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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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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 phytocomposition (PC-1) containing oregano (*Origanum vulgare*), wild thyme (*Thymus serpyllum*) and coltsfoot (*Tussilago farfara*) aerial parts, valerian (*Valeriana officinalis*) leaves and little-leaf linden (*Tilia cordata*) flowers. PC-1 extract exhibited the strongest anti-PLO and antihemolytic 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 phytocompositions. 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 phytocompositions.

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 (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 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 (α-tocopherol), can ameliorate the toxic effects of 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,

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NAD(P)H quinone oxidoreductase-1 (NQO1) and γ-glutamylcysteine synthetase (γGCS), 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 therapeutic 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 anticancer 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 rhamnoides*), dog rose (*Rosa canina*), garden sage (*Salvia officinalis*) and oregano (*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 CCl_4 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. Phytocompositions 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% CO_2 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'-TGACAAAGCACCC-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, Illkrich, France), as described previously [16]. Briefly, cells were seeded in 24-well plates (5×10^4 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 ($\sim 2 \times 10^6$) 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 γGCS/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 $CaCl_2$, 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 Na_2HPO_4 (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 KH_2PO_4 (pH 7.2) and 145 mM NaCl at 37 °C, for 10 min, under constant stirring. The basal and 0.02 mM Fe^{2+}/0.5 mM ascorbate-induced 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 formazan formation from WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]. SOD activity was expressed as U/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% Na_2CO_3.

2.12. CCl_4-induced acute and chronic hepatotoxicity models in rats

To study the preventive effects of PC-1 against CCl_4-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. On day 15, rats were treated with a single s.c. injection of either refined olive oil (groups 1–3) or 2 ml/kg of 50% CCl_4 in olive oil (groups 4–8), for another 24 h. To evaluate the hepatoprotective effects of PC-1 in rats with chronic CCl_4 intoxication (see Table 2), the animals were divided into 6 groups (6 rats/group) and treated p.o. with 100 µl of 50% ethanol in saline (groups 1 and 4), 200 mg/kg PC-1 extract (groups 2 and 5) or 100 mg/kg silymarin (groups 3 and 6) 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% CCl_4 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.

Table 1
Experimental protocol for the determination of preventive effects of PC-1 and α-tocopherol on CCl_4-induced acute hepatotoxicity in rats.

Group No.	Ethanol/ saline p.o.	PC-1 p.o. (mg/kg)	α-Tocopherol p.o. (mg/kg)	Olive oil s.c.	CCl_4 s.c. (ml/kg)
1	+	–	–	+	–
2	–	200	–	+	–
3	–	–	100	+	–
4	+	–	–	–	2
5	–	100	–	–	2
6	–	200	–	–	2
7	–	400	–	–	2
8	–	–	100	–	2

Table 2
Experimental protocol for the determination of hepatoprotective effects of PC-1 and silymarin on CCl_4-induced chronic hepatotoxicity in rats.

Group No.	Ethanol/ saline p.o.	PC-1 p.o. (mg/kg)	Silymarin p.o. (mg/kg)	Olive oil s.c.	CCl_4 s.c. (ml/kg)
1	+	–	–	+	–
2	–	200	–	+	–
3	–	–	100	+	–
4	+	–	–	–	1
5	–	200	–	–	1
6	–	–	100	–	1

2.13. Liver histology

Tissue samples from washed and saline-perfused livers were fixed with 10% buffered formalin (pH 7.4), for 24 h, and embedded in paraffin. Deparaffinized sections (~5 µm) were stained with hematoxylin and eosin (H&E) and examined under a Leica DM1000 light microscope equipped with a Leica DI C800 digital imaging color module (Leica Microsystems, Wetzlar, Germany) at 100× magnification.

2.14. Determination of biochemical parameters in rat serum

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-automatic BioChem SA Chemistry Analyzer (High Technology, Inc., Walpole, MA, USA) using the corresponding HTI kits.

2.15. Statistical analysis

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 Fe^{2+}/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 3
IC_{50} values for the LPO inhibitory effects of water-ethanol extracts from selected plants and their compositions.

