachloride (CCl\textsubscript{4})-induced liver injury is extensively applied as an animal model of hepatic failure and liver fibrosis (19). Severe reactive oxygen species (ROS) formation and its deleterious adverse events which finally lead to hepatic failure, are attributed to CCl\textsubscript{4}-induced liver failure animal model (19). The hepatoprotective properties of many herbal remedies have been evaluated in CCl\textsubscript{4}-induced hepatotoxicity model to find hepatoprotective molecules with potential therapeutic capabilities (20-23).

Several pharmacological effects have been attributed to *Avicennia marina* (Forssk.) Vierh. (*Avicenniaceae*; AM). It has been found that AM extract possesses antimicrobial, anti-inflammatory, anti-diabetic, antiviral, and antitumor activity (24-29). It has been found that the extract of *Avicenniaceae* species also showed protective effects in different experimental models (30-32). Some investigations also mentioned the antioxidant effects of *Avicenniaceae* species (33-35). Hence, AM extract might provide hepatoprotection against CCl\textsubscript{4}-induced hepatotoxicity and oxidative stress.

The current investigation was designed to evaluate the protective effects of AM leave extract and respective fractions against CCl\textsubscript{4}-induced toxicity in vitro and in vivo.

### 2. Materials and methods

#### 2.1. Plant Material and Chemicals

Fresh leaves of AM were collected during March 2016 from the Persian Gulf, Nayband, Bushehr, Iran. Samples were authenticated by a botanist (Botany Department, Shiraz University, Shiraz, Iran). The plant leaves were dried at the room temperature. HepG2 cells were obtained from Pasteur Institute (Tehran, Iran). RPMI-1640 and FBS were from GibCo (United States). Trypan blue, methyl thiazolyl diphenyl-tetrazolium bromide (MTT), Dithio-bis-nitrobenzoic acid (DTNB), CCl\textsubscript{4} and Na\textsubscript{2}HPO\textsubscript{4} were purchased from Merck (Darmstadt, Germany). Thiobarbituric acid (TBA) and Trichloroacetic acid (TCA) were obtained from Sigma Chemical Company (St. Louis, USA).

#### 2.2. Extract preparation

Approximately, 500 g of fine powder leave samples were extracted with 1 L 80\% V/V ethanol/water via maceration method for 24 hrs. Subsequently, the extract was concentrated by a rotary evaporator and gently dried via speed vacuum. The total solid yield of hydroalcoholic extract was 56 g/kg dry crude sample. The solid extract was subsequently dissolved in a mixture of water and ethanol and traditionally fractionated to the ether, chloroform, ethyl acetate and ethanol fractions, using a separation funnel. Fractions were orderly concentrated by rotary evaporator and subsequently dried in a speed vacuum system.

#### 2.3. Phenolic determination of total extract

Total polyphenol content was determined by a modification in the folin-ciocalteu method using ascorbic acid as standard (36). For preparation of a calibration curve, 0.5 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml ethanol-gallic acid solutions were mixed with 2.5 ml folin-ciocalteu reagent (diluted ten-fold) and 2 ml (75 g/l) sodium carbonate. The contents were mixed and allowed to stand for 30 min. The absorption at $\lambda$=765 nm was measured in a UV-Vis spectrophotometer (T80 plus, PG Instrument, UK). Half of one ml total extract was mixed with the same reagents as described above. One hour later, the absorption was measured for the determination of plant phenolics. All assessments were performed in triplicate, and the total phenolic content in plant total extract was calculated in ascorbic acid equivalents.

#### 2.4. Antioxidant assays of total extract

The antioxidant activities of total extract were measured in term of radical scavenging power by using stable free radical; 2, 2’-Diphenyl- 1-picrylhydrazyl (DPPH)(37). A 20 $\mu$L of the sample at various concentrations (50-800 $\mu$g/ml) were added to 200 $\mu$L of a methanol solution of DPPH (100 mM) in a microplate (7). Negative controls were prepared with 20 $\mu$L methanol and 200 $\mu$L DPPH in triplicate. Each well contained 200 $\mu$L solution of DPPH (100 mM) and 20 $\mu$L of extract. The microplates were incubated at 25 °C for 30 min, and the absorbance was measured at 492 nm using a microplate reader (Stat...
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2.5. Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was carried out according to the method described by Benzie using FeSO₄.7H₂O as the standard (38). Reagent A: 300 mM acetate buffer (pH=3.6), reagent B: 0 mM TPTZ in 40 mM HCl and reagent C: 20 mM FeCl₃.6H₂O was prepared. The FRAP reagent was prepared by mixing reagents A:B:C in 10:1:1 just before use. About 30 µL of FRAP reagent was introduced into 10 µL of samples and standard. The mixture was incubated at 37 °C for 10 minutes. The absorbance reading was set at 593 nm within 1 min interval for 6 min (38).

