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Anticancer Properties of *Eisenia Foetida* Proteins in Prostate Cancer Cells In Vitro

Asuman Deveci Ozkan[1] · Gamze Guney Eskiler[1] · Mehmet Sarihan[2] · Nur Kazan[3] · Ozlem Aksoy[4] · Burcu Yuksel[5] · Zeynep Betts[4]

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Abstract
Earthworms are tube-shaped, segmental worms and have a double transport system consisting of earthworm coelomic fluid (ECF) moving within the fluid-filled colon. As one of the naturally available extracts (NAEs), ECF exerts different biological functions, such as inhibiting the proliferation of cancer cells. Pharmacokinetic limitations in prostate cancer (PCa) treatment and undesirable side effects of chemotherapeutic drugs necessitate the discovery of new anticancer agents. Recent studies have focused on the antitumor activity of different regions of earthworms as a new potential anticancer agent. However, there are limited findings on the potential effects of ECF on PCa cells in vitro. Therefore, we aimed to evaluate the potential anticancer effects of ECF (obtained from *Eisenia foetida*) on two different types of PCa cells. In this study, the protein content of ECF, the most effective dose and incubation time and potentially genotoxic and apoptotic effects were determined in PC-3 and LNCaP cells for the first time. Bax and Bcl-2 gene and protein expression levels were determined by real-time polymerase chain reaction (RT-PCR) and western blotting, respectively. ECF increased apoptotic cell death; however, it did not have a significant genotoxic effect on PCa cells. The apoptotic effect of ECF in PCa cells varies according to the AR status and is more effective in AR-insensitive cells (PC-3). Therefore, our results will specifically support the pharmacological development of ECF as a potential anticancer molecule in the treatment of PCa in the future.

Keywords *Eisenia foetida* · Prostate Cancer · Coelomic fluid · Apoptosis

Introduction

Earthworms are tube-shaped, segmental worms found in the *Annelida* phylum. Earthworms have a double transport system consisting of earthworm coelomic fluid (ECF) moving within the fluid-filled colon and a simple, closed blood circulation system. ECF contains various bioactive compounds such as proteinases released by the tubule cells of metanephridium and exerts different biological functions such as antibacterial, anticancer, hemolytic, cytotoxic, hemagglutinating and proteolytic activities due to these active ingredients of worms such as *Eisenia foetida*, *Lumbricus Terrestris* and *Dendrobaena venata* Vaillier et al. 1985; Roch et al. 1991; Kauschke and Mohrig 1987; Yanqin et al. 2007; Augustine et al. 2017, 2018; Shafi and Faleh 2019; Singh et al. 2019). Although there are many studies on the ecological, physiological and other health effects of ECF, little is known about its medicinal value, pharmacological significance and cellular effects Reynolds and Reynolds 1972; Deng et al. 2018; Lin et al. 2018).

The development of PCa, characterized by uncontrolled cell growth and uncontrolled spread of cells, is considered a complex and multi-step disease. This complexity in the molecular mechanisms of PCa complicates the treatment options (Calabrò and Sternberg 2007; Yuksel and Ozkan 2021; Ozkan and Yuksel 2022). Therefore, new treatment strategies based

✉ Asuman Deveci Ozkan
deveci@sakarya.edu.tr

1 Department of Medical Biology, Faculty of Medicine, Sakarya University, Sakarya, Turkey

2 Department of Medical Biology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

3 Department of Medical Biology, Institute of Health Science, Sakarya University, Sakarya, Turkey

4 Department of Biology, Faculty of Science and Art, Kocaeli University, Kocaeli, Turkey

5 Department of Medical Services and Techniques, Kocaeli Vocational School of Health Services, Kocaeli University, Kocaeli, Turkey

