Original article

A composition of medicinal plants with an enhanced ability to suppress microsomal lipid peroxidation and a protective activity against carbon tetrachloride-induced hepatotoxicity

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\section*{ABSTRACT}

Treatment of liver injury induced by various toxicants represents a serious clinical challenge. Here, we utilized the ability of natural agents to inhibit microsomal lipid peroxidation (LPO) as the in-vitro screening paradigm for selecting efficacious tissue-protective combinations of cooperatively acting medicinal plants. Based on screening of 70 water-ethanol extracts obtained from different parts of 65 plants we prepared a highly active phyto-composition (PC-1) containing oregano (\textit{Origanum vulgare}), wild thyme (\textit{Thymus serpyllum}) and coltsfoot (\textit{Tussilago farfara}) serial parts, valerian (\textit{Valeriana officinalis}) leaves and little-leaf linden (\textit{Tilia cordata}) flowers. PC-1 extract exhibited the strongest anti-PLO and antimethylotic effects in vitro compared to those of the individual plants and other compositions tested. Using luciferase reporter assay and Western blotting in HepG2 human hepatocellular carcinoma cells, we found that PC-1 extract activated the Nrf2/antioxidant response element signaling pathway more effectively than the extracts of other phyto-compositions. Importantly, oral administration of PC-1 extract (100–200 mg/kg) markedly ameliorated liver injury in rats acutely or chronically intoxicated by carbon tetrachloride. This was evidenced by improved liver histology, blood chemistry parameters, and microsomal LPO status and superoxide dismutase activity. In addition, treatment with PC-1 extract salvaged the osmotic resistance of erythrocytes in carbon tetrachloride-intoxicated rats. Collectively, these data support the strategy of in-vitro plant selection for developing efficacious tissue-protective phyto-compositions.

1. Introduction

Different environmental toxic agents can target the liver and induce hepatic injury. Among these compounds are xenobiotics (e.g., carbon tetrachloride, thioacetamide), synthetic drugs (e.g., paracetamol, diclofenac), natural substances (e.g., plant and mushroom toxins) and alcohol. Carbon tetrachloride (\textit{CCl}_4), a highly reactive lipid-soluble toxicant, is widely used in animal studies to model acute and chronic hepatotoxicity in rodents [1,2]. The primary cellular metabolite of \textit{CCl}_4, trichloromethyl radical, and its oxidized derivative, trichloromethyl peroxy radical, can covalently bind to DNA, proteins and, particularly, to membrane phospholipids leading to lipid peroxidation (LPO), and thus to oxidative damage of the liver tissue [2]. LPO gives rise to multiple secondary metabolites, malondialdehyde (MDA) being one of the principal final products of polyunsaturated fatty acid peroxidation. MDA is a highly toxic metabolite which is capable of interacting with cellular macromolecules and may affect cell signaling pathways and metabolism [3].

It is well documented that antioxidant vitamins A, C and E (\textit{\alpha}-tocopherol), can ameliorate the toxic effects of \textit{CCl}_4 on the liver (e.g., [4,5]). Furthermore, numerous studies have shown that plant extracts and isolated antioxidant phytochemicals are capable of producing similar protective effects [6,7]. There is a vast body of evidence that the cytoprotective effects of different phytochemicals are mediated by the activation of the nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE) signaling pathway, a “master regulator” which induces the expression of various intracellular redox regulators in response to oxidative stress or plant antioxidants [8,9]. In particular, the Nrf2/ARE-driven cellular defense system includes antioxidant and detoxifying enzymes, such as superoxide dismutase (SOD), catalase,
NAD(P)H quinone oxidoreductase-1 (NQO1) and γ-glutamylcysteine synthetase (γ-GCS), the rate limiting enzyme of glutathione synthesis, as well as antioxidant proteins, e.g., thioredoxin and peroxiredoxin [9].

