Research Article

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Anticancer, antioxidant, and acute toxicity studies of a Saudi polyherbal formulation, PHF5

https://doi.org/10.1515/chem-2020-0047
received October 15, 2019; accepted February 26, 2020

Abstract: A popular polyherbal formulation prepared from five plants (PHF5) may have anticancer effects. However, there is a lack of adequate scientific evidence. We assessed the anticancer, antioxidant, and acute toxicity effects of PHF5. Cancer cells were treated with 0 to 300 μg/mL PHF5 extract. Established assays were used to assess cytotoxicity, apoptosis, and radical scavenging activities. In the acute toxicity study, mice were administered a single oral dose (2,000 mg/kg) of PHF5, and biochemical and histopathological parameters were assessed. The IC_{50} values of PHF5 on LoVo, HepG2, MCF-7, and MDA-MB 231 cells were 71.8, 64.8, 45.3, and 47.3 μg/mL, respectively. Fluorescence staining demonstrated that PHF5 induced MCF-7 cell apoptosis. After 48 h, the percentage of late apoptotic cells increased significantly compared with the control cells (74.16 ± 0.64 vs 3.7 ± 2.05, $P < 0.05$). No mortality or behavioral alterations were observed in mice treated with a single dose (2,000 mg/kg) of PHF5, indicating that the LD_{50} value exceeded 2,000 mg/kg. However, histopathological changes were observed in the liver tissues. PHF5 has potential as a therapeutic agent for the treatment of human carcinoma. Further safety data will be necessary before clinical use.

Keywords: polyherbal formulation, anticancer, apoptosis, phytochemical, acute toxicity

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1 Introduction

Cancer is a widespread disease in developed and developing nations. In 2018, cancer reportedly caused 9.6 million deaths and 18.1 million cases in 185 countries [1]. Cancer therapy continues to face numerous challenges in terms of disease management [2]. Chemotherapeutic drugs used in cancer treatment have different mechanisms of action. Taxanes inhibit the function of microtubules, anthracyclines block the topoisomerase activity, mitomycin inhibits tumors by crosslinking the DNA, and triazenes crosslink the guanine nucleobases in DNA [3]. Although many types of cancers are susceptible to chemotherapy, they eventually acquire resistance through mechanisms that include drug inactivation, alteration of drug targets, drug efflux, DNA damage repair, cell death inhibition, epithelial–mesenchymal transition and metastasis, and epigenetic modifications [4]. Cancer cells have developed resistance to many anticancer drugs, such as taxol [5], tamoxifen [6], cyclophosphamide [7], anastrozole [8], and methotrexate [9]. Furthermore, most chemotherapeutic drugs remain inadequate and are associated with several adverse effects [10] that include peripheral neuropathy, myelosuppression, and leukopenia [11]. To address these issues, investigators have been evaluating plant extracts and the combination of plant extracts to identify treatments with enhanced therapeutic effects and reduced side effects [12,13].

Globally, herbal products have been used as health supplements or therapeutic agents in the treatment of diseases. Herbal medicines remain popular owing to their ease of availability, affordability, and the belief that they are safe for consumption merely because they are plant based or natural. Herbalists mostly use a combination of different herbs, rather than a single herb, to prepare plant extracts to achieve the maximum desired therapeutic potential [14].

Traditional medicine in Saudi Arabia is based on herbal preparations and is commonly prescribed in most cities and villages. Additionally, they are generally used as home remedies for certain illnesses [15]. A polyherbal formulation composed of five plants (PHF5), such as *Peganum harmala* L. (*Zygophyllaceae*), *Saussurea lappa* (Decne.) (*Asteraceae*), *Boswellia carterii* (*Burseraceae*), *Commiphora myrrha* (*Burseraceae*), and *Artemisia judaica* L. (*Asteraceae*), is a popular polyherbal formulation in Saudi Arabia. Herbalists have claimed that PHF5 is effective in the treatment of cancer. However, despite its widespread use, the pharmacological properties, safety profile, and toxicity of PHF5 are unknown. The present study was performed to assess the anticancer and acute toxicity activities of PHF5. We successfully demonstrated and achieved results for the above-mentioned activities of PHF5.

2 Experimental

2.1 Preparation of methanol extract

The plants and plant parts were purchased as dry herbs from an herbal shop (Reef al Yamen Co., Al Morooj District, Riyadh, Saudi Arabia). The PHF5 herbal formulation consisted of 20 g each of *P. harmala* seeds, *S. lappa* roots, *B. carterii* resin, *C. myrrha* resin, and the aerial part of *A. judaica*. The herbs were powdered using a commercial blender (Stardust, Japan). The PHF5 powder was extracted with high-performance liquid chromatography (HPLC)-grade methanol (Fisher Scientific, UK) for 24 h using a Soxhlet apparatus and filtered using a Whatman filter paper no. 1. The solvent was dried using a rotary evaporator (Heidolph, Germany) at 45°C and 150 rpm. The extract was reconstituted in methanol to prepare a 300 mg/mL stock solution.

2.2 Phytochemical screening

The PHF5 extracts were assessed for the presence of phytochemicals using a standard method [16,17].

