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Original Research Article

Cytotoxic and Apoptotic Effect of the Decoction of the Aerial Parts of *Flueggea leucopyrus* on Human Endometrial Carcinoma (AN3CA) Cells

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Abstract

Purpose: *To evaluate the anti-cancer potentials of a decoction of Flueggea. leucopyrus (Willd.) on human endometrial carcinoma (AN3CA) cells.*
Methods: *Decoction was prepared by boiling 60 g of the ground plant material in 1.6 L of distilled water for about 3 h to reduce the volume to 200 mL and then freeze dried. The effect of the decoction on AN3CA cells was determined by evaluating its cytotoxicity by 3-(4, 5-dimethylthiazol-2yl) -2, 5-biphenyl tetrazolium bromide (MTT) and sulphorhodamine B (SRB) assays, as well as its ability to modulate apoptosis (microscopic observation of morphological changes, DNA fragmentation and caspase activity). The antioxidant activity of the decoction was also determined by DPPH assay, and its total polyphenolic and flavonoid content.*
Results: *The decoction exerted a significant dose-dependent cytotoxicity on AN3CA cells as evident from MTT assay IC_{50} values of 22.09 and 14.60 µg/mL at 24 and 48 h post-incubation, respectively; and SRB assay IC_{50} values of 28.60 and 15.09 µg/mL at 24 and 48 h post-incubation, respectively. The decoction also enhanced apoptosis as shown by enhanced DNA fragmentation, microscopic observation of nuclear condensation, fragmentation and apoptotic bodies and enhanced caspase 3 and 9 activities, as well as moderately increased radical scavenging activity.*
Conclusion: *The cytotoxic and apoptotic effects demonstrated by F. leucopyrus (Willd.) decoction provide supportive evidence for the ethnomedicinal use of this plant for cancer therapy.*

Keywords: *Fleuggea leucopyrus (Willd.), Endometrial carcinoma cells, Cytotoxicity, Apoptosis, Antioxidant, Anti-cancer*

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INTRODUCTION

Flueggea leucopyrus (Willd.) or "Bushweed" is a medicinal plant (family Phyllanthaceae) that grows commonly in certain regions of South East Asian countries, including Sri Lanka. Aerial parts of this plant, specifically the leaves, are used traditionally as an alternative to commonly used antibiotics to destroy maggots in sores to treat myiasis and promote wound healing, and also for the treatment of otitis media [1,2]. Recently, traditional medical practitioners of Sri Lanka have

become increasingly interested in the use of a decoction prepared from this plant as a treatment for a variety of cancers. However, the anticancer potential of this decoction has not been scientifically validated to date.Endometrial cancer is a common gynecological cancer in the world. In the United Kingdom alone, its incidence has increased by about 40 % since 1993 [3]. AN3CA cells derived from a hormone and chemotherapy resistant endometrial cancer, thus presents an ideal model to assess potential novel anti-cancer properties. Cytotoxicity, apoptosis, and antioxidant activity have been reported to be some important mechanisms utilized by many plants and phytochemicals to mediate anticancer effects [4].

The present study was carried out to investigate anti-cancer properties of *F. leucopyrus* (Willd.) by evaluating its effects on chemo-endocrine resistant AN3CA human endometrial carcinoma cells [5]. The main aim was to determine whether the *F. leucopyrus* (Willd.) decoction can induce cytotoxicity and apoptosis in AN3CA cells and exert antioxidant activity.

EXPERIMENTAL

Chemicals and other reagents

Powdered Dulbecco's Modified Eagle Medium was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). MTT, SRB and other reagents used in antioxidant activity determination, fetal bovine serum (FBS), streptomycin/penicillin, dimethyl sulfoxide (DMSO), agarose, and trypsin/EDTA were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA) and BDH Chemicals, VWR International Ltd (Leicestershire, UK), and were used according to the manufacturer's instructions.

Collection of plant material

Whole plants of *F. leucopyrus* (Willd.) were collected from Wewaldeniya, Western province, Sri Lanka and identity authenticated by the Botanist, National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka. Voucher specimens have been deposited in the National Herbarium, Peradeniya, Sri Lanka (NO: S 03) and the Institute of Biochemistry, Molecular Biology & Biotechnology, University of Colombo, Sri Lanka (Nos. UOC/IBMBB/S 02).