2.6. In vitro hepatoprotective assay

HepG2 were cultured and maintained in an RPMI-1640 medium (pH=7.3, containing 0.37% NaHCO₃ supplemented with 10% fetal bovine serum albumin, 100 IU/ml penicillin, and 100 IU/ml streptomycin) and a humidified 5% CO₂-95% O₂ mixture at 37 °C (39). Cells were seeded in 96-well microplates (30000 cells/well/90 µl) and routinely cultured in a humidified incubator for 24 hrs. The cells were then treated with concentrations 10 and 50 mg/ml of the extracts (Ether, Chloroform, Ethyl acetate, and Ethanol 10 µl/well.) 1 hr. before CCl₄ 100 mM (CCl₄/ethanol; 1/10).

After 24 hrs. of incubation, 10 µl of the MTT solution was added to every well. Cells were re-incubated for an additional 4 hrs. The cell culture media and MTT solution were removed, and the cells remained in the bottom of the wells. Then, 100 µl of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of the purple dye was measured at a λ=570 nm.

2.7. In vivo hepatoprotective activity studies

2.7.1. Animals

Male Sprague-Dawley rats (200-250 g) were purchased from the Laboratory Animals Research Center of Shiraz University of Medical Sciences. Animals were maintained under controlled conditions of 12 h light/12 h dark, 50±5% humidity, and ambient temperature of 23±2 °C. Rats had free access to a standard laboratory chow diet (Behparvar®, Tehran, Iran) and tap water. All the experiments were performed in conformity with the guidelines for care and use of experimental animals approved by a local ethics committee in Shiraz University of Medical Sciences, Shiraz, Iran.

Rats were randomly divided into four groups (n=6 in each). Group I received olive oil (1 ml/kg, i.p); group II received CCl₄/olive oil (1:1, 1 ml/kg, i.p.) as treated control; groups III and IV received the extract with doses of 20 and 40 mg/kg respectively, by i.p. injection for 3 days and CCl₄/olive oil (1:1, 1 ml/kg, i.p.) on the third day. Twenty four hours after CCl₄ injection animals’ blood and liver samples were collected.

2.7.2. Serum biochemical analysis and liver histopathology

Blood was collected from the abdominal inferior vena cava under thiopental anesthesia (70 mg/kg, i.p) and the liver was removed. The blood samples were allowed to clot at 25 °C, and serum was prepared by centrifugation (3,000 g, for 20 minutes, 4 °C). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) activities were measured with commercial kits (Pars Azmun®, Tehran, Iran) (17, 40, 41). For histopathological evaluation, samples of liver were fixed in buffered formalin solution (10% formaldehyde, 0.4% sodium phosphate monobasic, NaH₂PO₄, and 0.64% sodium phosphate dibasic, Na₃HPO₄, in double distilled water) (42). Paraffin-embedded sections of liver were prepared and stained with hematoxylin and eosin (H&E) before light microscope viewing (17, 43). Histological damages were scored as follows: 0: absent; +: mild; ++: moderate; and +++: severe (44).
2.7.3. Lipid peroxidation in liver tissue

Thiobarbituric acid reactive substances (TBARS) assay was employed to measure the amount of lipid peroxidation in the liver tissue. Briefly, the reaction mixture consisted of 0.5 mL of 10% liver tissue homogenate, 1 mL of 1% thiobarbituric acid, and 3 mL of 1% phosphoric acid. The mixture was thoroughly mixed and then heated in boiling water (100 °C) for 45 minutes (40, 41, 45). Samples were centrifuged (3,000 g for 5 minutes) and the absorbance of developed pink color was read at λ=532 nm (Ultrospec 2000® UV spectrophotometer) (45).

2.7.4. Hepatic glutathione content

Liver glutathione contents were measured by determining non-protein sulphydryls with the Ellman reagent (46). Liver tissue samples (200 mg) were homogenized in 8 mL of EDTA (20 mM). Then, 5 mL of the tissue homogenate was mixed with 4 mL of double distilled water and 1 mL of trichloroacetic acid (TCA) (50 % w/v) (47). The mixture was vortexed and centrifuged (20 minutes, 700 g, 4 °C). Then, 2 mL of the samples supernatant was mixed with 0.1 mL of the Ellman reagent (DTNB, 10 mM in methanol), and the absorbance of developed yellow color was measured at 412 nm with an Ultrospec 2000® UV spectrophotometer (41, 48, 49).