Plant extracts	Plant parts	IC_{50} (μg/ml)
PC-1		**4.2 ± 1.2**
Origanum vulgare	aerial parts	25.1 ± 3.4**
Thymus serpyllum	aerial parts	124.5 ± 14.3***
Tilia cordata	flowers	37.4 ± 4.8**
Tussilago farfara	aerial parts	65.1 ± 10.1**
Valeriana officinalis	leaves	131.3 ± 12.7***
α-Tocopherol		11.4 ± 2.3*
PC-2		**40.8 ± 2.1**
Betula pendula	gemmae	35.5 ± 7.1
Hedysarum neglectum	roots	25.4 ± 4.4*
Rosa canina	leaves	115.3 ± 17.4**
Salvia officinalis	aerial parts	57.1 ± 8.3*
PC-3		**52.5 ± 6.2**
Dracocephalum integrifolium	aerial parts	28.8 ± 5.1*
Hyppophae rhamnoides	leaves	104.2 ± 12.8**
Viburnum opulus	fruits	36.4 ± 2.3*

The data are expressed as the mean ± SD from 5 (PC-1) or 3 (PC-2 and PC-3) independent experiments performed in duplicate. *, $p < 0.05$; **, $P < 0.01$; ***, $p < 0.001$ (individual plants *vs.* PC-1, PC-2 or PC-3, respectively; unpaired *t* test). The IC_{50} values for the PC-1 effects are calculated from the data shown in Fig. 1.

extracts produced 80–100% maximal inhibition (Suppl. Table S1) at 200–500 μg/mg. Following a more detailed testing we identified 12 individual extracts with the lowest IC_{50} 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 denoted Phytocomposition-1 (PC-1; Tables , 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)

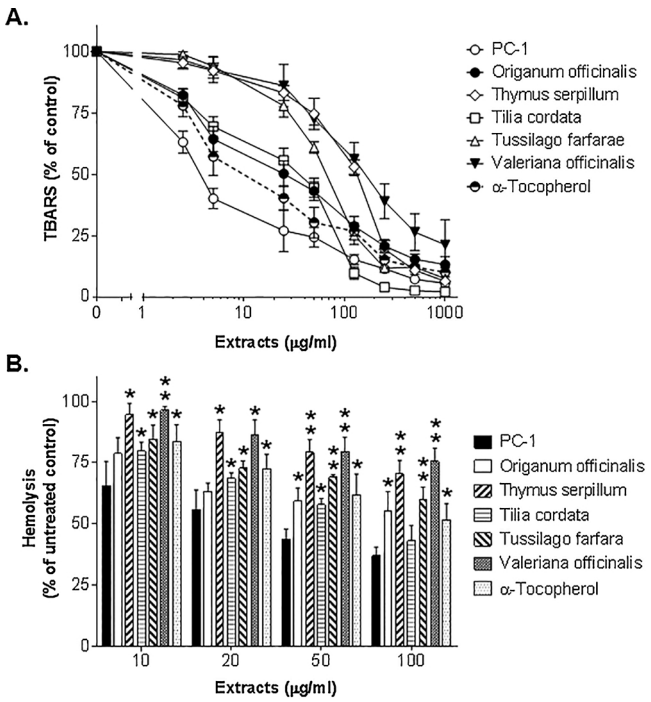

Fig. 1. Concentration-dependent effects of the extracts from Phytocomposition-1 and its individual components on microsomal lipid peroxidation (LPO) and osmotic resistance of erythrocytes (ORE). Liver microsomes (A) and erythrocyte suspensions (B) were prepared from healthy rats. (A) Microsomes were preincubated with the indicated extracts or α-tocopherol (positive control) for 10 min followed by the induction of LPO by the Fe^{2+}/ascorbate system. TBARS levels in treated samples were measured spectrophotometrically and were normalized to untreated controls (100%). (B) Erythrocytes were preincubated with extracts or α-tocopherol for 10 min followed by induction of hemolysis by hypotonic 0.4% NaCl solution. The extent of hemolysis was estimated by measuring hemoglobin absorbance in supernatants. The percent of hemolysis in treated samples was normalized to that in untreated controls (100%). The data are the means ± SD of 5 experiments performed in triplicate. The averaged IC_{50} values for dose-response LPO curves are presented in Table 3. *, $P < 0.05$; **, $P < 0.01$ *vs.* PC-1.