While the majority of experimental studies have described therapeuetic and preventive activities of single plant extracts or their individual components in different models of human pathologies, mounting evidence demonstrates superior beneficial actions of blended extracts or plant compositions (e.g., [10,11]). We and others have shown that both different plant extracts and purified bioactive phytochemicals can cooperate with one another [12-16] and with other natural agents, such as vitamin D derivatives [17,18], in producing enhanced antinocer and other beneficial effects in preclinical models. Particularly, we have recently demonstrated that different combinations of water-ethanol extracts from the leaves of sea buckthorn (Hippophae rhamnoïdes), dog rose (Rosa canina), garden sage (Salvia officinalis) and origano (Origanum vulgare), caused a marked cooperative cytostasis in acute myeloid leukemia cells at minimally effective doses of each extract [16]. In the present study we screened the extracts from the above and 61 other plants collected in the foothills of the Trans-Ili Alatau mountains (Kazakhstan) for their ability to inhibit LPO in rat liver microsomes. Based on this testing, we composed a blend of 5 plants which produced an enhanced LPO inhibition and anti-hemolytic and Nrf2/ARE-stimulating effects in vitro. Importantly, this composition had a marked hepatoprotective activity in both acute and chronic models of CCl4 intoxication in vivo. The above effects were comparable or even superior to those of the well-known tissue-protective agents α-tocopherol or silymarin, a standardized extract of milk thistle seeds [19].

2. Materials and methods

2.1. Plant materials

Samples of 65 plants listed in Suppl. Table S1 were collected in the foothills of the Trans-Ili Alatau Mountains (Almaty region, Kazakhstan) during June-August, 2016. The plants were identified and samples were deposited at the herbarium of the Institute of Botany and Phytointroduction (Almaty, Kazakhstan).

2.2. Preparation of plant extracts

Each g of crushed and powdered dried parts of the tested plants was extracted with 10 ml of 50% (v/v) aqueous ethanol at room temperature, for 20 h in the dark, as described previously [16]. The mixture was then centrifuged at 20,000 × g for 10 min and the supernatant was dried at 37 °C in a rotary evaporator. Stock solutions of the dried extracts (100 mg/ml) were freshly prepared in 50% ethanol before experiments. Phytoconstituents were prepared by mixing powdered parts of individual plants at different weight ratios (e.g., see Table 4) and were extracted as described above.

2.3. Cell culture

The HepG2 human hepatocellular carcinoma cell line (ATCC HB-8065) was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were grown in MEM-Eagle medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 12.5 μg/ml nystatin (Biological Industries, Beit-Haemek, Israel) at 37 °C in a humidified atmosphere of 5% CO2 in air.

2.4. Transient transfection and luciferase reporter gene assay

The 4 × ARE-Luc construct containing four tandem repeats of the ARE sequence from the glutathione S-transferase Ya subunit (5’-TGACAAGAAGCC-3’) was a gift from Dr. M. Hannink (University of Missouri, Columbia, MO). Renilla luciferase expression construct (pRL-null vector) was purchased from Promega (Madison, WI, USA) and served as an internal transfection standard. HepG2 cells were transfected using jetPEI reagent (Polyplus Transfection, Illkirch, France), as described previously [16]. Briefly, cells were seeded in 24-well plates (5 × 104 cells/well) in growth medium containing 3% FCS. One day later, cells were transfected with 0.2 μg of 4 × ARE-Luc plasmid and 0.05 μg of Renilla luciferase plasmid mixed with jetPEI reagent at a charge ratio of 1:2. Four hours later medium was replaced with one supplemented with 3% FCS containing test compounds and cells were incubated for an additional 20 h. Luciferase activity was measured in cell lysates using the Dual Luciferase Reporter Assay System (Promega). The data are presented as the normalized ratios of firefly luciferase to Renilla luciferase activity (relative luminescence units, RLU).