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on new ingredients or substances are required due to pharmacokinetic limitations in treating PCa and undesirable side effects of chemotherapeutic drugs (docetaxel, enzalutamide) (Nader et al. 2018). Naturally available extracts (NAEs) have a natural mechanism to inhibit the proliferation of cancer cells due to their antioxidant and free radical scavenging properties (Abraham et al. 2017). Earthworm extracts containing protease, such as ECF, are one of the NAEs that exhibit antiproliferative potential effects on cancer cells in vitro (Augustine et al. 2018). In one study, *Lumbricus Terrestris* is pulverized, and its extract potentially inhibits the proliferation of breast and PCa cells via changes in the mitochondrial membrane permeability, cytochrome c release and apoptosis (Shafi and Faleh 2019). Additionally, time- and dose-dependent significant antiproliferative effects of ECF are obtained from *Eudrilus eugeniae, Eisenia foetida* and *Perionyx excavatus* in oral cancer cell lines (KB3-1 and SCC-9) (Augustine et al. 2017). Furthermore, ECF from *E. foetida* significantly reduces the proliferation of the human lung adenocarcinoma cell line, LTEP-A2, HeLa cells leukaemia (Yanqin et al. 2007; Hua et al. 2011). In another study, ECF from *Dendrobaena veneta* has increased the apoptotic cell death in lung cancer cell line A549 (Hung et al. 2008). Therefore, the potential therapeutic feature of ECF is based on the biological substances, including lectins, polysaccharides, proteases, antibacterial peptides and fibrinolytic enzymes in the literature (Augustine et al. 2018). However, there are limited findings on the potential therapeutic effects of ECF on PCa cells in vitro. Therefore, we aimed to evaluate the potential cytotoxic and apoptotic effects of ECF from *E. foetida* on two different types of PCa cells in terms of androgen receptor (AR) status.

Materials and Methods

Preparation and Protein Content Determination of ECF LC/MS-MS Analysis

In this study, we used *Eisenia foetida* as earthworms and were purchased from a commercial company (WHM organic Co.). Earthworms were cultured and fed in cattle manure in an EkosolFarm CompostBox unit with a relative humidity of 65% in the darkness at 25 °C. Before collecting coelomic fluid, approximately 400 mg of earthworms were transferred to wet filter paper to empty their intestinal tract. The ECF was collected by the electric shock method (Hu et al. 2018). Afterwards, ECF was sterilized by filtration (0.22 mm diameter) and centrifuged under 1000 rpm at 4 °C for 5 min. The protein concentration of the collected ECF was measured using the Bradford method (Bradford 1976) and stored at −20 °C before the experiments. Additionally, the protein content of ECF was determined by LC-MS/MS analysis according to Akpinar et al. (2021).

Cell Culture

In this study, we used PC-3 (AR-independent) and LNCaP (AR-dependent) cell lines as PCa cells in all analysis also human umbilical vein endothelial cells (HUVEC) as a control cell for only cell viability assay. All cells were obtained from American Type Culture Collection (ATCC) commercially. PC-3 and LNCaP cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) and HUVEC cells in Dulbecco's Modified Eagle Medium (DMEM) cell culture medium with 0.1% penicillin and streptomycin, 10% FBS and cultured at 37 °C with 5% CO_2.

Cell Viability Assay

To determine the cytotoxic effects of ECF on PC-3, LNCaP and HUVEC cells, WST-1 assay was conducted. For this purpose, an equal number of cells were seeded on 96 well plates (2×10^4 cells/well) and incubated for 24 h. After incubation, the cells were treated with different concentrations of ECF (1, 10, 20 and 40 µg/mL) for 24 h at 37 °C with 5% CO_2. Forty-eight hours later, WST-1 reagent (Biovision) was added to each well and incubated for 45 min. Absorbance was measured at 450 nm with a microplate reader (Allsheng). According to the WST-1 analysis results, the two most effective ECF concentrations were selected for further experiments.

COMET and Cytokinesis Blocked Micronuclei (CBMN) Analysis

The genotoxic effects of ECF were evaluated by COMET and CBMN analysis. For this purpose, PC-3 and LNCaP cells were seeded in 6 well plates (1×10^5 cells/well) and incubated for 24 h. Then, the cells were treated with the two most effective concentrations of ECF (1 and 10 µg/mL) for 24 h. After incubation with ECF, the alkaline comet assay was performed according to the protocol of Comet Assay Kit (Abcam). For each slide, 25 randomly selected nuclei were examined under a fluorescence microscope (Olympus BX51). Analyzes were performed by the Cleavier Scientific Comet version computer package program. Median values for comet parameters were calculated by evaluating four slides for each treatment group. After repeated experiments, tail length (µm), tail momentum (µm), and percentage of DNA seen in comet form were calculated for each treatment group.