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.

Table 4
Plant content of selected phytocompositions.

Scientific name	Plant parts	Quantity (mg/g)[a]
PC-1		
Origanum vulgare	aerial parts	100
Thymus serpyllum	aerial parts	150
Tilia cordata	flowers	350
Tussilago farfara	aerial parts	300
Valeriana officinalis	aerial parts	100
PC-2		
Betula pendula	gemmae	225
Hedysarum neglectum	roots	100
Rosa canina	leaves	225
Salvia officinalis	aerial parts	450
PC-3		
Dracocephalum integrifolium	aerial parts	290
Hyppophae rhamnoides	leaves	290
Viburnum opulus	fruits	140
Vitis vinifera	seeds	280

[a] The quantity is presented as mg per each gram of a composition.

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

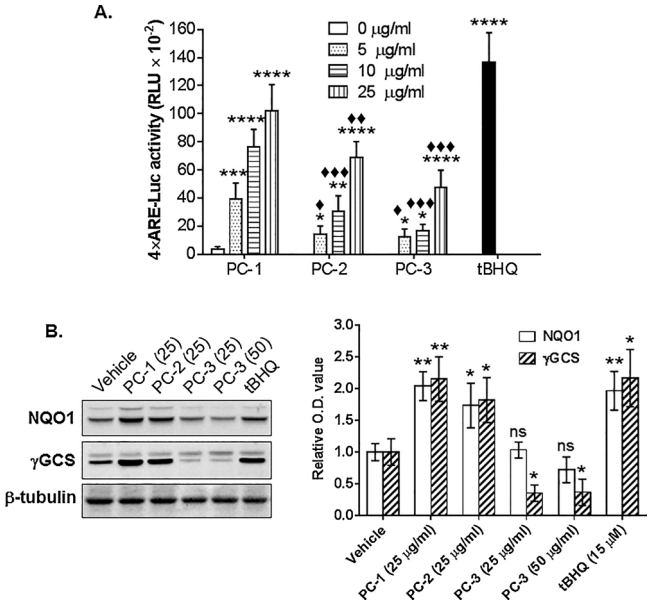

Fig. 2. Phytocomposition extracts transactivate the antioxidant response element and modulate NQO1 and γGCS protein levels in HepG2 cells. (A) Cells were transiently transfected with 4 × ARE-Luc and *Renilla* constructs and incubated with the extracts at the indicated concentrations, for 24 h, followed 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). ♦, $P < 0.05$; ♦♦, $P < 0.01$; ♦♦♦, $P < 0.001$ *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. *, $P < 0.05$; **, $P < 0.01$ *vs.* vehicle control (n = 3).

extract applied at the same or higher concentration (50 μg/ml) not only failed to enhance the expression of these proteins but even tended to reduce their basal levels, which was particularly significant for γGCS (Fig. 2B). This negative effect of PC-3 extract was unexpected since the extract was capable of significantly transactivating the ARE in the luciferase reporter plasmid (Fig. 2A), suggesting that certain components of PC-3 or their combinations may inhibit at least some Nrf2-driven signaling events, *e.g.* the downstream targets, in a promoter context-dependent manner.

Based on the above *in vitro* data showing superior effectiveness of the PC-1 extract, this preparation was chosen for further evaluation in animal models of CCl_4-induced acute and chronic hepatotoxicity.

3.3. Hepatoprotective effects of phytocomposition-1 in rat models of acute and chronic CCL_4-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 CCl_4. 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 CCl_4. 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. CCl_4 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 CCl_4, ALT levels remained comparable with those in the untreated control group. While 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 CCl_4 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 CCl_4-alone treated animals and were comparable with those observed in untreated controls (Fig. 3A,B).