2.2.1 Alkaloids

Five hundred milligrams of PHF5 was dissolved in chloroform. Next, 10 mL of a 3:10 mixture of ammonia and chloroform was added and filtered. One milliliter of sulfuric acid (2 M) was added and left to stand for 5 min. The aqueous layer was mixed with Mayer's reagent. The formation of precipitation confirmed the presence of alkaloids.

2.2.2 Triterpenoids

Five hundred milligrams of PHF5 was mixed with 4 mL of acetic anhydride and boiled. The extract was cooled and 1 mL of sulfuric acid (concentrated) was added to the test tube. The formation of a pink color confirmed the presence of triterpenoids.

2.2.3 Phenolic compounds

Five hundred milligrams of PHF5 was dissolved in 10 mL of ethanol (70%) and mixed with an equal volume of iron(III) chloride solution (5%). A deep bluish-green solution confirmed the presence of phenolic compounds.

2.2.4 Saponins

Distilled water and PHF5 extract (2 mL each) were mixed and shaken vigorously. The formation of a persistent froth confirmed the presence of saponins.

2.2.5 Determination of total phenolic and flavonoid contents

The total phenolic content of PHF5 extract was measured by the Folin–Ciocalteu method. Gallic acid was used as the reference compound to prepare the calibration curve. Briefly, 25% of the Folin–Ciocalteu reagent (125 µL) was mixed with 12.5 µL of the PHF5 extract (1 mg/mL) and left to stand for 5 min. Next, 12.5 µL of Na_2CO_3 (7%) was added and kept for 1.5 h in the dark. Blanks used 12.5 µL of methanol instead of the plant extract and were processed as described in the preceding sentences. The absorbance of the reaction mixture was measured at 760 nm using a plate reader (Thermo Fisher Scientific, USA).

Total flavonoid content was assessed using the aluminum chloride colorimetric assay. In a 96-well plate, 100 µL of PHF5 extract (1 mg/mL) was mixed with 100 µL of aluminum chloride (2%). After 10 min at 25°C, the absorbance was measured against the reagent blank at 368 nm. The calibration curve for total flavonoids

was plotted using quercetin as the standard reference as per previous method [18].

2.3 DPPH radical scavenging activity

The free-radical scavenging activity of the PHF5 extract was evaluated using a 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH)-based assay (Sigma-Aldrich, USA). Briefly, 5, 10, and 20 µL of the PHF5 extracts (1 mg/mL) were mixed with 160 µL of DPPH (100 mM) in methanol (Fisher Scientific, UK). The reaction mixture was placed in wells of a 96-well plate at 25°C for 30 min in the dark. After incubation, the changes in absorption were read at 515 nm using a microplate reader. All the tests were conducted in triplicate. Ascorbic acid was used as positive control. Methanol (5, 10, and 20 µL) was used as a control [19]. The scavenging ability of the extract was estimated using the below-mentioned formula:

$$\text{Scavenging } \% = \frac{\text{A517 control} - \text{A517 extract}}{\text{A517 control}} \times 100$$

2.4 Cell culture

Human cancer cells (MCF-7, MDA-MB 231, LoVo, and HepG2) were obtained from the German Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

2.5 Cell viability assay

Cells (5×10^5) were seeded into 24-well plates (1 mL/well) for 24 h and then treated with increasing concentrations of PHF5 (0–300 µg/mL). After 48 h, cells were incubated with 100 µL/well of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL; Invitrogen, USA) and incubated for 2 h. Subsequently, cells were dissolved in 0.01% HCl–isopropanol (1 mL). The optical density at 570 nm (OD_{570nm}) was measured using a microplate reader (Biochrom, England). Methanol (0.01%) was used as control.

2.6 Lactate dehydrogenase (LDH) assay

The release of LDH into the culture medium was used to assess the growth inhibitory effects of the PHF5 extract on MCF-7 cells. Cells treated with different concentrations of the PHF5 extract were incubated for 24 h. The supernatant (100 µL) was transferred to a 96-well plate and mixed with the LDH assay mixture (200 µL) in each well (Sigma-Aldrich, USA). The plate was incubated for 30 min at 25°C, and the absorbance was measured using a microplate reader at 490 nm. The intensity of the red color (formazan salt) indicated LDH release in the treated and untreated cells. The amount of LDH released was reported as the optical density of the control and treated groups.

2.7 Cellular morphological changes

Cellular morphology was analyzed after 48 h of incubation using phase-contrast microscopy (Leica, Germany).

2.8 Apoptosis

Fluorescent staining was performed to study the effect of PHF5 on the cellular morphology. MCF-7 cells were allowed to attach for 24 h to a 24-well plate. After 24 h of incubation, cells were treated as described in the subsequent sections.

2.9 DNA staining

Following treatment with PHF5 for 24 h, MCF-7 cells were washed with PBS and fixed in chilled methanol for 10 min at 25°C. Cells were washed twice with PBS and incubated for 10 min with 4′,6-diamidino-2-phenylindole (DAPI; Life Technology, USA). The images were captured using an EVOS® imaging system (Thermo Fisher Scientific, USA).

2.9.1 Acridine orange/ethidium bromide (AO/EB) staining

MCF-7 cells were treated with PHF5 (100 µg/mL) for 24 h, then stained with acridine orange/ethidium bromide (2 µg/mL) for 5 min and directly imaged using fluorescence microscopy.