Preparation of the decoction

Aerial parts of the plants were dried at room temperature, and ground into powder using an electrical grinder. An aqueous decoction was prepared according to the method recommended traditionally for administration to cancer patients (personal communication, Ayur Dr. N. Jayathilake, BMARI, Nawinna, Sri Lanka). Sixty grams (60 g) of the ground plant material was boiled in 1.6 L of distilled water for about 3 hours, until the volume reduced to 200 mL. The plant extract was then filtered and centrifuged at 3000 rpm for 15 min to remove any debris. Subsequently, the supernatant was freeze dried (freeze drier-Labconco freeze dry system, LCC 77500-01, USA) and stored at -20 ^{o}C until used.

Cell culture maintenance

Human endometrial carcinoma (AN3CA) cells purchased from the American Type Culture Collection (ATCC) in Rockville, MD, USA, were grown in monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, and 50 IU/mL penicillin and 50 µg/mL streptomycin. Cells were maintained at 37 0C in a humidified atmosphere (95 %) containing 5 % CO_2 and subcultured every 2 – 3 days, upon reaching 80 % confluence.

Evaluation of cytotoxicity by Sulphorhodamine (SRB) assay

The SRB cytotoxicity assay was performed according to the method described in Samarakoon *et al*, 2010 to determine cell survival [6]. After 24 h incubation with different concentrations (0 - 400 µg/mL) of the decoction re-dissolved in DMEM medium, and different concentrations (0 - 25 µg/mL) of thymoquinone (positive control) dissolved in 1 % DMSO, cells were fixed with 50 µL of ice-cold 50 % trichloroacetic acid solution for 1 h at 4 °C. Wells were rinsed five times with distilled water and allowed to air dry. Cells were then stained with 0.4 % SRB solution (100 µL stain/well) for 15 min at room temperature. Unbound SRB dye was removed by washing plates quickly with 1 % v/v acetic acid solution five times. The bound SRB dye was then solubilized by adding unbuffered Tris-base solution (200 µl/well), and shaking for 1 h on a plate shaker at room temperature. Plates were then read at OD 540 nm, using a microplate reader (ELx 800 Universal Micro Plate Reader, BIO-TEK instruments, USA) and the results expressed as a percentage of control values.

Evaluation of cytotoxicity by MTT assay

The cytotoxicity of the decoction on AN3CA cells was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium

(MTT) assay as described by Samarakoon *et al* [6]. Cultured cells were exposed to different concentrations (0 - 400 µg/mL) of the decoction re-dissolved in DMEM medium and different concentrations (0 - 25 µg/mL) of thymoquinone (positive control) dissolved in 1 % DMSO for 24 and 48 h. This was followed by addition of 20 µL of MTT solution into the 200 µL medium in each well of the 96-well plate, and the plate was incubated at 37 ^{o}C for 4 h. The medium was then removed by aspiration and each well received 100 µL isopropanol / HCl. The plate was shaken for 30 min and the absorbance at 620 nm was measured using a microplate reader and the results expressed as a percentage of control values.

Detection of morphological changes related to apoptosis by light microscopy

AN3CA cells (4 × 10^5 cells/mL) maintained in DMEM for 24 h were exposed to different concentrations (25 - 400 µg/mL) of the decoction (test cells) or 0.1 % DMSO (control cells) respectively, for further 24 h. Morphological changes of the cells were then observed under an inverted light microscope (Olympus CKX41SF, Japan).

Detection of morphological changes related to apoptosis

AN3CA cells were grown on cover slips at a final concentration of 4 × 10^5 cells/mL in 24-well culture plates for 24 h prior to the treatment for apoptosis detection by acridine orange/ethidium bromide (AO/EB) staining. The cells were then exposed to different concentrations of the decoction (25 - 400 µg/mL) (test cells) or 0.1 % DMSO (control cells) respectively for another 24 h. The cells were fixed by 4 % formaldehyde at room temperature and plated onto glass slides and subjected to apoptosis analysis by acridine orange/ethidium bromide (AO/EB) staining as described in Samarakoon *et al* [7]. Changes in the nuclei of cells were observed within 15 minutes after AO/EB staining under a fluorescence microscope (Olympus, BX51TRF, Japan).