In the model of chronic CCl_4-induced 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 CCl_4 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 CCl_4 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 CCl_4 injury, as manifested by significantly less pronounced changes in the above biochemical parameters compared to CCl_4-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 CCl_4, alone or together with PC-1. Liver sections from untreated control rats showed normal tissue architecture, with distinct hepatocytes and sinusoidal spaces (Fig. 4A). CCl_4-induced intoxication was accompanied by pronounced tissue damage with the signs of fibrotic changes (Fig. 4B), hepatocellular ballooning, necrotic cell death, lipid 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 CCl_4 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 CCl_4-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 CCl_4-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, CCl_4 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

Table 5
Changes in serum biochemical parameters in a rat model of CCl_4-induced acute hepatotoxicity following pretreatment with Phytocomposition-1 (PC-1) or α-tocopherol.

No.	Experimental group	ALT(IU/L)	AST(IU/L)	ALP(IU/L)	Total bilirubin (μmol/L)	Direct bilirubin(μmol/L)
1	Control	94.1 ± 6.6	127.6 ± 10.4	114.2 ± 12.4	2.93 ± 0.43	0.79 ± 0.04
2	PC-1-200	87.8 ± 5.9	117.0 ± 7.9	108.2 ± 9.2	3.09 ± 0.29	0.70 ± 0.10
3	α-Tocopherol-100	93.7 ± 11.5	122.9 ± 13.6	111.9 ± 14.1	3.23 ± 0.18	0.75 ± 0.07
4	CCl_4	138.9 ± 7.1**	285.3 ± 18.0***	195.7 ± 23.7**	4.73 ± 0.40*	3.27 ± 0.48**
5	PC-1-100 + CCl_4	123.3 ± 18.1	253.5 ± 17.3♦	173.9 ± 14.9♦	3.95 ± 0.43♦	1.57 ± 0.42♦♦
6	PC-1-200 + CCl_4	91.9 ± 15.7♦♦	161.2 ± 33.3♦♦	127.4 ± 22.3♦♦	3.39 ± 0.45♦♦	1.30 ± 0.54♦♦♦
7	PC-1-400 + CCl_4	88.4 ± 17.8♦♦	155.6 ± 43.9♦♦	130.3 ± 25.9♦♦	3.41 ± 0.33♦♦	1.28 ± 0.34♦♦♦
8	α-Tocopherol + CCl_4	98.1 ± 16.4♦♦	176.8 ± 27.2♦	138.8 ± 23.4♦	3.55 ± 0.54♦	1.02 ± 0.52♦♦♦

Rats were treated with PC-1 (100, 200 and 400 mg/kg), α-tocopherol (100 mg/kg) and CCl_4 (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.* CCl_4 (unpaired *t* test).