2.9.2 Annexin-V assay

Apoptosis was evaluated using Muse Annexin-V and a dead cell kit (MCH100105) using the Muse Cell Analyzer

(Merck Millipore, USA) according to the manufacturer's guidelines. MCF-7 cells (5×10^5 cells/well) were plated in 6-well plates and treated with propolis for 48 h. Cells were harvested, washed with PBS, and 100 µL Muse™ Annexin-V Dead Cell reagent was added to each sample. Cells were incubated at 25°C for 30 min in a dark box. Apoptosis was analyzed with the Muse analysis software (EMD Millipore, USA).

2.9.3 Determination of caspase-3/7 activity

MCF-7 cells were cultured in 24-well plates as mentioned above for the detection of caspase-3/7 activity by fluorescence microscopy using the caspase-3/7 green detection reagent (Invitrogen, USA) as per previously described method [20].

2.10 Acute toxicity assay

The acute toxicity of the PHF5 extract was assessed following the OECD guideline 423. The PHF5 extract was tested using three randomly selected female albino mice (28 ± 2 g). Mice were kept under standard conditions for 5 days. The limit test was carried out at 2,000 mg/kg body weight as a single oral dose. Mice had *ad libitum* access to water but were starved for 3 h before dosing. The animals were observed for the first 30 min, then for 4 h, and then daily for 14 days. After survival of the treated mice, three additional mice were administered the same dose under the same conditions. The same procedure was performed in a vehicle-treated control group of six mice that administered corn oil in an equivalent volume (200 µL) as that in the treatment group. After 14 days, blood samples were collected using a cardiac puncture. Following cervical dislocation, the kidneys and the liver were excised, weighed, and preserved in formalin (10%) for histopathological observation.

2.11 Histological processing

Fresh sections of the liver and the kidney tissues were obtained from experimental animals, fixed in buffered formalin (10%), and then dehydrated with ethanol (70%, 80%, 90%, 95%, and 100%). Dehydration was followed by the placement of the samples in xylene and impregnation with paraffin wax. Samples were embedded and sectioning of each sample block was performed using a HistoCore BIOCUT manual rotary microtome (Leica Biosystems, Germany). Paraffin sections 4 to 5 µm in thickness were stained using the ST5010 Autostainer XL (Leica, Germany) and examined for alterations in the renal and hepatic tissues using a model BP73 optical microscope (Olympus, Japan) equipped with a digital camera.

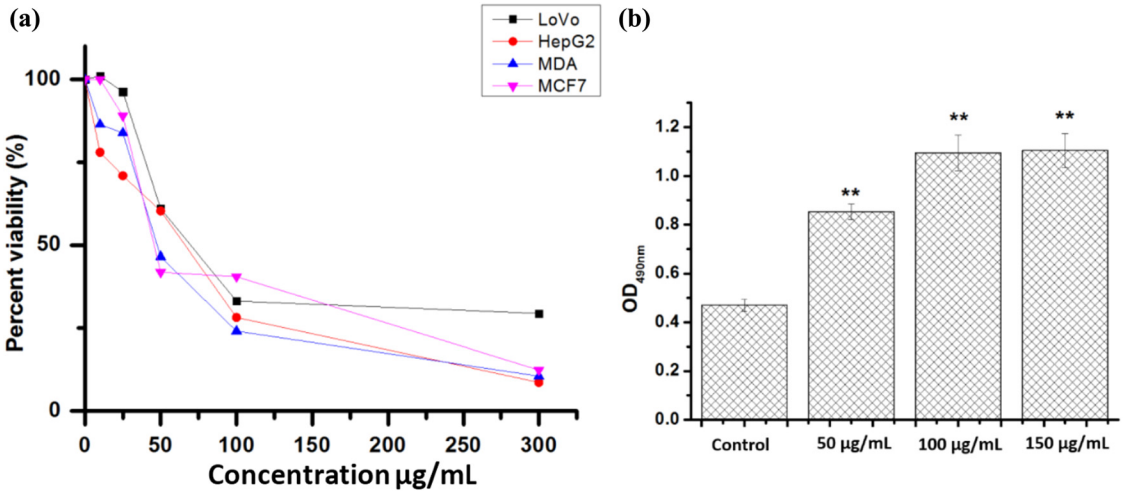

Figure 1: *In vitro* cytotoxic activity of the PHF5 methanolic extract against MCF7, MDA-MB 231, LoVo, and HepG2 cell lines. (a) Cytotoxicity (% viability) was measured by MTT assay. (b) LDH assay revealed cytotoxicity of the PHF5 methanolic extract against MCF7 cells. The results are presented as percentage of the control. Data are presented as mean ± SD of three independent experiments. **Statistically significant difference compared with the untreated control (Student's *t*-test, $P < 0.05$).