Additionally, we performed the CBMN analysis according to the protocol of Kalantzi et al. (2004) with minor modifications. Slides were imaged with the EVOS Floid

Cell Imaging Station (Thermo Fisher Scientific). For each treatment, micronuclei (MNi), micronucleated binuclear cells (MN), and total micronuclei distribution (TMN) in 1000 binucleotic cells from a minimum of three experiments were determined. Also, the mitotic rate was evaluated as the percentage of binuclear cells (mean ± SD, n = 3500 cell count).

Annexin V Assay

The apoptotic effects of ECF were evaluated by Annexin V assay. For this purpose, PC-3 and LNCaP cells were seeded in 6 well plates (1×10^5 cells/well) and incubated for 24 h. Then, the cells were treated with the two most effective concentrations of ECF (1 and 10 µg/mL) for 24 h. After incubation, the cells were collected using trypsin/EDTA solution (Gibco) and centrifugated at 1500 rpm for 5 min. The cell pellet was washed with PBS twice and treated with Muse™ Annexin V and Dead Cell Kit (Merck Millipore) according to the kit instructions and also analyzed by Muse™ Cell Analyzer (Merck Millipore).

Acridine Orange (AO) Assay

To further evaluate the apoptotic effects of ECF on PCa cells, the changes in cell morphology were observed by AO staining. For this purpose, PC-3 and LNCaP cells were seeded in 6 well plates (1×10^5 cells/well) and incubated for 24 h. Then, the cells were treated with the two most effective concentrations of ECF (1 and 10 µg/mL) for 24 h. After incubation, the cells were fixed with 4% paraformaldehyde solution for 30 min. After fixation, the cells were washed twice and staining with AO dye (100 mg/mL) for 30 min and visualized by EVOS FL Cell Imaging System (Thermo Fisher Scientific).

Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

To support the apoptotic effects of ECF on PCa cells, *Bax* and *Bcl-2* gene expression levels were determined by using RT-PCR analysis. For this purpose, PC-3 and LNCaP cells were seeded in 6 well plates (1×10^5 cells/well) and incubated for 24 h. Then, the cells were treated with the two most effective concentrations of ECF (1 and 10 µg/mL) for 24 h. After incubation, total RNA was isolated from the cells using an RNA extraction kit (Omega Bio-Tek) according to the manufacturer's instructions. The concentration of total RNA was measured by a Qubit 4 Fluorometer (Thermo Fisher Scientific) and converted to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). After cDNA conversion, the relative gene expression levels of *Bax* and *Bcl-2* were analyzed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Additionally, as an endogenous reference gene, *β-actin* was used.

Western Blotting

To further support the apoptotic effects of ECF on PCa cells, Bax and Bcl-2 protein expression levels were determined by western analysis. For this purpose, PC-3 and LNCaP cells were seeded in 6 well plates (1×10^5 cells/well) and incubated for 24 h. Then, the cells were treated with the two most effective concentrations of ECF (1 and 10 µg/mL) for 24 h. After incubation, total protein was extracted with RIPA lysis buffer (Santa Cruz). The concentration of total protein was measured by a Qubit 4 Fluorometer (Thermo Fisher Scientific) and separated at 12% SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad) and then blocked in 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T). Afterwards, the membranes were incubated with the primary antibodies of Bax and Bcl-2. Finally, the membranes were incubated with HRP-conjugated anti-mouse IgG, and chemiluminescent bands were visualized and photographed. β-actin protein was used as a standard.

Statistical Analysis

All statistical analysis was assessed with GraphPad version 6.0 (La Jolla, CA) with a statistically significant level of $p < 0.05$. One-way ANOVA analysis with variance Tukey's test was used for multiple comparisons. For analyzing the gene expression results, the software of Qiagen (https://www.qiagen.com/tr/shop/genes-andpathways/data-analysis-center-overviewpage/other-real-time-pcrprobes-orprimersdataanalysis-center/).