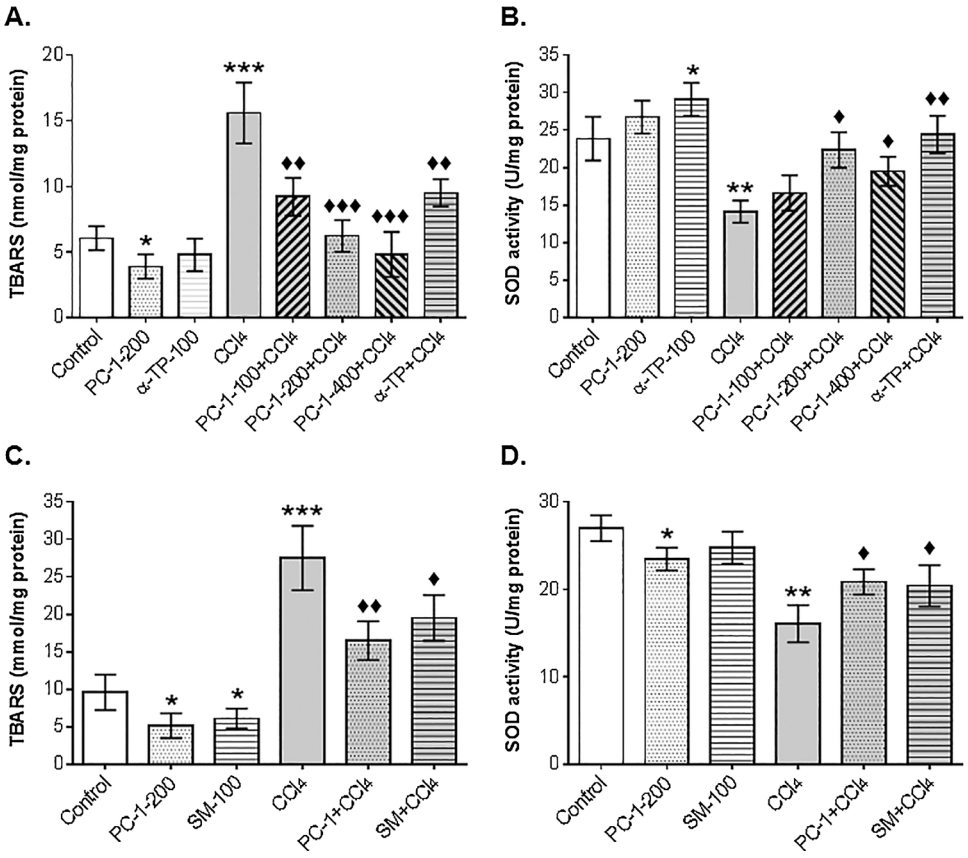

Fig. 3. Treatment with Phytocomposition-1 inhibits LPO and restores SOD activity in liver microsomes from acutely and chronically CCl_4-intoxicated rats. (A, B) Rats were pretreated p.o. with vehicle (control), the indicated doses of PC-1 (100, 200 and 400 mg/kg) or α-tocopherol (α-TP, 100 mg/kg) daily, for 2 weeks, followed by treatment with 2 ml/kg, for 24 h. (C, D) Rats were treated p.o. with vehicle (control), 200 mg/kg PC-1 or 100 mg/kg silymarin (SM) for 2 weeks, with or without simultaneous s.c. injections of CCl_4 (1 ml/kg) every other day, for 2 weeks. (A, C) Microsomal TBARS measured following Fe^{2+}/ascorbate induction and expressed as nmol/mg protein. (B, D) Microsomal SOD activity (U/mg protein). The data are the means ± SD (n = 6). *, $p < 0.05$; **, $P < 0.01$; ***, $p < 0.001$ *vs.* Control. ♦, $p < 0.05$; ♦♦, $P < 0.01$; ♦♦♦ $p < 0.001$ *vs.* CCl_4 alone.

Table 6
Effects of Phytocomposition-1 (PC-1) and silymarin on serum biochemical parameters in a rat model of CCl_4-induced chronic hepatotoxicity.

No.	Experimenta lgroup	ALT(IU/L)	AST(IU/L)	ALP(IU/L)	Total bilirubin (μmol/L)	Direct bilirubin (μmol/L)
1	Control	104.8 ± 11.3	135.5 ± 33.2	117.7 ± 8.0	2.90 ± 0.16	0.71 ± 0.05
2	PC-1-200	92.8 ± 6.9	124.3 ± 30.1	110.56 ± 8.9	2.86 ± 0.15	0.68 ± 0.06
3	Silymarin-100	96.2 ± 13.2	112.2 ± 17.1	107.6 ± 9.4	2.95 ± 0.16	0.67 ± 0.05
4	CCl_4 (1 ml/kg)	171.5 ± 12.5***	345.5 ± 85.2***	148.0 ± 11.4***	5.67 ± 0.51***	4.59 ± 0.28***
5	PC-1 + CCl_4	134.0 ± 26.3♦	173.7 ± 48.5♦♦	126.0 ± 18.0♦	3.98 ± 0.77♦	1.55 ± 0.56♦♦♦
6	Silymarin + CCl_4	124.0 ± 19.3♦	154.8 ± 47.4♦♦	129.5 ± 11.5♦	3.79 ± 0.64♦♦	1.70 ± 0.58♦♦♦

Rats were treated with PC-1 (200 mg/kg), silymarin (100 mg/kg) and CCl_4 (1 ml/kg) as described in Materials and methods (see Table 2). The data are expressed as the mean ± SD (n = 6). ***, $p < 0.001$ *vs.* Control. ♦, $p < 0.05$; ♦♦, $P < 0.01$; ♦♦♦ $p < 0.001$ *vs.* CCl_4 (unpaired *t* test).