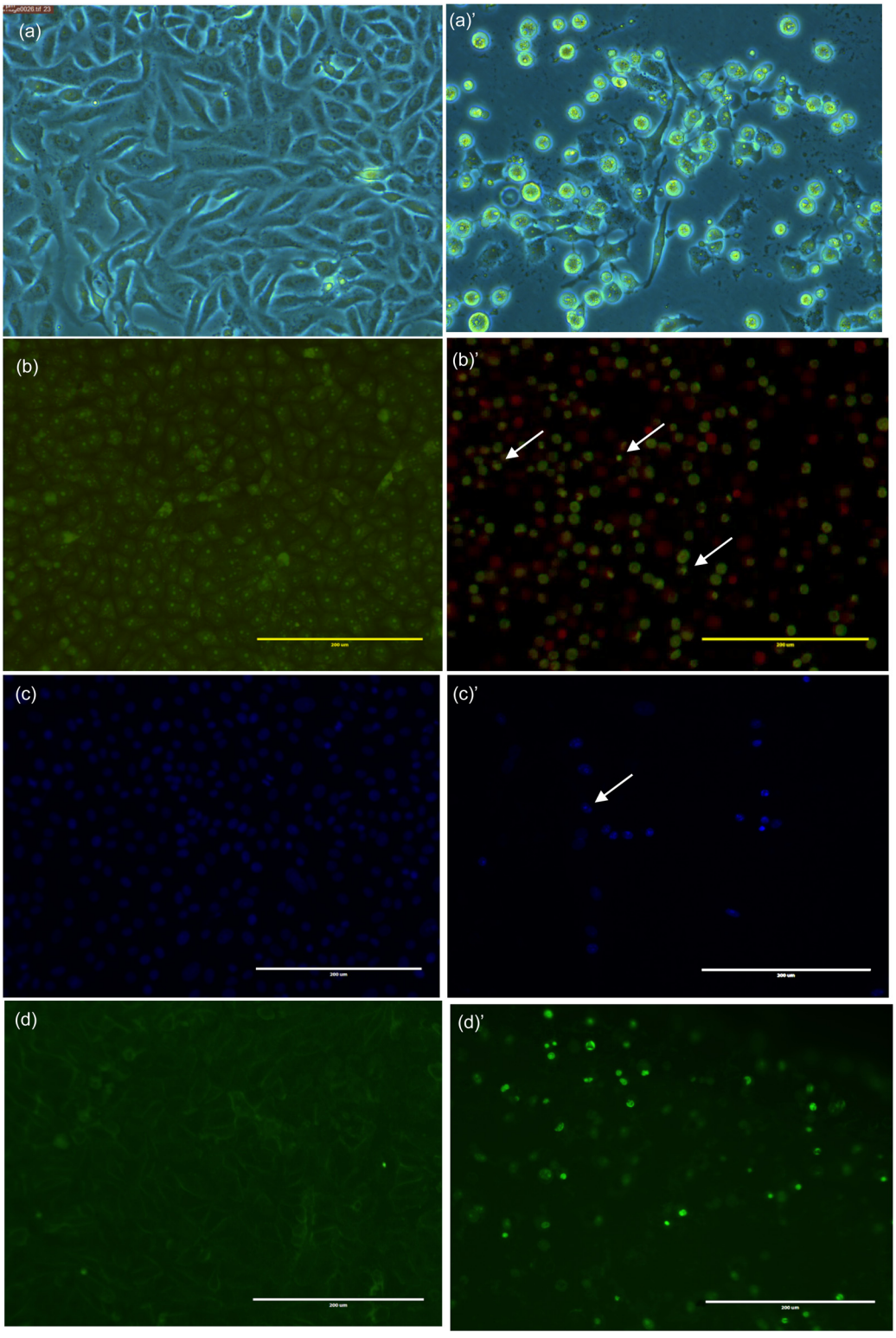

Figure 2: MCF7 cells were treated with 100 µg/mL PHF5 extract for 48 h. (a) Morphological changes in treated MCF7 cells where cell death occurred only in the treated cell. The number of cells decreased and floating cells were observed; (a) control cells, (a′) treated cells. (b) Cell apoptosis observed by acridine orange/ethidium bromide staining at 24 h. Cells containing normal nuclear chromatin showed green nuclear staining (b). Cells containing fragmented nuclear chromatin showed orange to red nuclear staining (b′). (c) Apoptosis evaluated in MCF7 cells by nuclear staining with DAPI. Control cells treated with corn oil vehicle showed uniform nuclear staining (c). Treated cells showed DNA condensation (c′). The arrows indicate apoptotic cells. (d) Cell apoptosis was demonstrated by green fluorescence using the caspase-3/7 green detection reagent. Cells were stained for 30 min and caspase-3/7 activation was observed by fluorescence microscopy; (d) control cells, (d′) treated cells.

2.12 Statistical analysis

Results have been presented as mean ± standard deviation. Data analysis was conducted using Excel software (Microsoft, USA). The analysis was performed using one-sample Student's *t*-test. Significance was indicated at $P < 0.05$.

3 Results and discussion

Chemotherapy is a principle means of cancer treatment. However, most chemotherapeutic agents have adverse effects. The anticancer activity of medicinal plants and their isolated components are the focus of several investigations. This is the first report on the toxicity and the apoptotic effect of the PHF5 extract using different cancer cell lines. Cytotoxicity was evaluated on LoVo, HepG2, MCF-7, and MDA-MB 231 cells using the MTT assay. After 48 h, the PHF5 extract showed a promising inhibitory effect against all cancer cells. The IC_{50} values of the PHF5 extract were 71.8, 64.8, 45.3, and 47.3 µg/mL against LoVo, HepG2, MCF-7, and MDA-MB 231 cells, respectively, as compared with that of the control. The PHF5 extract reduced the viability of all cell lines in a dose-dependent manner (Figure 1a). Since MCF-7 was the most sensitive among the cell lines tested, the subsequent assays were performed using only the MCF-7 cell line. LDH is an enzyme present in the cytosol of cells and is considered as a bio-indicator for cytotoxicity and loss of the cell membrane integrity. After 24 h, the PHF5