DNA fragmentation assay

Cells (4 × 10^5 cells/mL) exposed to the characterized decoction for 24 h were scraped off by trypsinization and harvested by centrifugation at 4 ^{o}C for 7 min. at 1000 rpm. The cell pellets were incubated for 60 min at 55 ^{o}C in 300 µL lysis buffer [5 mM Tris–HCl pH 8, 1 M NaCl and 5 mM EDTA pH 8, proteinase K (0.1 mg/mL), RNAse (0.03 mg/mL]. DNA was extracted with phenol–chloroform–isoamyl alcohol, subjected to 2.0 % of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light using a gel-doc system (Quantum-ST4 1100/20M).

Evaluation of caspase 3 and caspase 9 activities

Caspase activity assays were performed using the colorimetric assay kits for caspase-3 and caspase-9 (GenScript USA) according to the method described by Samarakoon *et al* [7]. AN3CA cells (4 × 10^5 cells/mL) were incubated with different concentrations of the decoction (50 and 100 µg/mL) for 24 h. Cells were trypsinized, washed twice with ice-cold PBS buffer (pH 7.4) and lysed in the supplied lysis buffer containing dithiothreitol (DTT). The protein concentration of each sample was determined using Bradford's reagent and equal amount of protein (100 µg) was incubated with the supplied reaction buffer containing dithiothreitol (DTT) and substrates (5 µL) at 37 ^{o}C for 4 h. Following the incubation, released chromophore pNA was spectrophotometrically quantified at the wavelength of 405 nm using the micro plate reader (ELx 800 Universal Microplate Reader, BIO-TEK instruments, USA). Average caspase 3 or 9 activities of treated cells in six repeated experiments were calculated relative to the control cells and expressed as percentage differences.

Determination of radical scavenging activity

The free radical scavenging capacities of plant decoction were determined by the DPPH (2,2-Diphenyl-1-picryl hydrazyl) radical assay. Aliquots of decoction (dissolved in 100 µL methanol, with amounts of sample ranging from 1 to 100 µg/mL) were mixed with 200 µL of 0.1 mM DPPH in methanol and these solution mixtures were kept in the dark. Ascorbic acid was used as a positive control. The change in optical density was monitored after 30 min at 517 nm using a Molecular Devices SpectraMax Plus multi plate reader. Scavenging activity was determined using Eq 1.

$$\text{Scavenging activity (\%)} = \{(Ac - As)/Ac\}100 \quad \ldots\ldots\ldots (1)$$

where Ac and As are the absorbance of control and test samples, respectively. The data are presented are mean ± SD (n = 3) and expressed as IC_{50} (The half maximal inhibitory concentration) value.

Determination of polyphenolic content

The freeze-dried decoction re-dissolved in distilled water (0.05 mL) was mixed with 5 mL of 10-fold diluted solution of 2 N Folin-Ciocalteau reagent and the total polyphenolic content determined according to the Folin-Ciacalteau method described by Samarakoon *et al* [6]. Gallic acid (0 - 100 mg/L) was used as a standard to prepare a calibration curve. The total phenolic content was expressed in mg of gallic equivalent (GAE)/100 g of extract.

Determination of total flavonoid content

The dried decoction was re-dissolved in 95 % methanol to a final concentration of 10 mg/mL. The total flavonoid content was determined using the Dowd method as adapted by Samarakoon *et al* [6]. Total flavanoid contents are expressed as mg of quercetin equivalent (QE)/100 g of extract.

Statistical analysis

Statistical analysis was carried out by using Prism 2.01 software (Graphpad Prism, San Diego, CA). The IC50 values were obtained through linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals in DPPH assay. Non-linear regression was used to determine IC_{50} values of cytotoxicity assays. Effect of the decoction on caspase activities was analysed by using one way ANOVA with Bonferroni post test.