2.5. Preparation of whole cell lysates and western blotting

Western blot analysis of protein expression was performed, as described previously [14,16]. Briefly, HepG2 cells (~ 2 × 106) were lysed in buffer containing 1% (v/v) Triton X-100 at 4 °C, subjected to SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were exposed to the following primary antibodies overnight at 4 °C: NQO1 (C-19) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and γ-GCS/Glutamate-cysteine Ligase (Ab-1; RB-1697-P) from ThermoFisher Scientific (Waltham, MA, USA). Blots were washed and incubated with horse-radish peroxidase-conjugated anti-rabbit secondary antibodies (Promega, Madison, WI, USA). Membranes were then stripped and reprobed for β-tubulin (H-235) from Santa Cruz Biotechnology Inc., as the internal loading control. The protein bands were visualized using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA). The absorbance of each band was determined using the Image Quant LAS 4000 system (GE Healthcare, Little Chalfont, UK).

2.6. Laboratory animals

Male Wistar rats (190 ± 25 g) were housed in the animal facility of the Institute of Human and Animal Physiology (Almaty, Kazakhstan) under standard conditions of light and dark cycle, with free access to food and water. The blood and livers were obtained from both healthy and treated rats, as described below. The experimental protocols were approved by the Committee for the Ethical Care and Use of Animals in Experiments of the Institute of Human and Animal Physiology, Ministry of Education and Science of the Republic of Kazakhstan.

2.7. Preparation of rat liver microsomes

Rats were euthanized by cervical dislocation under isoflurane anesthesia. The livers were isolated, washed, and perfused with chilled saline. Tissue was minced and homogenized (1:10 w/v) in 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA on ice. The homogenate was centrifuged at 10,000 × g at 4 °C, for 20 min. The supernatant was further centrifuged at 100,000 × g, for 60 min, to obtain the microsomal fraction. Microsomes were suspended in a buffer containing 10 mM histidine (pH 7.2), 25% (v/v) glycerol, 0.1 mM EDTA and 0.2 mM CaCl2, and were kept at −20 °C. The protein content was measured by the Lowry assay using bovine serum albumin as a standard.

2.8. Isolation of rat plasma and erythrocytes

The blood was collected from rats by cardiac puncture under isoflurane anesthesia followed by humane euthanasia. The blood was centrifuged at 1000 × g, for 10 min, white blood cells were removed, and plasma was collected and stored frozen at −20 °C until use. Erythrocyte pellets were washed twice with a buffer containing 5 mM Na2HPO4 (pH 7.4) and 150 mM NaCl, and were used immediately for osmotic resistance tests (see 2.10).
2.9. Determination of microsomal lipid peroxidation

LPO was assessed by measuring malondialdehyde content in the form of thiobarbituric acid-reacting substances (TBARS) by the method of Ohkawa et al. [20], as described previously [16]. Briefly, liver microsomes were preincubated with vehicle or test agents in a buffer containing 50 mM K2HPO4 (pH 7.2) and 145 mM NaCl at 37 °C, for 10 min, under constant stirring. The basal and 0.02 mM Fe2+/0.5 mM ascorbate-incubated microsomal LPO was then determined in a reaction mixture containing 0.9 M sodium acetate buffer (pH 3.5), 0.4% SDS and 20 mM thiobarbituric acid following incubation at 95 °C for 60 min. After cooling to room temperature, the mixture was extracted by n-butanol:pyridine (15:1, v/v) and centrifuged at 3000 × g for 5 min. The organic layer was collected and its absorbance was measured at 532 nm using a PD-303UV spectrophotometer (Apel, Saitama, Japan). The MDA concentration was expressed as nmol of TBARS per mg protein.

2.10. Superoxide dismutase activity assay in liver microsomes

SOD activity of the microsomal fraction was determined using SOD Assay Kit-WST (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturer’s recommended protocol. SOD-mediated decrease in superoxide levels was measured at 450 nM as a decrease in the absorbance at 540 nm. In the animal experiments, 0.4% NaCl-followed by centrifugation. Hemoglobin absorbance was then measured in the supernatant at 540 nm. In the animal experiments, 0.4% NaCl-followed by centrifugation. Hemoglobin absorbance was then measured in the supernatant at 540 nm. The organic layer was collected and its absorbance was measured at 532 nm using a PD-303UV spectrophotometer (Apel, Saitama, Japan). The MDA concentration was expressed as nmol of TBARS per mg protein.