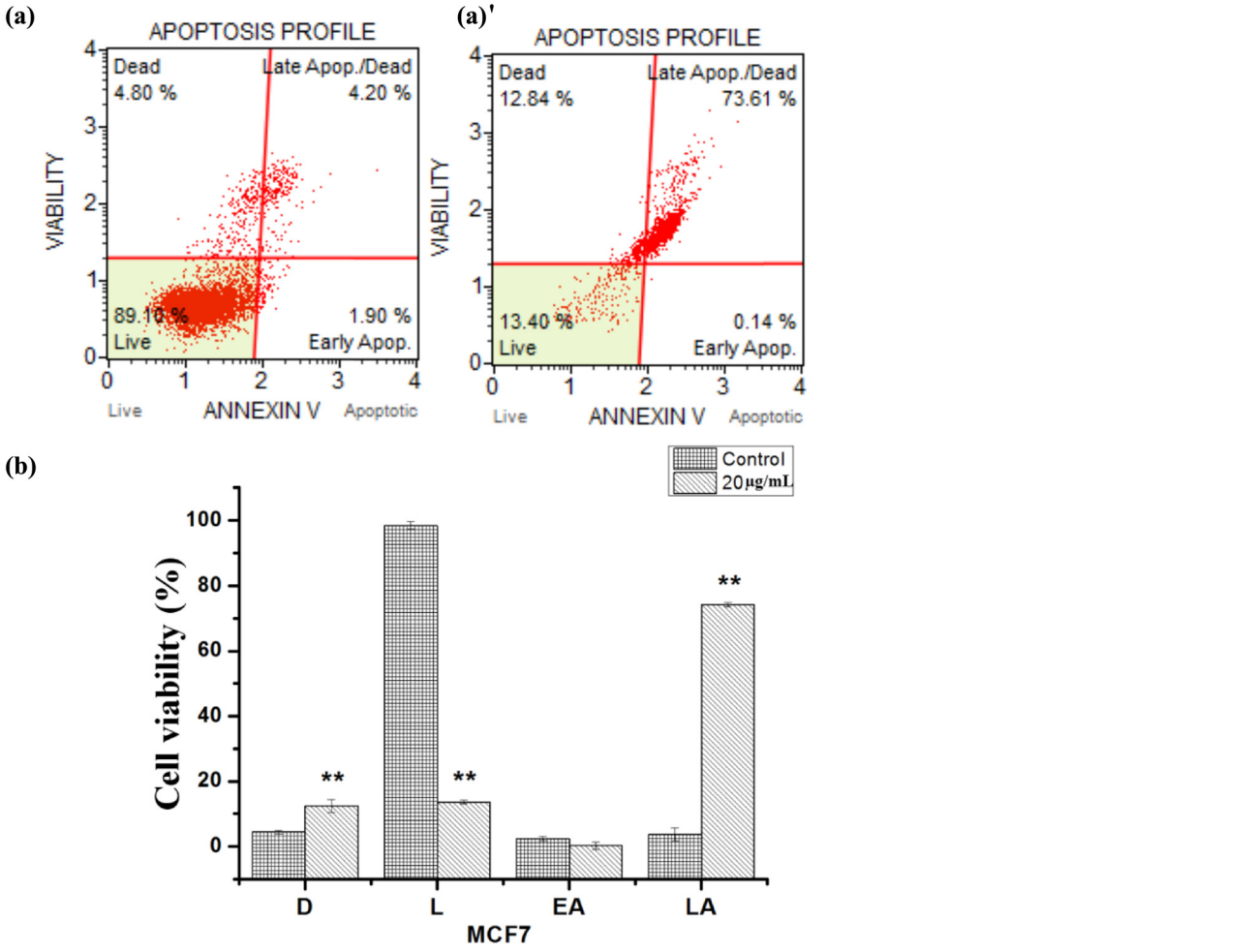

Figure 3: PHF5-induced apoptosis in MCF7 cells. (a) The distribution of the apoptotic cells using the Muse cell analyzer. The cells were incubated with 0.01% MeOH and PHF5 at concentrations of 100 µg/mL for 48 h. The percentage of early and late apoptotic cells was estimated in comparison with the MeOH control. Each sample was run in triplicate. (b) Error bars represent standard deviations. **Significant differences relative to the control ($P < 0.05$).

extract induced the significant release of the LDH enzyme at concentrations of 50, 100, and 150 µg/mL, confirming the cytotoxic potential of the PHF5 extract on MCF-7 cells (Figure 1a).