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 CCl_4-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 CCl_4-induced acute liver toxicity in mice compared to

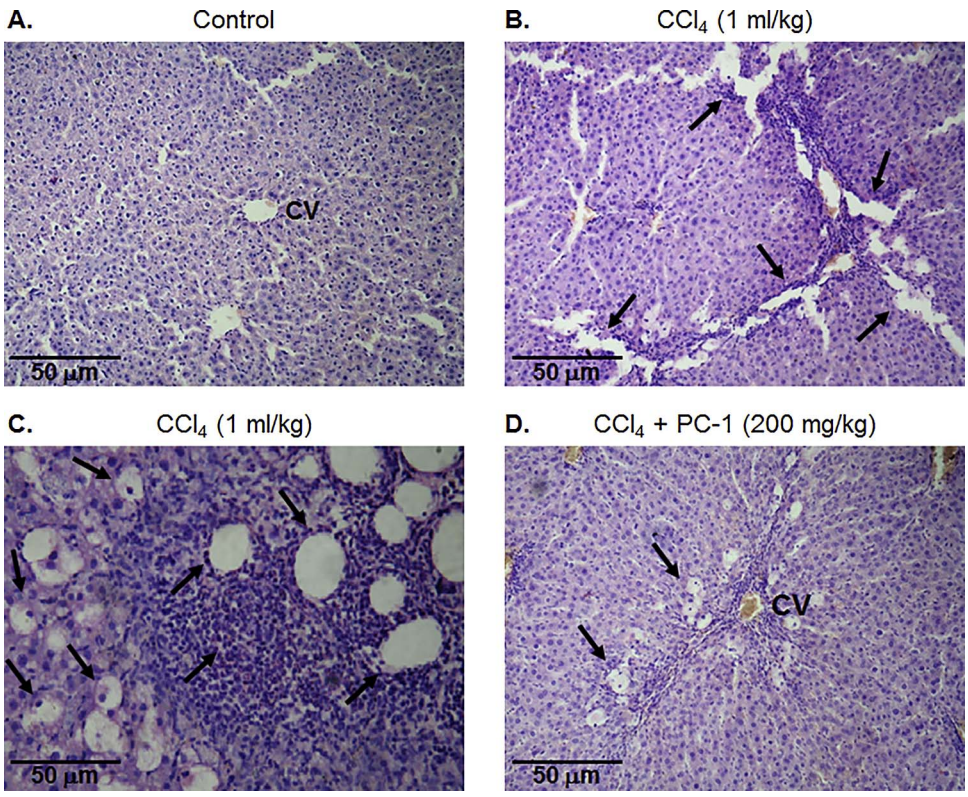

Fig. 4. Treatment with Phytocomposition-1 improves liver histology in chronically CCl_4-intoxicated rats. H&E-stained issue sections from the livers of untreated control rats (A) and those treated with 1 ml/kg CCl_4 alone (B, C) or together with 200 mg/kg PC-1 (D), for 2 weeks. *Arrows* indicate: (B) fibrotic changes; (C, D) hepatocellular ballooning, lipid droplets and leukocyte infiltration. CV, central vein. Magnification, 100 × .

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^R (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, antiinflammatory and antiapoptotic 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 hepatotoxicants, including CCl_4. 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