2.11. Determination of osmotic resistance of erythrocytes

Osmotic resistance of erythrocytes was measured as described previously [16,21]. Briefly, in the in vitro studies, isolated erythrocytes were preincubated with vehicle or test agents at 37 °C, for 10 min, and subjected to a hypotonic solution of NaCl (0.4%) at 37 °C, for 20 min, followed by centrifugation. Hemoglobin absorbance was then measured in the supernatant at 540 nm. In the animal experiments, 0.4% NaCl-induced hemolysis of isolated erythrocytes was measured directly, without preincubation. The extent of hemolysis was calculated as the percentage of total hemolysis caused by 0.1% Na2CO3.

2.12. CCl4-induced acute and chronic hepatotoxicity models in rats

To study the preventive effects of PC-1 against CCl4-induced acute hepatotoxicity, rats were divided into 8 groups (6 rats/group) and were treated as indicated in Table 1. The animals received oral gavage (100 μl) of 50% ethanol in saline (vehicle control; groups 1 and 4), PC-1 extract at 100 mg/kg (group 5), 200 mg/kg (groups 2 and 6) and 400 mg/kg (group 7) or 100 mg/kg α-tocopherol (groups 3 and 8) in ethanol/saline, daily for 14 days. These animals were simultaneously injected s.c. with either refined olive oil (groups 1–3) or 1 ml/kg of 50% CCl4 in olive oil (groups 4–6) every other day, for 14 days. At the end of experiments, all animals were anesthetized with isoflurane followed by blood collection using cardiac puncture and euthanasia by cervical dislocation. The livers were excised for the preparation of microsomes and tissue sections.

2.13. Liver histology

Frozen plasma samples were thawed and kept at room temperature for 30 min. Serum was then separated by centrifugation at 3000 rpm, for 15 min. Serum alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) activities as well as total and direct bilirubin levels were determined in a semi-automated BioChem SA Chemistry Analyzer (High Technology, Inc., Walpole, MA, USA) using the corresponding HTI kits.

2.14. Determination of biochemical parameters in rat serum

In vitro experiments were repeated at least three times. In vivo experiments were performed in groups of 6 rats. The data are reported as the means ± SD. The significance of the differences between the means of experimental groups was assessed by unpaired two-tailed Student’s t test. P < 0.05 was considered statistically significant. Statistical analysis was performed with the GraphPad Prism 6.0 Program (GraphPad Software, San Diego, CA).

3. Results

3.1. In vitro screening of plant extracts for the preparation of active phytocompositions

To identify plant preparations with potential hepatoprotective features we first screened 70 water-ethanol extracts from different dried parts of 65 plants collected in the Trans-Ili Alatau region of Kazakhstan. A two-step selection procedure was based on the ability of the extracts to inhibit Fe2+/ascorbate-induced LPO in rat liver microsomes. The initial results demonstrated that while most of these extracts applied at 200–2000 μg/ml were capable of inhibiting LPO to a varying extent, 23

Table 2

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Ethanol/ saline p.o.</th>
<th>PC-1 p.o. (mg/kg)</th>
<th>α-Tocopherol p.o. (mg/kg)</th>
<th>Olive oil s.c.</th>
<th>CCL4 s.c. (mg/kg)</th>
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<tr>
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</table>
extracts produced 80–100% maximal inhibition (Suppl. Table S1) at 200–500 μg/mL. Following a more detailed testing we identified 12 individual extracts with the lowest IC₅₀ values (listed in Table 3), which were selected for further examination.