Apoptotic cells are morphologically distinguished by shrinkage, budding, membrane blebbing, and apoptotic body formation [21]. To examine the morphological changes induced by the PHF5 extract, morphological changes in MCF-7 cells were assessed by phase-contrast microscopy. MCF-7 cells were treated with 100 µg/mL PHF5 for 24 h. Floating cells, low cell confluency, and membrane blebbing were observed. In the control group, cells were attached with more than 90% confluency (Figure 2a and a′). The morphological alterations in apoptotic cells were also observed using fluorescence microscopy. Nuclear fragmentation is one of the distinguishing features of apoptosis. DAPI and acridine orange/ethidium bromide dual staining have been successful in studying the death and morphological changes of cells during apoptosis [22]. We evaluated the morphological changes in MCF-7 cells treated with the PHF5 extract using fluorescence microscopy. Control cells displayed an intact nuclear structure with normal nuclear chromatin and uniformly green nuclear staining. Cells treated with the PHF5 extract displayed chromosomal condensation and the formation of apoptotic bodies. Cells with fragmented nuclear chromatin appeared orange to red with nuclear staining (Figure 2b and b′). However, MCF-7 cells stained with DAPI showed an altered DNA nuclear staining pattern with condensed chromatin and apoptotic bodies, which are hallmarks of apoptosis. In the untreated MCF-7 cells, the nuclei were found to be round and evenly stained (Figure 2c and c′).

During apoptosis, phosphatidylserine is translocated from the inner leaflet to the outer leaflet of the plasma membrane, which is recognized by phagocytes [23,24]. To assess the effect of PHF5 in apoptosis induction, the Muse™ Annexin-V staining kit was used with the Muse Cell Analyzer. After treatment with PHF5, the percentage of the live MCF-7 cells declined significantly compared with that in the control (13.6 ± 0.54 vs 98.5 ± 1.14, $P < 0.05$). Analysis of MCF-7 cells revealed variation in the percentage of apoptotic cells. The percentage of late apoptosis with IC_{50} after 48 h increased significantly in treated cells compared with control cells (74.16 ± 0.64 vs 3.7 ± 2.05, $P < 0.05$; Figure 3a and a′). This staining helped in distinguishing intact cells, dead cells, early apoptosis, and late apoptosis, and an increase in the percentage of apoptosis in MCF-7 cells was observed. The use of the Muse Cell Analyzer revealed that the PHF5 extract increased the percentage of apoptotic cells. The collective results confirmed that the inhibition of MCF-7 cell proliferation by the PHF5 extract was capable of inducing apoptosis.

Apoptosis induction is often associated with the activation of caspases. In the next experiment, we evaluated the effect of the PHF5 extract on caspase-3/7 activation. Treatment of MCF-7 cells with PHF5 induced an increase in caspase-3/7 activity in the treated cell lines, as indicated by a bright green fluorescence when compared with the control cells after 48 h (Figure 2d and d′). Thus, apoptosis was induced by the PHF5 treatment and was caspase dependent. The induction of caspases-3, -7, -8, and -9 were due to the induction of intrinsic and extrinsic pathways. In both pathways, the initiator caspase cleaves and activates downstream effector caspases, such as caspase-3 and caspase-7. Apoptosis plays a vital role in cancer treatment as it is a common target in treatment strategies. Agents that help to induce apoptosis are considered candidates for cancer treatment.

Oxidative stress caused by reactive oxygen species is a characteristic feature of cancer cells [25] owing to metabolic and genetic changes. Accordingly, antioxidant supplements have been recommended to reduce the risk of cancer in humans [26]. Plant secondary metabolites, particularly phenolics and flavonoids, are the most abundant plant metabolites involved in defense against oxidative stress, prevention of diseases [27,28], including diabetes, cardiovascular diseases, cancer, and inflammatory conditions, and in reducing age-related deleterious effects.

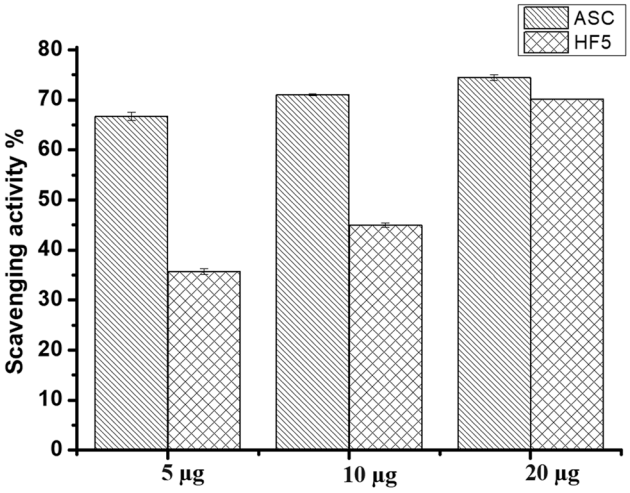

Figure 4: Antioxidant activity of PHF5 extracts. Antioxidant activity was assessed by measuring the reduction in DPPH radicals detected at 517 nm. Ascorbic acid was used as a positive control. Results are presented as mean ± SD of three independent experiments. ASC: ascorbic acid, PHF5: polyherbal formula composed of five plant extracts.

Antioxidants play a significant role against oxidative stress by inhibiting lipid peroxidation and scavenging free radicals, among many other mechanisms, and hence may prevent disease [29]. We assessed the antioxidant activity of PHF5 as the percentage of DPPH scavenging activity and showed a dose-dependent antioxidant activity (Figure 4). At concentrations of 5, 10, and 20 µg/mL, the percentage scavenging activity was 35%, 45%, and 70%, respectively. The scavenging activity of different concentrations of the PHF5 extract on the DPPH free radical was compared with that of ascorbic acid (standard antioxidant). Percentage scavenging activity of ascorbic acid was 66%, 71%, and 74.5% at concentrations of 5, 10, and 20 µg/mL, respectively. However, the scavenging activity of PHF5 in comparison to ascorbic acid was insignificant ($P > 0.05$).