RESULTS

Effect on overall cell activity

The effect of the decoction on overall activity of AN3CA cells as evaluated by MTT assay are summarized in Figure 1. A dose-dependent reduction in the overall activity of AN3CA cells was mediated by the decoction, with the maximum effect at concentrations > 400 µg/mL. The doses causing 50 % inhibition (IC_{50}'s) at 24 h and 48 h post-incubation were 22.09 µg/mL, and 14.60 µg/mL, respectively. IC_{50} values of thymoquinone at 24 h and 48 h post-incubation were 8.32 µg/mL and 5.21 µg/mL respectively.

Relative cell survival

Results of the SRB assay are summarized in Figure 1B. On incubation of the cells for 24 h or 48 h with the decoction, a dose dependent inhibition of cell survival was observed with IC_{50} values of 28.60 and 15.09 µg/mL at 24 and 48 h post incubation periods, respectively. IC_{50} values of thymoquinone at 24 and 48 h post-incubation were 8.95 and 5.37 µg/mL, respectively.

Total polyphenol and flavonoid contents and radical scavenging activity of the decoction

Results demonstrated that the total phenolic content of the decoction was 10.32 ± 0.48 mg GAE/100 g of decoction. Total flavonoids content was 0.775 ± 0.53 mg QE/100 g. Radical scavenging activity (IC_{50}) of the decoction assessed by DPPH assay was 25.63 ± 0.98 ppm* and IC_{50} values of positive control (L-ascorbic acid) of radical scavenging activity was 3.513 ± 0.23 ppm.

Apoptosis by DNA fragmentation

UV illuminated gel image of DNA extracted from cells treated and untreated with the decoction are shown in Figure 2. A very clear DNA band was observed in the control cells (lane 2). A characteristic ladder pattern related to apoptosis was exhibited by DNA extracted from decoction treated cells. The higher dose (400 µg/mL) of the decoction induced a significantly greater laddering of DNA when compared to the lower dose (300 µg/mL).

Effect of the decoction on caspase 3 and caspase 9 protein activities in AN3CA cells

As evident from Figures. 3A and 3B, compared to control cells, cells treated with 50 and 100 µg/mL decoction showed caspase 3 and caspase 9 activities that were significantly higher ($p < 0.001$), indicating that caspases 3 and 9 protein expressions were enhanced in AN3CA cells in response to the decoction in a dose-dependant manner.

Apoptotic morphological changes

Phase contrast micrographs (Figure 4A) shows that exposure of AN3CA cells to the decoction for 24 h resulted in (a) a dose-dependent reduction of cell number and (b) significant changes in cell morphology, when compared to control cells.

Fluorescent microscopic observations of AO/EB stained AN3CA cells after 24 h of incubation, following the treatment with different concentrations of the decoction are shown in Fig 4B. Nuclei of viable cells were stained uniformly green by acridine orange, while those of apoptotic cells exhibited yellow to orange coloration, depending on the degree of loss of membrane integrity, due to co-staining with

ethidium bromide. In this experiment, yellow staining represented early apoptotic cells, while reddish orange staining represented late apoptotic cells. A dose-dependent increase in induction of apoptosis was observed in the AN3CA cells treated with the different doses of *F. leucopyrus* decoction for 24 h, as indicated by alterations in cell staining (described above).