As combinations of either isolated phytochemicals or whole plant extracts have proven more effective than their individual components in various disease models (e.g. [12,13]), we set out to determine the in-vitro protective activity of several compositions containing 4–5 different plants of the selection shown in Table 3. In these experiments, we compared the effects of the extracts of such compositions and their individual components on both microsomal LPO and osmotic resistance of erythrocytes (ORE). As a result of several pilot experiments performed to optimize the content of these compositions we established a highly effective preparation denominated Phytocomposition-1 (PC-1; Tables 3, Fig. 1) which contained aerial parts of oregano (Origanum vulgare), wild thyme (Thymus serpillum), coltsfoot (Tussilago farfara) and valerian (Valeriana officinalis) as well as flowers of small-leaved lime (Tilia cordata).

As shown in Fig. 1 and summarized in Table 3, the concentration-dependent inhibitory effects of PC-1 on LPO (Fig. 1A) and ORE (Fig. 1B) were substantially greater compared to its individual components. Interestingly, PC-1 efficiency in the above assays was mildly though significantly higher than that of α-tocopherol, the known antioxidant and intramembrane chain-breaking scavenger of lipid radicals, applied at the same concentrations (Fig. 1A,B). The other tested compositions (e.g., PC-2 and PC-3; Tables) were less potent as compared to PC-1 and to the majority of their respective components.

3.2. Effects of different phytocompositions on the Nrf2/ARE signaling pathway in HepG2 hepatocarcinoma cells

We next determined the ability of PC-1, PC-2 and PC-3 (5–25 μg/mL) to activate the Nrf2/ARE cytoprotective transcriptional system in HepG2 hepatocarcinoma cells using the luciferase reporter gene assay. A classical Nrf2/ARE inducer, tert-butylhydroquinone (tBHQ) [22], was used in this assay as the positive control. The results demonstrated that untreated control cells had a very low 4×ARE-Luc transcriptional activity which was markedly stimulated by increasing concentrations of all three extracts or by 15 μM tBHQ. When applied at equal concentrations, PC-1 extract was a significantly stronger inducer compared to PC-2 or PC-3 extracts (Fig. 2A). To further characterize the effects of the phytocompositions on the Nrf2/ARE pathway in HepG2 cells, we determined the expression of two proteins encoded by Nrf2 target genes: NQO1, a typical phase II detoxification enzyme, and the catalytic subunit of γGCS, the first rate-limiting enzyme of glutathione synthesis [9]. Similar to tBHQ, treatment with 25 μg/mL PC-1 or PC-2 extract resulted in a marked upregulation of both NQO1 and γGCS levels, PC-2 extract being slightly less effective than PC-1 (Fig. 2B). In contrast, PC-3...
CCl4 hepatotoxicity was evident by marked increases in ALT, or 200 mg/kg PC-1 followed by a subsequent injection of olive oil separate groups of animals were administered with vehicle, P < 0.01; ***, P < 0.001; ****, P < 0.0001 calculated from the data of 3 experiments performed in quadruplicate. *, P < 0.05; **, indicated concentrations, for 24 h, followed by luciferase activity assay. tBHQ (15 μg/mg). 3.3. Hepatoprotective effects of phytocomposition-1 in rat models of acute and chronic CCL4-induced hepatotoxicity

We first determined whether prolonged oral administration of PC-1 may protect the liver from the damaging effect of a subsequent acute exposure to high-dose CCL4. To this end, rats were gavaged with vehicle, 100 mg/kg α-tocopherol (the positive control) or increasing dosages of PC-1 (100–400 mg/kg) daily, for two weeks, followed by a 24-h treatment with a single s.c. injection of 2 ml/kg CCL4. For comparison, separate groups of animals were administered with vehicle, α-tocopherol or 200 mg/kg PC-1 followed by a subsequent injection of olive oil alone. CCL4 hepatotoxicity was evident by marked increases in ALT, AST, ALP, and total and direct bilirubin levels, as compared to untreated controls. Notably, following pretreatment with either α-tocopherol or PC-1 at all the doses tested these parameters were significantly less affected by the toxicant (Table 5). For instance, after administration of 200 mg/kg or 400 mg/kg PC-1 before CCL4 ALT levels remained comparable with those in the untreated control group.