Results

Protein Content of ECF Determined by LC-MS/MS

Protein identification was performed with the raw data obtained from LC-MS/MS analysis for peptide and protein identification for ECF. The minimum number of peptides identified for each protein was assumed to be two, and data were searched according to the organism (*E. foetida*) specific data found in the UniProt/SwissProt database. The functional and biological grouping of proteins was analyzed according to Gene Ontology data, an extension of Proteome Discoverer 2.2 (Table 1). Our result showed that the presence of these proteins (Lysenin and Lumbrokinase) exerted

Table 1 List of proteins of ECF obtained with LC-MS/MS analysis

Accession	Description	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI
O18425	Lysenin-related protein 2	62.33	16	67	9	1	300	34,1	5,91
O18423	Lysenin	55.89	13	68	7	1	297	33,4	6,24
A0A2I7YV73	Actin	63.82	17	25	17	1	376	41.9	5.48
A0A410RGS5	Extracellular globin (Fragment)	55.62	8	11	8	1	169	18.9	6.99
A0A410RH35	Extracellular globin (Fragment)	63.12	10	13	10	1	160	18	6.58
A0A2I7YV68	Superoxide dismutase [Cu-Zn]	47.09	6	7	6	1	155	15.9	6.71
A0A088BZ83	SCBP2-like protein (Fragment)	18.33	3	8	3	1	120	13.8	4.41
P13579	Extracellular globin-4	15.23	3	4	3	1	151	17.5	6.42
A0A410RF39	Haemoglobin linker chain (Fragment)	30.08	4	6	4	1	246	27.5	4.92
A0A410RH77	Extracellular globin (Fragment)	10.69	1	1	1	1	159	18.3	6.16
A0A410RH42	Extracellular globin (Fragment)	29.67	3	6	3	1	182	20.6	6.74
A6XIJ5	Elongation factor 1-alpha (Fragment)	9.22	4	4	4	1	401	44.4	7.78
O61253	Elongation factor 1-alpha (Fragment)	9.86	4	4	4	1	375	41.3	7.2
A6XII3	Elongation factor 1-alpha (Fragment)	9.78	4	4	4	1	378	41.8	7.47
D0EP16	Pi-class glutathione S-transferase (Fragment)	12.08	1	1	1	1	91	10	9.5
H9MEL4	Glutathione-S-transferase (Fragment)	13.25	1	1	1	1	83	9.2	9.39
A0A2D1PJH7	Lysenin-like protein (Fragment)	44.61	4	7	3	1	130	14.5	5.11
A0A410RFC9	Haemoglobin linker chain (Fragment)	18.53	3	3	3	1	259	29	5.22
E9NPR7	Glutathione S-transferase	19.41	2	3	2	1	206	23.5	9.88
B8YCQ7	Ferritin	10.46	1	1	1	1	172	19.7	5.3
B7SHS5	Coactosin-like protein	25.17	3	3	3	1	143	15.9	4.88
A0A6B9ITD0	NADH dehydrogenase subunit 4	5.30	1	2	1	1	452	50.5	9.2
Q9GRJ1	Calmodulin	11.40	2	3	2	1	149	16.8	4.22
Q6DKQ2	Lumbrokinase	6.50	1	1	1	1	246	26.3	4.92
G3M459	Catalase	1.58	1	1	1	1	505	57.8	8.6
Q7JQD3	Gelsolin-like protein 1	8.44	3	4	3	1	367	41.6	6.54
E9NPR6	Heat shock protein 70	4.88	2	2	2	1	655	72.9	8.72
O18477	Translationally-controlled tumour protein homolog	4.79	1	1	1	1	167	18.8	5.06
A0A1B1LTZ1	Ubiquitin (Fragment)	19.70	2	2	2	1	137	15.5	9.41
Q5D2M8	Eisenstasin II	9.62	1	1	1	1	187	21.3	8.07
A0A2I7YV10	Aminopeptidase	2.953	1	1	1	1	237	25.4	7.59
A0A088BZM2	Haemoglobin linker chain (Fragment)	6.27	1	2	1	1	239	26.7	5.99
Q34947	Eisenstasin I	15.38	1	2	1	1	156	17.3	7.91
Q2I6A1	Glyceraldehyde-3-phosphate dehydrogenase	4.49	1	1	1	1	334	36.2	7.91
Q2I695	Neurocalcin-like protein (Fragment)	13.92	1	1	1	1	79	8.9	6.55
Q2I6A3	NADH-ubiquinone oxidoreductase chain 5	4.00	1	1	1	1	574	64.3	8.53
B9TY07	Ubiquitin (Fragment)	22.31	2	2	2	1	121	13.7	7.4
D1MJA5	TCTp (Fragment)	8.08	1	1	1	1	99	11.5	7.33
P84589	Catalase (Fragment)	5.5	1	1	1	1	144	16.6	6.68
A0A2D1PJE4	Translationally-controlled tumour protein (Fragment)	8.08	1	1	1	1	99	11.5	6.54
Q2I6A7	Calmodulin (Fragment)	10.84	1	1	1	1	83	9.5	4.3
Q1ZZB7	Fibrinolytic protease 1	15.70	1	1	1	1	242	24.9	4.98
Q0Q596	Peptidyl-prolyl cis-trans isomerase (Fragment)	11.53	1	1	1	1	78	8.3	7.28