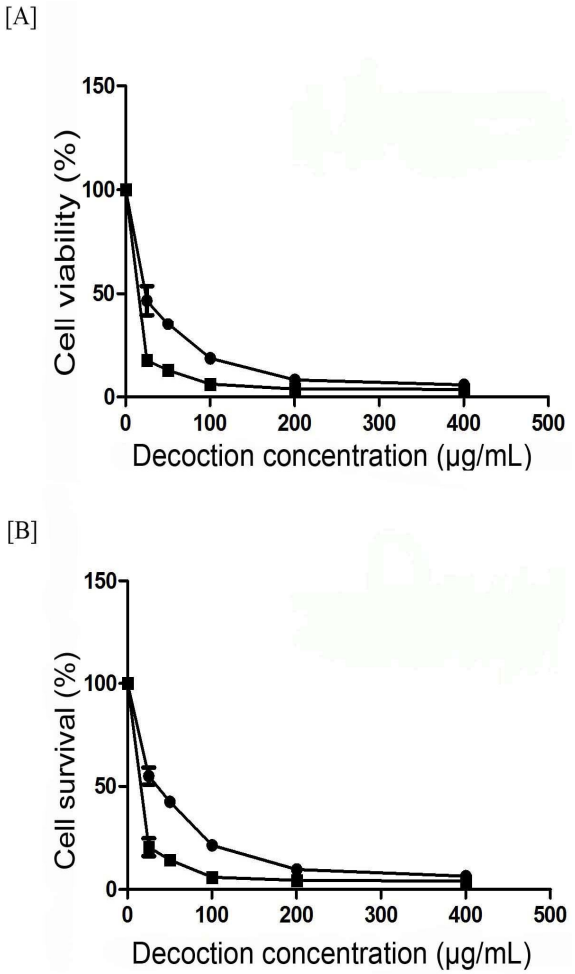

Figure 1: (A) Cell viability (by MTT assay) and (B) cell survival (by SRB assay of AN3CA cells treated with the *F. leucopyrus* decoction for 24 (●) and 48 h (■). Data values are expressed as mean ± SD

DISCUSSION

Results of the present study demonstrate conclusively that the decoction prepared from aerial parts of *F. leucopyrus* can exert a strong dose dependent cytotoxicity to human endometrial carcinoma (AN3CA) cells, when compared to the cytotoxic effects of thymoquinone (positive control) as assessed by its inhibitory effects in the MTT and SRB assays. Thymoquinone, a terpinoid that is the most abundant constituent of the *Nigella sativa* seed

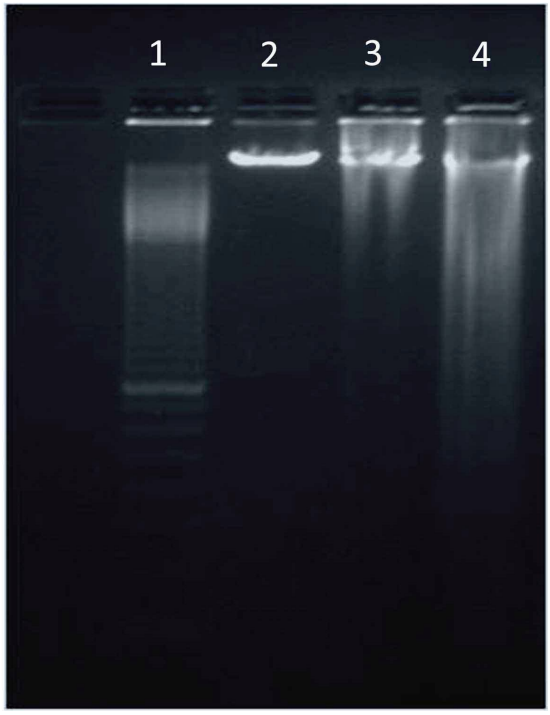

Figure 2: Detection of apoptosis by DNA fragmentation. (1) 100 bp ladder (2) Control cells (3) 300 µg/mL decoction, (4) 400 µg/mL decoction

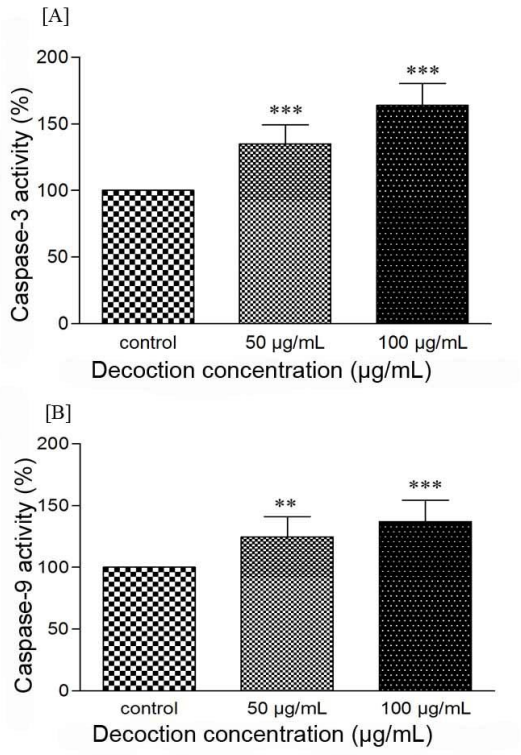

Figure 3: Effect of the decoction on caspase-3 and caspase-9 activities (mean ± SEM); ** $p < 0.01$, *** $p < 0.001$ when compared with the control; (A) caspase 3 activity and (B) caspase 9 activity

