Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading

Ameliorative effects of *Spinacia oleracea* L. seeds on carbon tetrachloride (CCl_4) - induced hepatotoxicity: *In vitro* and in *vivo* studies

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ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 20 February 2012
Accepted 28 March 2012
Available online 28 April 2012

Keywords:
Spinacia oleracea
Chenopodiaceae
CCl_4
Hepatoprotective
HPTLC

ABSTRACT

Objective: To investigate the *in vitro* and *in vivo* protective effects of *Spinacia oleracea* L. (Chenopodiaceae) seeds on carbon tetrachloride (CCl_4)-induced hepatic toxicity.**Methods:** In the in vitro studies, different extracts (*i.e.* petroleum ether, ethanol and aqueous) and fractions derived from ethanol extract (*i.e.* chloroform, ethyl acetate and *n*-butanol) of *Spinacia oleracea* seeds were screened at a concentration of 100 μg/mL against carbon tetrachloride (CCl_4)-toxicity in rat hepatocyte culture. *In vivo* hepatoprotective activity was assessed in rats intoxicated with CCl_4. Level of biochemical markers along with histological changes were monitored to evaluate the extent of hepatoprotection. Silymarin was taken as reference drug.**Results:** In the *in vitro* screening, *n*-butanol fraction of *Spinacia oleracea* seeds was found to be more potent than other screened plant samples, hence selected further for phytochemical and *in vivo* studies. In the *in vivo* studies, the *n*-butanol fraction of *Spinacia oleracea* showed significant protection against CCl_4-induced hepatotoxicity as evident by restoration of biochemical and histological changes caused by CCl_4 intoxication. HPTLC fingerprinting of the *n*-butanol fraction of *Spinacia oleracea* confirmed the presence of 20-hydroxyecdysone (20-HE) besides other phytochemicals, which partially may explain the effects.**Conclusions:** The results of present study indicates the significant *in vitro* and *in vivo* hepatoprotective activity of *n*-butanol fraction of *Spinacia oleracea* on CCl_4-induced hepatotoxicity, and hence suggests its use as a potential therapeutic agent in liver diseases.

1. Introduction

Liver is an organ of paramount importance and its disorders are numerous with no effective remedies. Therefore, search for new medicines is still ongoing. In recent years, much interest has been developed in therapeutic evaluation of traditionally used herbals with that of the modern concept of evidence based evaluation[1].

Spinacia oleracea Linn. (Family-Chenopodiaceae), commonly known as "Paalak" in Hindi, is an erect herb with about 30-60 cm height. It is native to South-West Asia and cultivated throughout world as vegetables. Several parts of this plant are used in traditional Indian medicine for numerous therapeutic effects. The leaves are cooling and useful in febrile conditions, urinary calculi and lung inflammation. The seeds are cooling, laxative, and useful in difficult breathing, liver inflammation and jaundice[2,3]. Presence of 20-hydroxyecdysone, polypodine B and protein has been shown in *Spinacia oleracea* seeds[4, 5].

Literature review reports that very little work has been done on *Spinacia oleracea* seeds. Moreover, no scientific report is available regarding its hepatoprotective action, to the best of our knowledge. Therefore to validate the traditional claims, the present study was aimed to examine the potential hepatoprotective effects of *Spinacia oleracea* seeds against hepatotoxicity induced carbon tetrachloride (CCl_4), a most widely used hepatotoxin.

2. Material and methods

2.1. Chemicals and drugs

CCl_4, ethylene glycol tetraacetic acid (EGTA), hydroxyethyl piperazine ethane sulfonic acid, William's E medium, collagenase, thiobarbituric acid and 5, 5'-dithiobis-2-nitrobenzoic acid were procured from Sigma Chemical Co. (St. Louis, MO, USA). 20-hydroxyecdysone was purchased

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Foundation project: It is supported by All India Council for Technical Education (AICTE), New Delhi (No. 1-10/RID/NDF-15/2009-10 Dated- 26/02/10)

from Altavista Phytochemicals Pvt. Ltd, Hyderabad. All other chemicals and reagents used were of analytical grade and purchased from commercial sources.

2.2. Experimental animals

Wistar albino rats (200-250 g) of either sex were used for the studies. The animals were maintained under standard laboratory conditions of temperature [(25±2) ℃] and relative humidity [(55±5) %] with 12 h light-dark cycle. The animal studies were approved by the Institutional Animal Ethics Committee (379/01/ab/CPCSEA).

2.3. Plant material

The seeds of *Spinacia oleracea* were procured from the seed market, Sagar and authenticated by Dr. P. Tiwari, Botanist, Department of Botany, Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India. The voucher specimen has been preserved (No. Bot/Her/889) for future reference.