Table 1 (continued)

Accession	Description	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI
A8ILP1	Lumbrokinase (Fragment)	16.17	1	1	1	1	235	24.1	4.98

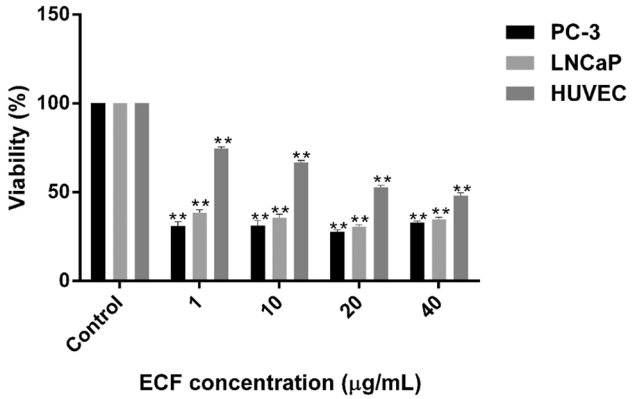


Fig. 1 Determination of the cytotoxic effect of ECF in PC-3 LNCaP and HUVEC cells. The percentages of viability for 24 h in PC-3 and LNCaP cells treated with ECF at different concentrations (1, 10, 20 and 40 µg/mL) were statistically analyzed by comparing them with the control group (*$p < 0.05$, **$p < 0.01$)

toxic properties in cancer (Kobayashi et al. 2006; De Colibus et al. 2012; Hu et al. 2018).

The Cytotoxic and Genotoxic Effects of ECF on PCa Cells

We determined the cytotoxic effect of ECF on the PC-3 and LNCaP cells compared with HUVEC control cells (Fig. 1). After treatment with different concentrations of (1, 10, 20, and 40 µg/mL) ECF for 24 h, the viability rates were 30.89 ± 2.04%, 31.00 ± 2.40%, 27.76 ± 0.89%, and 32.87 ± 0.69%, respectively in PC-3 cells, while the viability of LNCaP cells reduced to 35.65%±1.58%, 38.55 ± 1.22%, 30.68 ± 0.82%, and 34.71 ± 0.86%, respectively ($p < 0.01$, Fig. 1). In HUVEC cells, the viability of cells decreased in a dose-dependent manner (74.44 ± 0.84%, 66.71 ± 0.93%, 52.71 ± 0.77 and 47.97 ± 1.28% at 1, 10, 20 and 40 µg/mL ECF treatment, respectively) ($p < 0.01$, Fig. 1). Thus, 1 and 10 µg/mL ECF were selected as the most effective doses for further analysis to evaluate the effects of ECF on both PCa cells.

Additionally, we performed COMET and CBMN analysis to demonstrate the genotoxic effect of ECF on PCa cells. ECF did not cause DNA breaks in PCa cells and therefore did not show genotoxic effects with little change and statistically insignificant results compared to the control group (Fig. 2; Table 2).

The Apoptotic Effects of ECF on PCa Cells

The apoptotic effects of ECF on PC-3 and LNCaP cells were analyzed by Annexin V analysis (Fig. 3). The obtained results demonstrated that there was a significant increase in the percentage of early apoptotic cells in a dose-dependent manner in PCa cells ($p < 0.01$, Fig. 3A). Following the treatment of 1 and 10 µg/mL ECF, a significant increase (from 2.53 ± 0.67% to 72.95 ± 0.89% and 75.45 ± 0.45%, respectively) was detected in the number of total apoptotic cells in PC-3 cells for 24 h ($p < 0.01$, Fig. 3B). Additionally, 1 and 10 µg/mL ECF treatment increased total apoptotic cells from 7.12 ± 0.67% to 59.90 ± 0.78% and 52.0 ± 0.65%, respectively, in LNCaP cells ($p < 0.01$, Fig. 3B). As a result, ECF caused early apoptotic cell death in PCa cells. However, ECF more induced apoptotic death in PC-3 cells than hormone-sensitive LNCaP cells.