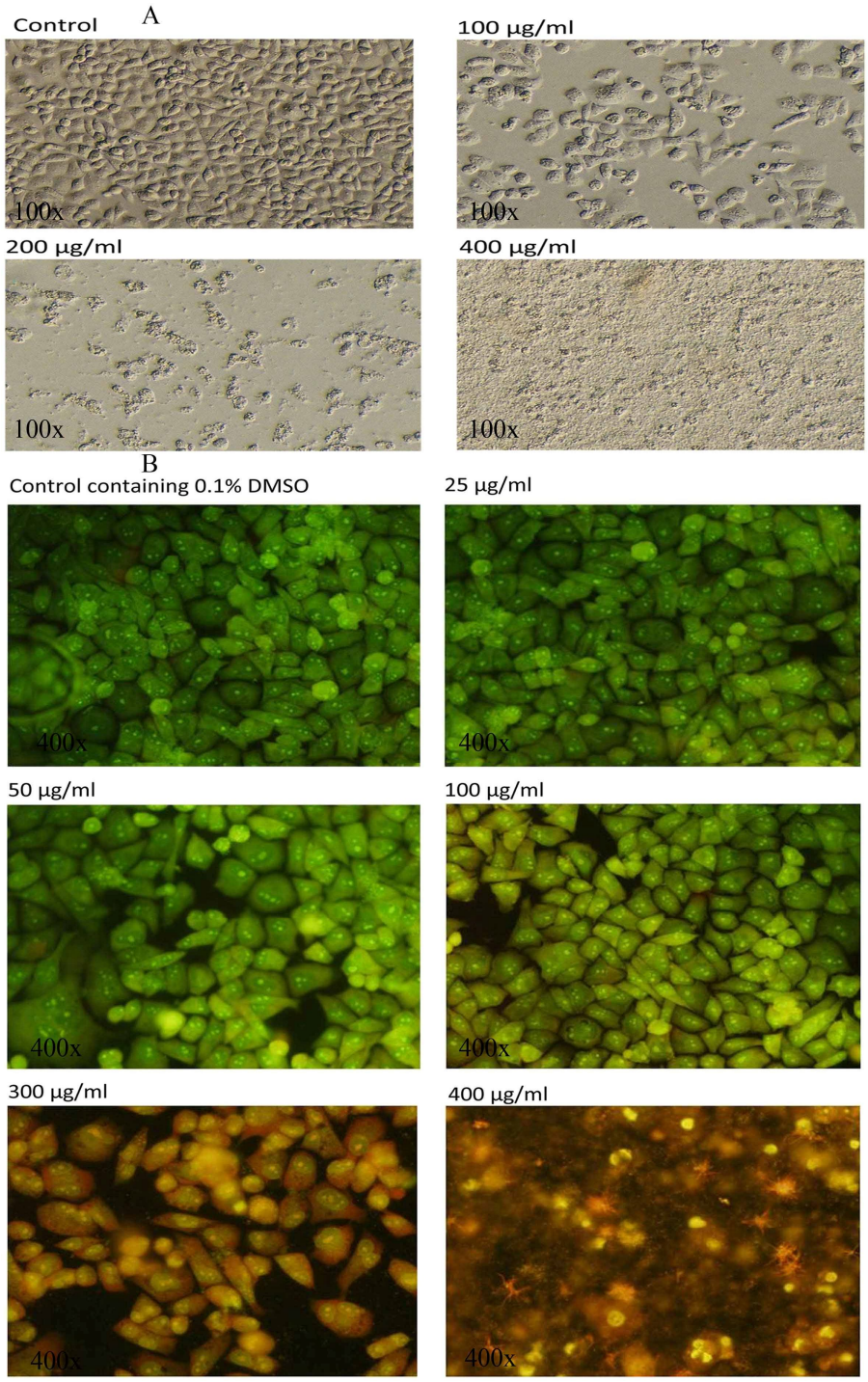

Figure 4: Morphological changes related to apoptosis, as indicated by (A) phase contrast microscopy and (B) induction of apoptosis in AN3CA cells by different doses of decoction after 24 h post-incubation (magnification: 400X). Under fluorescent microscopy using blue filter, live cells were stained uniformly green, whereas apoptotic cells were characterized by yellow-orange staining due to chromatin condensation and loss of membrane integrity