2.4. Extraction and fractionation

The coarsely powdered seeds (800 g) were successively extracted with petroleum ether (60-80℃) and 95% ethanol using Soxhlet extractor. The marc left after the ethanol extraction was macerated with distilled water for 24 h. The solvents were removed by distillation under reduced pressure below 45℃ to afford petroleum ether extract of *Spinacia oleracea* seeds [yield 4.05% (w/w)], ethanol extract [yield 9.07% (w/w)] and aqueous extract, [yield 10.5% (w/w)], respectively.

The ethanol extract (30 g) was suspended in water (300 mL) and fractionated successively with chloroform (4 × 300 mL), ethyl acetate (4 × 300 mL) and *n*-butanol (4 × 300 mL) to afford chloroform fraction (3.7 g), ethyl acetate fraction (4.8 g) and *n*-butanol fraction (6.9 g), respectively.

2.5. Preliminary phytochemical screening

Preliminary phytochemical analysis was performed to identify the nature of phytoconstituents in different extracts and fractions[6].

2.6. In vitro hepatoprotective evaluation

2.6.1. Isolation and culture of rat hepatocytes

The rat hepatocytes were isolated by the two step collagenase perfusion technique[7]. Briefly, the rats were anaesthetized by pentobarbital sodium (50 mg/kg, *i.p.*). After opening the abdomen, livers were perfused via the portal vein with Ca^{2+}- free phosphate buffer (pH 7.4), containing 135 mM NaCl, 15 mM $NaHCO_3$, 5 mM glucose, 5.9 mM KCl 0.74 mM KH_2PO_4 and 0.1 mM EGTA, at a flow rate of 25 mL/min to remove blood. After 10 min, the liver was reperfused for another 10 min with the same phosphate buffer containing 50 mg collagenase, 3 mM $CaCl_2$ and 22 mg pyruvate, at a flow rate of 35 mL/min. To produce a single cell suspension of hepatocytes, the collagenase-digested liver was removed, passed through a nylon mesh (mesh size, 0.3 nm), washed and centrifuged at 500 rpm for 5 min at 4℃. Hepatocytes were then resuspended and washed twice with washing medium. The cell viability was more than 85% as determined by trypan blue exclusion.

The freshly isolated hepatocytes (viability > 85%) were seeded at a density of 2 to 3×10^3 cells/60 nm tissue cultures plates in Williams medium E consisting 10% fetal calf serum, and 0.1 μM insulin at 37℃ in humidified atmosphere of 5% CO_2 in a CO_2 incubator. After 3 h of plating, the fresh Williams medium E containing 3% FCS, 0.1 μM dexamethasone, 5nM epidermal growth factor and 1 nM glucagons was added.

2.6.2. Toxicity induction and drug treatment

The hepatocytes monolayer was exposed to CCl_4 (2.5 mM) [7] with or without plant samples (100 μg/mL) or silymarin (10 μM) and incubated for another 24 h at 37℃ in a humidified atmosphere of 5% CO_2 in a CO_2 incubator. After 24 h incubation, the leakage of alanine transaminase (ALT)[8] and lactate dehydrogenase (LDH)[7] in culture medium was determined.

2.7. Chromatographic studies

Precoated and preactivated TLC plates (E. Merck No. 5548) of silica gel 60 F_{254} with the support of aluminium sheets 0.1 mm thick and 20 cm × 10 cm were used. The SOBF (10 mg) was weighed accurately and dissolved in 10 mL of methanol. The sample was applied in the form of a band using CAMAG LINOMAT V, an automatic sample applicator, maintaining a band width 6 mm, space 10 mm, 250 nL/s. The volume of sample applied was 10 μL. The mobile phase optimized and used was ethyl acetate: ethanol: water (16:2:1). 20-hydroxyecdysone (10 mg) was dissolved in methanol (10 mL). Vanilin-sulphuric reagent was used as detecting reagent.

2.8. Acute oral toxicity studies

The acute oral toxicity studies were performed on *n*-butanol fraction of *Spinacia oleracea* (SOBF) following OECD guideline[9]. On the basis of studies, the oral doses of 50, 100 and 200 mg/kg, b.w. were selected for the *in vivo* experiments.

2.9. In vivo hepatoprotective evaluation

2.9.1. Experimental protocol

The experiment was conducted according to the method described previously[10]. Rats were randomly divided into seven groups, each consisting of six rats. Group I (normal control) rats received distilled water (1 mL/kg, *p.o.*) daily for 5 days and olive oil (1 mL/kg, *s.c.*) on days 2 and 3. Group II (CCl_4 control) rats received distilled water (1 mL/kg, *p.o.*) daily for 5 days and CCl_4: olive oil (1:1, 2 mL/kg, *s.c.*) on days 2 and 3. Group III (SOBF control) rats were treated with the SOBF (200 mg/kg, *p.o.*) daily for 5 days. Group IV rats were treated with silymarin (50 mg/kg, *p.o.*) daily for 5 days and received CCl_4: olive oil (1:1, 2 mL/kg, *s.c.*) on days 2 and 3,