The Effects of ECF on PCa Cell Morphology

To further evaluate the apoptotic effect of ECF on PC-3 and LNCaP cells, AO staining was performed (Fig. 4). It was observed that the general morphological structure of the cells changed depending on the increasing concentration of ECF in PCa cells. Moreover, blebbing in the cell membrane and nucleus and shrinkage of the cell and cytoplasm were observed, especially in the cells treated with 10 µg/mL ECF in the cells (Fig. 4). However, ECF mediated more damage was observed in PC-3 cells than in LNCaP cells.

The Effects of ECF on mRNA and Protein Expression Levels of PCa Cells

To further confirm the apoptotic effect of ECF in PC-3 and LNCaP cells at the mRNA level, *Bax* and *Bcl-2* mRNA and protein expression levels were analyzed by RT-PCR and Western Blot (Fig. 5). Following the treatment of 1 and 10 µg/mL ECF, the mRNA level of *Bax* significantly upregulated 1.66- and 1.93-fold, respectively, while there was no statistically significant difference from the control group in the expression of *Bcl-2* (1.09- and 1.10-fold, respectively) in PC-3 cells (Fig. 5A). On the other hand, after treatment with 1 and 10 µg/mL ECF, the mRNA levels of *Bax* were 0.64- and 0.75-fold, respectively ($p < 0.01$), whereas the expression of *Bcl-2* (0.65- and 0.62-fold, respectively) was downregulated in LNCaP cells (Fig. 5A). Furthermore, we also