oil, has been demonstrated to mediate significant *in vitro* and *in vivo* anti-neoplastic activities against different cell lines [8]. During the past few years, much attention has been focused on exploiting the cytostatic and cytotoxic effects of phytochemicals to discover novel and effective treatment modalities for different types of endometrial cancers [9,10]. Although, some plant extracts such as essential oil extracted from *Anemopsis californica* [11] and plant derived anti carcinogenic compounds have recently been demonstrated to inhibit the proliferation of a

variety of endometrial cancer cells [12,13], they are mainly hydrophobic agents, while the present investigation has been carried out with an aqueous extract.

Apoptosis has been demonstrated to be a major mechanism employed by many natural agents to mediate anticancer effects [4]. Results obtained in the present investigation confirm that apoptosis could indeed be a major mechanism through which the *F. leucopyrus* decoction can also mediate anti-proliferative effects in AN3CA cells. Typical features of apoptosis such as chromatin condensation, and nuclear fragmentation of decoction treated AN3CA cells were clearly evident from light microscopic and fluorescent microscopic studies, and DNA fragmentation analysis by agarose gel electrophoresis.

Apoptosis can occur through a death receptor (extrinsic) pathway or a mitochondrial (intrinsic) pathway. Both pathways will result in the activation of caspases, a family of enzymes that act as death effector molecules in various forms of cell death [14]. Two classes of caspases are generally involved in the regulation and execution of apoptosis: the initiator caspases, which include caspases- 2, -8, -9 and -10 and the effector caspases, which include caspases-3, -6 and -7 [15]. Results of the present study demonstrate that the decoction under investigation can significantly enhance the activities of both caspase-3 and caspase-9 in AN3CA cells in a dose dependent manner, although a higher dose of the decoction is required for a statistically significant enhancement of caspase 9 when compared to caspase 3.

It is well known that an imbalance in the oxidant (ROS in particular) – antioxidant status (ROS) in biological systems can result in the development of many types of disease including cancer. Agents that can exert significant antioxidative activity are considered to protect against carcinogenesis by combating ROS-induced oxidative tissue damage and improving host antioxidant defence mechanisms [16]. Antioxidant activity has been reported to be to be a major mechanism by which many plants and plant compounds mediate anti-cancer effects [17-19]. Results of the DPPH-radical scavenging assay carried out in the present study demonstrates that the *F. leucopyrus* decoction also has the potential to exert radical scavenging activity.

In the last few years, the identification and development of phenolic compounds or extracts from different plants has become a major area of health and medical research. Potent antioxidant properties in plant polyphenols are considered to be responsible for their ability to protect against various oxidative stress associated diseases such as cancer [20]. Many herbal extracts have been reported to contain moderate levels of polyphenolics [20]. Results of the present study shows that the *F. leucopyrus* decoction also contains moderate polyphenolic content and can mediate a considerable radical scavenging activity.

Overall finding of this study indicates that the *F. leucopyrus* decoction has the potential to be exploited for development as a therapeutic agent for endometrial cancer. Apart from surgery, hormone therapy is used as an effective option for the treatment of patients with low-grade estrogen and/or progesterone receptor positive cancers. However, estrogen receptor negative endometrial carcinoma cells such as AN3CA cells are less sensitive to hormone therapy and develop resistance. Therefore, the development of a non-hormonal type of chemotherapy would be beneficial for the treatment of this type of endometrial cancer.

CONCLUSION

The cytotoxicity and apoptotic effects of *Flueggea leucopyrus* (Willd) decoction observed in the present study provide supportive evidence for the ethnopharmacological use of this plant for cancer therapy.

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