2.8. Statistical analysis

Results are shown as Mean±SEM for at least six animals in each group. Comparisons between multiple groups were made by a one-way analysis of variance (ANOVA) followed by Turkey’s post hoc test. Differences between groups were considered significant when P<0.05.

3. Results

The results of DPPH radical-scavenging activity, total phenolic content, and FRAP assay of total extract of AM are presented in Table 1. The Phenolic content (mg/g dry weight) of methanolic extract was measured as ascorbic acid equivalent. The level of phenolic content was 0.9±0.06 mg/g (Table 1). The percent deterrence of DPPH radical by AM leaves extract (10.7±3.3) was compared to a known antioxidant, quercetin (Table 1). AM extract demonstrated significant antioxidant capacities with FRAP test (Table 1). This extract showed 42±9 mM Fe$^{2+}$/mg sample values somehow similar to diminishing power of ascorbic acid (60.75 mM Fe$^{2+}$/mg sample) (Table1).

CCl$_4$ administration to rats caused hepatoxicity as judged by elevated serum transaminases level (Figure 1), serum lactate dehydrogenase (LDH) activity (Figure 1). The serum biochemical changes after CCl$_4$ administration was accompanied with significant alterations in liver tissue histopathology (Table 2). Severe liver fatty changes, centrilobular necrosis, and inflammatory cells infiltration were detected in CCl$_4$-treated animals (Table 2). It was found that total extract of AM (20 and 40 mg/kg, i.p, three consecutive days before CCl$_4$) effectively mitigated CCl$_4$-induced hepatotoxicity as revealed by lower serum transaminase activity and LDH level (Figure 1). Administration of the AM extract (20 and 40 mg/kg, i.p, three consecutive days before CCl$_4$) also attenuated liver histopathological changes in CCl$_4$-treated rats (Table 2).

CCl$_4$ caused a significant amount of thiobarbituric reactive substances (TBARS) formation in rat liver tissue (Figure 2). On the other hand, it was found that AM extract significantly decreased CCl$_4$-induced lipid peroxidation in rat liver (Figure 2). Significant depletion of liver glutathione reservoirs was also detected in CCl$_4$-intoxicat-ed rats (Figure 2). It was found that administration of a total extract of AM (20 and 40 mg/kg, i.p, three consecutive days before CCl$_4$) preserved liver glutathione stores at a higher level (Figure 2).

| Table 1. Total phenolic content and antioxidant activity as IC$_{50}$ (μg/mL) and Fe (μg/mL) DPPH for FRAP assays of Avicennia marina total extract, respectively (Mean±SD). |
|---------------------------------|---------------------------------|---------------------------------|
| Total phenolic contents (mg ascorbic acid/g dry weight) | DPPH IC$_{50}$ (mg/g) | FRAP |
| 0.9±0.06 | 10.7±3.3 | 42±9 |

In another part of this research on HepG2 cells, different AM extract fractions (Petroleum ether, Chloroform, Ethyl acetate, and Ethanol) were tested on HepG2 cells to assess their cytoprotective effects. 

CCl₄ administration caused more than 50% loss of cell viability in the concentration of 100 mM in 24 hrs. of incubation. When cells were pre-incubated with AM extract fractions (Chloroform, Ethyl acetate, and Ethanol, 10 and 50 mg/ml), CCl₄-induced cytotoxicity was significantly attenuated (Figure 3). Petroleum ether fraction of AM extract did not have protective effects against CCl₄-induced cell death (Figure 3). On the other

<table>
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<th>Necrosis</th>
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<th>Hepatocytes Deformation</th>
<th>Lymphocyte Infiltration</th>
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0: absent +: mild ++: moderate +++: severe.

Rats received the total extract of Avicennia marina (i.p. for three consecutive days; AM) before CCl₄.

Table 2. Effect of Avicennia marina extract on histopathological damages induced by CCl₄ injection in rats.

**Figure 1.** Effect of different doses of the Avicennia marina extract on biomarkers of liver injury in CCl₄-intoxicated rats. AM: Total Extract of Avicennia marina. Data are given as Mean±SD (n=6).

*** Indicates significantly different from control group (P<0.05).

* Indicates significantly different from CCl₄-treated group (P<0.001).
hand, the AM extract petroleum ether fraction itself caused cytotoxicity towards HepG2 cells (10 and 50 mg/ml) (Figure 3). Other fractions of AM extract were not cytotoxic when administered alone (10 and 50 mg/ml) (Figure 3).