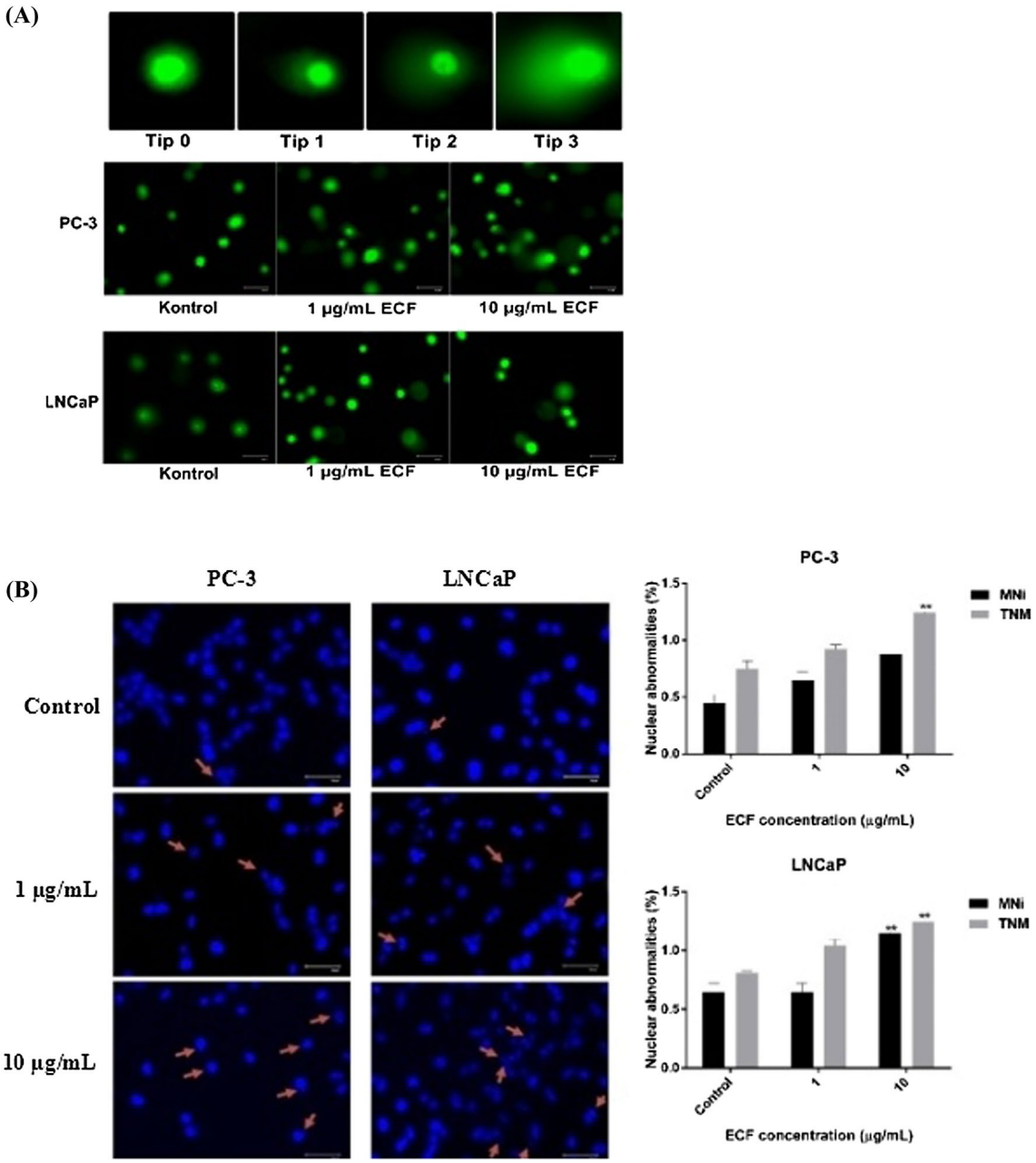


Fig. 2 The genotoxic effect of ECF on PC-3 and LNCaP cells. **A** Determination of genotoxic effect by single-cell gel electrophoresis (SCGE) analysis in PCa cells. It was scored as being visually scored into four classes according to tail length; Type 0: undamaged, no tail, Type 1: tail shorter than the diameter of the head (core), Type 2: tail, 1–2 times the diameter of the head, and Type 3: ultra-highly damaged, with a tail longer than the diameter of the head. **B** DAPI staining imaging of PC-3 and LNCaP cells by CBMN analysis. Micronucleus formation and binuclear cells were visualized. The data obtained after the CBMN analysis were compared with the control group and statistically analyzed (n = 3 and 1000 cells were counted, *p < 0.05, **p < 0.001)

performed the western blot analysis to determine the Bax and Bcl-2 protein levels (Fig. 5B). Following the treatment of 1 and 10 µg/mL ECF, the expression of Bax increased in PC-3 cells, whereas decreased Bax and Bcl-2 protein levels were analyzed in LNCaP cells due to low mRNA levels (Fig. 5B). These findings were consistent with RT-PCR analysis results.

Discussion

In this study, the protein content of ECF (as an NAE) was determined, and its potential cytotoxic and apoptotic effects were revealed in PCa cells in terms of AR status. According to our findings, ECF proteins associated with its anti-cancer effects increased apoptotic cell death in PCa

Table 2 The alkaline comet assay analysis details to determine the DNA damage in PCa cells

	ECF concentration	Tail length (μm) ± SD	P value	Tail momentum ± SD (μm)	P value	Tail DNA (%) ± SD	P value
PC-3	Control	14.39 ± 5.25	–	5.89 ± 3.90	–	29.69 ± 21.90	–
	1 μg/mL	15.66 ± 5.11	0.819	6.68 ± 3.37	0.398	30.52 ± 16.80	0.292
	10 μg/mL	17.34 ± 8.66	0.292	7.41 ± 4.76	0.527	30.78 ± 17.97	0.399
LNCaP	Control	14.81 ± 8.04	–	5.50 ± 4.74	–	23.60 ± 15.42	–
	1 μg/mL	16.20 ± 6.52	0.753	6.11 ± 3.82	0.529	27.24 ± 18.36	0.093
	10 μg/mL	18.05 ± 5.54	0.834	8.25 ± 4.24	0.401	32.19 ± 19.23	0.248

* *Tail DNA%* per cent of DNA in the comet tail to comet head, *Tail moment* tail DNA %×tail length [(per cent of DNA in the tail) × (tail length)], *μm* micrometer, *SD* standard deviation

cells without genotoxic effects. Furthermore, the apoptotic effect of ECF in PCa cells was more profound in AR-insensitive PC-3 cells than in AR-sensitive LNCaP cells.