Phytochemical analysis of the PHF5 extract demonstrated the presence of major classes of phyto-compounds that included phenols, flavonoids, triterpenoids,

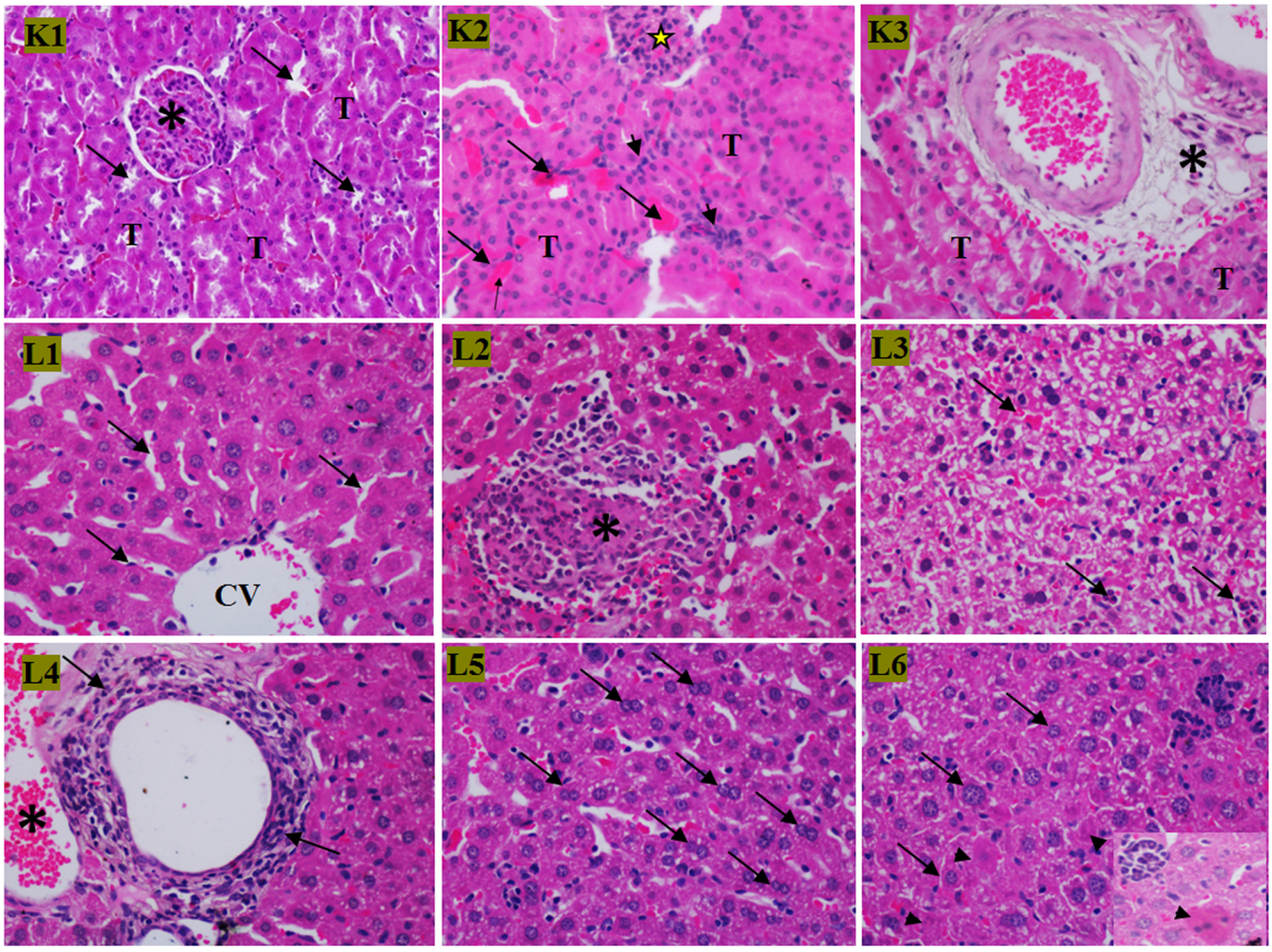

Figure 5: K(1–3) Histological sections of the kidney of control and PHF5-treated mice. (1) Control mice receiving corn oil vehicle (200 µL/kg/body weight) demonstrating normal histological architecture of kidney, glomeruli (*), kidney tubules (T), and intercellular tissue (arrows). H&E stain 400×. (2) PHF5-treated group (2,000 mg/kg/day) showing mild congestion of intertubular blood vessels (arrows), mild focal interstitial inflammation (arrow heads), and normal cortical kidney tubules (T). H&E stain 400×. (3) PHF5-treated group (2,000 mg/kg/body weight) showing perivascular edema (*), hydropic degeneration changes in the kidney tubules (T). H&E stain 400×. L(1–6) Histological sections of the liver of control and PHF5-treated mice. (1) Control mice receiving corn oil vehicle (200 mL/kg/body weight) showing normal morphology of liver and absent lesion area, with normal-appearing central vein (CV), parallel cords of hepatocytes radiating from the central vein toward the periphery of the hepatic lobule, separated by sinusoidal spaces (arrows). H&E stain 400×. (2) PHF5-treated group (2,000 mg/kg/body weight) showing granulomas (*). H&E stain 400×. (3) PHF5-treated group (2,000 mg/kg/body weight) showing hydropic degenerative changes in the ballooning hepatocytes and infiltration of neutrophils (arrows) in blood sinusoids. (H&E; 400×, original magnification.) (4) PHF5-treated group (2,000 mg/kg/body weight) showing congested hepatic portal vein (*) with infiltration of inflammatory cells around the branch of the hepatic artery (arrows) in the portal area (H&E; 200×, original magnification). 5PHF5-treated group (2,000 mg/kg/body weight) showing binucleation (arrows) in the hepatocytes (H&E; 400×, original magnification). (6) PHF5-treated group (2,000 mg/kg/body weight) showing anisokaryosis (arrows), focal necrosis (arrowheads), and apoptotic bodies (inset) (H&E; 400×, original magnification).

Table 1: Effect of PHF5 extract at the dose of 2,000 mg/kg body weight on total protein, creatinine, uric acid, ALT, and AST in female mice

Parameters	Unit	Vehicle control group corn oil	Acute toxicity group (PHF5: 2,000 mg/kg)
S.G.P.T (ALT)	U/L	46.8 ± 0.6	44.55 ± 8.2
S.G.O.T (AST)	U/L	133.2 ± 4.7	139.9 ± 13.4
Total protein	g/dL	6.18 ± 0.52	5.72 ± 0.43
Creatinine	mg/dL	0.49 ± 0.02	0.49 ± 0.07
Uric acid	mg/dL	2.53 ± 0.23	2.033 ± 0.16*

The data are expressed as mean ± standard deviation of the mean (n = 5). For statistical analysis, Student's t-test was applied. *Significance at $P < 0.05$.

and alkaloids. However, saponin was not detected in the extract. The total phenolic and flavonoid concentration of the PHF5 extract was 61.5 and 24.4 µg/mL, respectively. The total phenolic and flavonoid content was calculated using linear equations based on the calibration curves of gallic acid ($Y = 0.0011X + 0.04$, $R^2 = 0.9918$) and quercetin ($Y = 0.00264X + 0.0644$, $R^2 = 0.9953$), where Y is the absorbance and X is the amount of gallic acid or quercetin in microgram per milliliter. The phenolic and flavonoid contents found in PHF5 may justify their antioxidant activities and support their potential as anticancer molecules.