4. Discussion

CCl$_4$-induced liver injury is widely used as an experimental model to study the effects of hepatoprotective agents (50-52). CCl$_4$ is metabolized by liver cytochrome P4502E1 (CYP2E1) system to produce a highly reactive metabolite

![Graphs showing cell viability and liver markers](image-url)

Figure 2. Effect of *Avicennia marina* extract on markers of oxidative stress in CCl$_4$-treated rats. Data are given as Mean±SD (n=6). AM.: Total Extract of *Avicennia marina*.

*** Indicates significantly different from control group (P<0.05).

a Indicates significantly different from the CCl$_4$-treated group (P<0.001).

Figure 3. The effect of different extracts of *Avicennia marina* on CCl$_4$-induced toxicity in HepG2 cells. P: Petroleum ether, C: Chloroform, E: Ethyl acetate, and ET: Ethanol fractions of AM extract.

Data are given as Mean±SD (n=4).

*** Significant difference as compared with control cells (P<0.05).

a Significant difference as compared with CCl$_4$-treated group (P<0.05).

ns: not significant as compared with CCl$_4$-treated group
which starts a chain of free radical-mediated reactions resulting in peroxidation of biomembrane lipids and damage to other components such as intracellular proteins (53). Hence, the dominant mechanism involved in CCl₄ hepatotoxicity is believed to be associated with oxidative stress and its consequences including GSH depletion and lipid peroxidation (54). On the other hand, herbal medicines have been widely studied for their hepatoprotective activity (55). The current study was designed to assess the antioxidant and hepatoprotective effect of a total extract of AM as well as its fractions against CCl₄-induced toxicity.

In this study, CCl₄ caused a significant elevation in serum biomarkers of liver injury and histopathological changes in rat liver. Moreover, CCl₄ also decrease the hepatic glutathione reservoirs and increased lipid peroxidation in the liver tissue. These changes specify the occurrence of oxidative stress following CCl₄ administration to rats. On the other hand, the phytochemical screening of AM revealed that the extract contains alkaloids, flavonoids, triterpenoids, glycosides, and steroids (24, 26, 34, 56, 57). Several studies mentioned the antioxidant potential of Avicennaceae species in different experimental models (33-35). As oxidative stress and its deleterious consequences play a significant role in CCl₄-induced liver injury (58), a part of the hepatoprotective mechanism of AM extract might be related to its antioxidant properties (Figure 4).

Different chemicals are characterized in AM (57). As mentioned, flavonoids are essential constituents of AM extract (57). There is a sizeable circumstantial evidence implicating the hepatoprotective properties of flavonoids in different experimental models (59, 60). Polyphenol compounds such as quercetin, gallic acid, and curcumin efficiently prevented CCl₄ induced liver injury in previous investigations (61-64). On the other hand, chemicals such as sterols present in AM extract might act as growth factors which enhance liver tissue regeneration (56). Hence, these chemicals could be responsible for hepatoprotective effects of AM extract (Figure 4).

Indeed the polarity of AM extract constituents is different. Hence, these chemicals might distribute in different fractions of AM extract. Therefore, the different cytoprotective profile of AM extract fractions was observed in this study. The hepatoprotective fractions (Polar fractions; Figure 4) of AM extract in the current study, preserved cell viability against CCl₄-induced toxicity. Hence, we might be able to propose that more polar chemicals of AM extract are responsible for its hepatoprotective effects (Figure 4).

Several studies proposed the role of natural products in inhibiting CYP2E1 and blocking CCl₄ bioactivation (65). Although not investigated in the current study, such mechanism might also be involved in the hepatoprotective properties of the AM extract and its constituents.

Figure 4. Different chemicals such as antioxidant flavonoids present in Avicennia marina extract might counteract CCl₄-induced oxidative stress and its deleterious consequences.
Collectively, it is proposed that the hepatoprotective effects of AM extract might be attributed to the presence of protective compounds such as flavonoids and sterols (Figure 4). These chemicals provide antioxidant and tissue regenerating properties. On the other hand, these compounds have different physicochemical properties and might distribute in different extract fractions due to their polarity. As polar fractions of AM extract alleviated CCl₄-induced toxicity, we might be able to propose that the hepatoprotective chemicals of AM extract are dissolved in these fractions. The exact chemical(s) responsible for the hepatoprotective properties of AM and the mechanism(s) of hepatoprotection deserves more investigations to be cleared.

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**Conflict of Interest**

None declared.

5. References

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