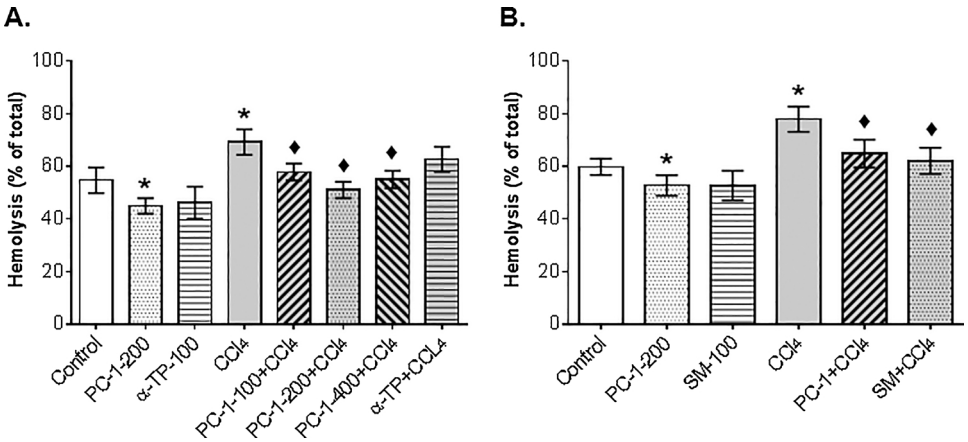

Fig. 5. Treatment with Phytocomposition-1 alleviates changes in osmotic resistance of erythrocytes induced by acute and chronic CCl_4 intoxication. (**A**) Rats were pretreated p.o. with vehicle (control), the indicated doses of PC-1 (100, 200 and 400 mg/kg) or α-tocopherol (α-TP, 100 mg/kg) daily, for 2 weeks, followed by treatment with 2 ml/kg, for 24 h. (B) Rats were treated p.o. with vehicle (control), 200 mg/kg PC-1 or 100 mg/kg silymarin (SM) for 2 weeks, with or without simultaneous s.c. injections of CCl_4 (1 ml/kg) every other day, for 2 weeks. Hemolysis of erythrocytes induced by 0.4% NaCl is expressed as the percent of total hemolysis caused by 0.1% Na_2CO_3. The data are the means ± SD (n = 6). *, $p < 0.05$; *vs.* Control. ♦, $p < 0.05$ *vs.* CCl_4 alone.

increases in microsomal SOD activity observed in CCl_4-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 phytocomposition 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 (*e.g.* [37–40]), primarily since abnormally activated Nrf2 signaling has been strongly implicated in carcinogenesis and drug resistance [41].

The fact that the high *in-vitro* LPO inhibitory potency of the PC-1 extract ($IC_{50} \approx 4$ μg/ml) was found to be much greater compared to that of its individual plant components (Fig. 1 and Table 3) indicates a clearly cooperative nature of action, which was not the case for any other phytocompositions tested here. Such concerted effects may reflect the ability of different phytochemicals, plant extracts or whole foods to cooperate with one another in various processes [12,42]. The underlying mechanisms include chemical stabilization of one compound by another due to the antioxidant effect; mutual improvement of bioavailability and facilitation of intracellular accumulation through inhibition of cellular mechanisms responsible for drug efflux and/or targeting distinct cellular regulatory pathways, which would then converge on a common target. In addition, some constituents of extracts or mixtures may neutralize adverse effects of the others, thus improving the effectiveness [12,13,42].

The finding that, in addition to its strong anti-LPO activity, PC-1 treatment also exhibited a noticeable antihemolytic activity in both *in-vitro* and *in-vivo* models suggests that the tissue-protective effects of this phytocomposition may be associated, at least in part, with an improvement in the cell membrane structure and/or function. LPO is known to cause damage to biomembranes due to the accumulation of oxidized phospholipids and other LPO products which disturb membrane structure and increase permeabilization [3,43,44]. Numerous studies have shown that various phytochemicals, particularly polyphenols [45–47], and medicinal plant extracts can interact with both the hydrophobic regions and polar headgroups of membrane phospholipids, thereby protecting biomembranes against LPO and disruption of the lipid bilayer structure [48,49].

5. Conclusions

In summary, this study was designed to develop an efficient tissue-protective phytocomposition from select medicinal plants with the strongest anti-LPO activities. Accordingly, we composed the PC-1 formulation containing *O. vulgare*, *T. serpyllum*, *T. cordata, T. farfara* and *V. officinalis* which exhibited more pronounced LPO-inhibitory and antihemolytic effects *in vitro* than its individual components and had a marked hepatoprotective effect in CCl_4-intoxicated rats. Further research is needed to elucidate the modes of cooperation between PC-1 constituents. Chemical and functional characterization of these constituents may lead to the development of novel therapeutic and preventive approaches against liver damage. Furthermore, our results warrant the exploration of PC-1 and PC-3 as well as their molecular components and their combinations in preclinical models of other pathologies, *e.g.*, neoplasia, associated with impaired redox balance and regulatory signaling.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2017.11.085

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