At the dosage of 200 mg/kg the preventive effect of PC-1 was significantly stronger than at 100 mg/kg (p < 0.05), there was little difference between the effects of 200 mg/kg and 400 mg/kg PC-1 (Table 5).

As expected, the increases in the blood chemistry markers of liver injury in CCL4 only-treated rats were accompanied by a substantial (~3-fold) elevation of lipid peroxidation levels and a significant reduction in SOD activity in isolated liver microsomes, as compared to control animals (Fig. 3A, B). However, in rats pretreated with α-tocopherol or PC-1, the levels of lipid peroxidation and SOD activity were much lower than in CCL4-alone treated animals and were comparable with those observed in untreated controls (Fig. 3A,B).

In the model of chronic CCL4-inhibited hepatotoxicity, rats were orally treated with vehicle, 200 mg/kg PC-1 or 100 mg/kg silymarin (the positive control), daily for 14 days, with or without s.c. injections of CCL4 at 1 ml/kg, every other day (a lower dosage than in the model of acute toxicity). Similar to the results described above, treatment with CCL4 alone resulted in a substantial elevation of diagnostic serum markers of liver dysfunction (Table 6) and microsomal lipid peroxidation levels (Fig. 3C) while microsomal SOD activity was markedly inhibited (Fig. 3D). Importantly, a simultaneous treatment with PC-1 or silymarin protected the liver from CCL4 injury, as manifested by significantly less pronounced changes in the above biochemical parameters compared to CCL4-alone treated rats (Table 6 and Fig. 3C,D).

The protective effect of PC-1 was also evident upon histological examination of H&E-stained tissue sections from the livers of untreated rats and those treated with CCL4, alone or together with PC-1. Liver sections from untreated control rats showed normal tissue architecture, with distinct hepatocytes and sinusoidal spaces (Fig. 4A). CCL4-induced intoxication was accompanied by pronounced tissue damage with the signs of fibrotic changes (Fig. 4B), hepatocellular ballooning, necrotic cell death, lipoid accumulation and leukocyte infiltration (Fig. 4B, C). However, simultaneous treatment with PC-1 resulted in fewer necrotic zones, ballooning degeneration and lipid droplets, and leukocyte infiltrated areas (Fig. 4D).

Interestingly, in addition to its protective effects against CCL4 intoxication, PC-1 (200 mg/kg) treatment resulted in a moderate reduction in most of the tested blood biochemical parameters (Tables) and the improvement of the microsomal LPO status and SOD activity (Fig. 3) in control animals, as compared to the corresponding basal values. Furthermore, we found that this treatment moderately increased ORE in both control and CCL4-intoxicated rats in the models of acute (Fig. 5A) and chronic (Fig. 5B) hepatotoxicity. These data suggest that PC-1 may protect other tissues from CCL4-induced injury and may also beneficially influence normal untreated rats.

4. Discussion

In this study, we utilized the ability of natural agents to inhibit microsomal LPO as the in-vitro bioactivity-based screening paradigm for selecting medicinal plants capable of acting in combination to produce enhanced tissue-protective effects. The LPO inhibition test was utilized primarily because, being a highly hydrophobic compound, CCL4 accumulates in and cause severe degenerative changes to lipid-rich organs, e.g., liver, kidney or brain, via free radical-triggered self-propagating chain reactions of membrane phospholipid oxidation [2]. The major outcome of this work was that screening of 70 plant extracts led us to the preparation of PC-1, the plant composition which had the strongest LPO inhibitory and anti-hemolytic activities in vitro, compared to the individual plants and several other phytocompositions tested, as well as a marked hepatoprotective effect in vivo. Extracts from the PC-1 components O. vulgare [23] and T. serpyllum [24] have already been reported to inhibit LPO and/or to exhibit hepatoprotective activities. To our knowledge, such effects have not yet been reported for V. officinalis and T. farfara aerial parts and T. cordata flowers as well as for compositions containing any the above 5 plants, and thus were