Female mice were orally treated with a single dose of PHF5 (2,000 mg/kg body weight). All females survived the 14-day treatment. No abnormal changes were noticed in behavior, tremors, salivation, skin, fur, and eyes. Therefore, the LD_{50} of PHF5 exceeded 2,000 mg/kg body weight. PHF5 has been classified as category 5 according to the Organization of Economic Cooperation and Development guidelines.

Histological sections of the kidneys of the PHF5-treated mice displayed mild congestion of intertubular blood vessels, mild focal interstitial inflammation, perivascular edema, hydropic degenerative changes in the kidney tubules, and normal cortical kidney tubules. In contrast, tissue sections obtained from the control mice receiving the corn oil vehicle (200 µL/kg/day) displayed normal histological architecture of the kidney, kidney tubules, and intercellular tissue (Figure 5K(1–3)). Similarly, histological sections of the liver of the PHF5-treated mice revealed hydropic degeneration, infiltration of neutrophils, congested hepatic portal vein, anisokaryosis, focal necrosis, and apoptotic bodies. The control mice showed normal morphology of the liver (Figure 5L(1–6)). Table 1 presents the acute toxicity potential data of the selected liver and kidney function tests. No significant alterations in the levels of alanine aminotransferase, aspartate aminotransferase, total protein, and creatinine were observed in the PHF5-treated mice compared with the corn oil vehicle-treated control group. However, there was a significant ($P < 0.05$) elevation in the level of uric acid in comparison with the control group.

The toxic effect of the PHF5 extract may be attributed to the presence of one or more phyto-compounds, which have a toxic impact on the animal model. Similar results were found in an acute and sub-acute toxicity study of a Pakistani polyherbal formulation [30,31] and in an investigation of Chinese traditional medicine when an herbal formula was used in patients. However, liver toxicity was attributed to overdosing [32]. These findings highlight that patients must adhere to the designated dosage limits and doses should also be prescribed according to the physique and health of the patient. Our results provide evidence for the toxicity profile of the PHF5 extract and reinforce the need to use the extract with caution. Further investigations evaluating the sub-acute toxicity and anticancer mechanism of action are necessary.

4 Conclusion

PHF5 displayed anticancer activities via apoptosis. The results of the acute toxicity test suggest the potential for liver damage of the PHF5 extract, while no damage to the kidney was evident. Further studies will be needed to assess the sub-acute toxicity using lower doses. Also, further evaluation of the possible mechanisms of action of PHF5 to support its therapeutic potential in cancer will be needed.

Acknowledgment: The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RG-1439-030.

Ethical approval: All procedures were performed according to the Animal Ethics Committee, King Saud University, Zoology Department (Animal Ethics Approval/2019/(392)(18).

Conflict of interest: The authors declare that they have no conflicts of interest.

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