![Fig. 2. Phytocomposition extracts transactivate the antioxidant response element and modulate NQO1 and γGCS protein levels in HepG2 cells. (A) Cells were transiently transfected with 4×ARE-Luc and Renilla constructs and incubated with the extracts at the indicated concentrations, for 24 h, followed by luciferase activity assay. tBHQ (15 μM) was used as the positive control. The relative 4×ARE-Luc activity (means ± SD) was calculated from the data of 3 experiments performed in quadruplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 vs. control (0 μg/ml). ♦, P < 0.05; ♦♦, P < 0.001 vs. respective concentrations of PC-1 extract. (B) Cells were treated with the extracts at the indicated concentrations (25 or 50 μg/ml) or tBHQ (15 μM), for 48 h, followed by Western blot analysis. β-Tubulin was used as a protein loading control. Representative blots of 3 similar experiments (left panel) and summarized absorbance values (means ± SD) for specific proteins normalized to those of β-tubulin (right panel) are shown. *, P < 0.05; **, P < 0.001 vs. vehicle control (n = 3).](image-url)
demonstrated here for the first time.

While various plant extracts and their isolated phytochemical constituents have been shown to suppress LPO and produce beneficial effects against CCl4-induced liver damage (e.g. [7,10,23]), accumulating evidence demonstrates that blended extracts from different plants [10,11] or extracts made from plant compositions [25,26] had more pronounced effects than their components in both in vitro and in vivo models of chemical liver injury. For instance, Yimam et al. [11] have shown that oral treatment with a blend of extracts from Myristica fragrans seeds, Astragalus membranaceus roots, and Poria cocos whole fruits (150–400 mg/kg) resulted in a greater amelioration of ethanol-, acetaminophen- and CCl4-induced acute liver toxicity in mice compared to

<table>
<thead>
<tr>
<th>No.</th>
<th>Experimental group</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Total bilirubin (μmol/L)</th>
<th>Direct bilirubin (μmol/L)</th>
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<tr>
<td>1</td>
<td>Control</td>
<td>94.1 ± 6.6</td>
<td>127.6 ± 10.4</td>
<td>114.2 ± 12.4</td>
<td>2.93 ± 0.43</td>
<td>0.79 ± 0.04</td>
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<tr>
<td>2</td>
<td>PC-1-200</td>
<td>87.8 ± 5.9</td>
<td>117.0 ± 7.9</td>
<td>108.2 ± 9.2</td>
<td>3.09 ± 0.29</td>
<td>0.70 ± 0.10</td>
</tr>
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<td>3</td>
<td>α-Tocopherol-100</td>
<td>93.7 ± 11.5</td>
<td>122.9 ± 13.6</td>
<td>111.9 ± 14.1</td>
<td>3.23 ± 0.18</td>
<td>0.75 ± 0.07</td>
</tr>
<tr>
<td>4</td>
<td>CCl4</td>
<td>138.9 ± 7.1**</td>
<td>285.3 ± 18.0***</td>
<td>195.7 ± 23.7**</td>
<td>4.73 ± 0.40*</td>
<td>3.27 ± 0.48***</td>
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<tr>
<td>5</td>
<td>PC-1-100 + CCl4</td>
<td>123.3 ± 18.1</td>
<td>253.5 ± 17.9*</td>
<td>173.9 ± 14.9*</td>
<td>3.95 ± 0.43*</td>
<td>1.57 ± 0.42***</td>
</tr>
<tr>
<td>6</td>
<td>PC-1-200 + CCl4</td>
<td>91.9 ± 15.3**</td>
<td>161.2 ± 33.3**</td>
<td>127.4 ± 22.9*</td>
<td>3.39 ± 0.45*</td>
<td>1.30 ± 0.54***</td>
</tr>
<tr>
<td>7</td>
<td>PC-1-400 + CCl4</td>
<td>88.4 ± 17.8**</td>
<td>155.6 ± 43.9**</td>
<td>130.3 ± 25.9*</td>
<td>3.41 ± 0.35**</td>
<td>1.28 ± 0.34***</td>
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<tr>
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<td>α-Tocopherol + CCl4</td>
<td>98.1 ± 16.4**</td>
<td>176.8 ± 27.2*</td>
<td>138.8 ± 23.4</td>
<td>3.55 ± 0.54*</td>
<td>1.02 ± 0.52***</td>
</tr>
</tbody>
</table>

Rats were treated with PC-1 (100, 200 and 400 mg/kg), α-tocopherol (100 mg/kg) and CCl4 (2 ml/kg), as described in Materials and methods (see Table 1). The data are expressed as the mean ± SD (n = 6). *, p < 0.05; **, P < 0.01; ***, p < 0.001 vs. Control. ♦, p < 0.05; ♦♦, P < 0.01; ♦♦♦, p < 0.001 vs. CCl4 (unpaired t test).
the same extracts applied separately. The oral PC-1 dosages (100–400 mg/kg) which were effective in our study are also similar to or lower than those used for other herbal compositions, e.g. Hepacare® (50–400 mg/kg) [26], AO-8 (500–750 mg/kg) [27] or Majoon-e-Dabeed-ul-ward (250–1000 mg/kg) [28].

The mechanism of tissue-protective effects of various plant preparations is generally attributed to their antioxidant, anti-inflammatory and anti-apoptotic activities (e.g. [7]). Crude plant extracts contain numerous phytochemicals, e.g., polyphenols and carotenoids, known for their antioxidant and cytoprotective activities in different cell and tissue types [7,29]. For instance, we have previously reported that water-ethanol extracts from H. rhamnoides, R. canina, S. officinalis, and O. vulgare, which were also used in the current study (see Table 3), contained significant amounts of polyphenolic/flavonoid compounds [16]. The PC-1 plant components T. serpyllum [24], T. cordata [30], and T. farfara [31] were also shown to contain high levels of phenolics. Different polyphenols and other medicinal plant constituents may act as direct and indirect antioxidants [32,33].

Accumulating evidence demonstrates that indirect antioxidant, detoxifying and cytoprotective effects of many of these agents are primarily mediated by the activation of the Nrf2/ARE signaling pathway, a major cellular defense system. Recently, the involvement of this pathway in the hepatoprotective effects of different phytochemicals and crude plant extracts has been reported [8,34,35]. Furthermore, using mice with genetically modified Nrf2 expression, Liu et al. [36] confirmed a critical role of this transcription factor in liver protection against various hepatotoxins, including CCl4. Our data showing a marked ARE-Luc transactivation in HepG2 cells treated with the extracts from the 3 tested phytocompositions are consistent with the above findings. Notably, the order of potency for LPO inhibition produced by the extracts (PC-1 > PC-2 > PC-3; Table 4) was similar to that for their activation of the ARE reporter (Fig. 2A). These data suggest that Nrf2-driven antioxidant and detoxification response may significantly contribute to the anti-LPO activity of at least some of these phytocompositions. Furthermore, significant upregulation of NQO1 and γGCS protein expression in PC-1- or PC-2- treated HepG2 cells as well as...
increases in microsomal SOD activity observed in CCL-intoxicated animals following PC-1 treatment may also be related to the involvement of Nrf2/ARE since these enzymes are major Nrf2 target gene products [9]. On the other hand, the fact that PC-3 extract negatively regulated NQO1 and, particularly, γGCS levels in HepG2 cells (Fig. 2B) implies that this phytophysiology may contain inhibitors of some components of the Nrf2 signaling pathway. This feature of PC-3 is intriguing and warrants further characterization. There has been growing interest in synthetic and natural Nrf2 inhibitors (warrants further characterization. There has been growing interest in the feature of PC-3 is intriguing and that this phytocomposition may contain inhibitors of some components.


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Appendix A. Supplementary data

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References