Different studies have a focused on the identification of various proteins and peptides with antitumor activity in the coelomic fluid due to the necrotic and apoptotic activities of the coelomic fluid of worms and the necessity of being related to the components in the coelomic fluid (Gachet et al. 1999; Kakkar et al. 2004; Klerk et al. 2005; Kobayashi et al. 2006; Chen et al. 2007; DeFeo et al. 2010; Hua et al. 2011; Chan et al. 2012; De Colibus et al. 2012; Hu et al. 2018). With the LC-MS/MS analysis, the presence of components of ECF content associated with its antitumor activity was determined. In our result, higher Lysenin, Lumbrokinase (LK) and translationally controlled tumour protein (TCTP) were found. Lysenin consists of the same protein family as Lysenin-related protein 1 (LRP1) and Lysenin-related protein 2 (LRP-2) and induces erythrocyte lysis and binds to sphingomyelin as a pore-forming toxin (De Colibus et al. 2012). Therefore, Lysenin induces hemolysis and has a toxic effect on cultured mammalian cells such as colon and breast cancer cells (Kobayashi et al. 2006). LK is another enzyme complex with antithrombotic, antioxidant, antitumor and immunomodulatory effects (Hu et al. 2018). In recent years, LK has been investigated as a potential antitumor and anti-metastatic agent (Kakkar et al. 2004; Klerk et al. 2005; DeFeo et al. 2010). LK shows antitumor activity on human hepatoma cells in vitro and in vivo (Chen et al. 2007). TCTP is a highly conserved and ubiquitously expressed protein in all eukaryotes and has very important functions in the cell (Chan et al. 2012). TCTP interacts with microtubules during the G1, S, G2 and early M phases of the cell cycle through binding to the mitotic spindle during mitosis and separates from the spindle during the metaphase-anaphase transition. Microtubule reorganization and growth inhibition are observed when TCTP is overexpressed in bovine mammary epithelial cells (Gachet et al. 1999). Therefore, these proteins could be related to the anti-cancer activity of ECF in cancer cells and PCa cells. However, the role of each protein should be further investigated at the molecular level.

Several in vitro studies show that ECF exerts potential anticancer effects on many cancer types (Hua et al. 2011; Augustine et al. 2017, 2018; Shafi and Faleh 2019). In one study, the antiproliferative effect of ECF from *E. foetida* is detected in KB3-1 and SCC-9 cells (Augustine et al. 2019). Additionally, *Lumbricus Terrestris* earthworm powder exerts cytotoxic effects against breast and PCa cells (Shafi and Faleh 2019). Our results demonstrated that ECF reduced PC-3 cell viability more than LNCaP cells, with a low cytotoxic effect on HUVEC cells. Furthermore, ECF obtained from *E. foetida* exerts an apoptotic induction effect by increasing the percentage of early apoptotic cells in HeLa and SCC-9 cells (Yanqin et al. 2007; Augustine et al. 2019). In our study, ECF caused especially early apoptosis in PCa cells, consistent with the literature and apoptotic changes of the cell morphology were more profound in PC-3 cells than in LNCaP cells.

In addition to the existing literature data, the apoptotic effect of ECF was also supported by gene and protein expression levels of Bax and Bcl-2. In our study, ECF increased apoptotic cell death by the over-expression of the Bax gene and protein levels and the down-regulation of Bcl-2 in only PC-3 cells. However, there was a slight increase in the Bax mRNA level in LNCaP cells. Therefore, the underlying molecular mechanisms of ECF induced apoptotic death should be further investigated. Particularly, cleaved and total caspase levels, as well as other pro-and anti-apoptotic Bcl-2 family members, are analyzed at both mRNA and protein levels. Finally, the effect of AR status on the effectiveness of ECF is further analyzed through AR associated signalling pathways.

The disadvantages of current treatment methods used in cancer treatment (toxic effects, serious side effects, lack of cell specificity, etc.) lead to the investigation of the effects of natural components as an alternative treatment strategy in cancer treatment (Yuksel et al. 2022). Therefore, our results will specifically support the pharmacological development of ECF as a potential therapeutic molecule with fewer side effects in the treatment of PCa in the future.

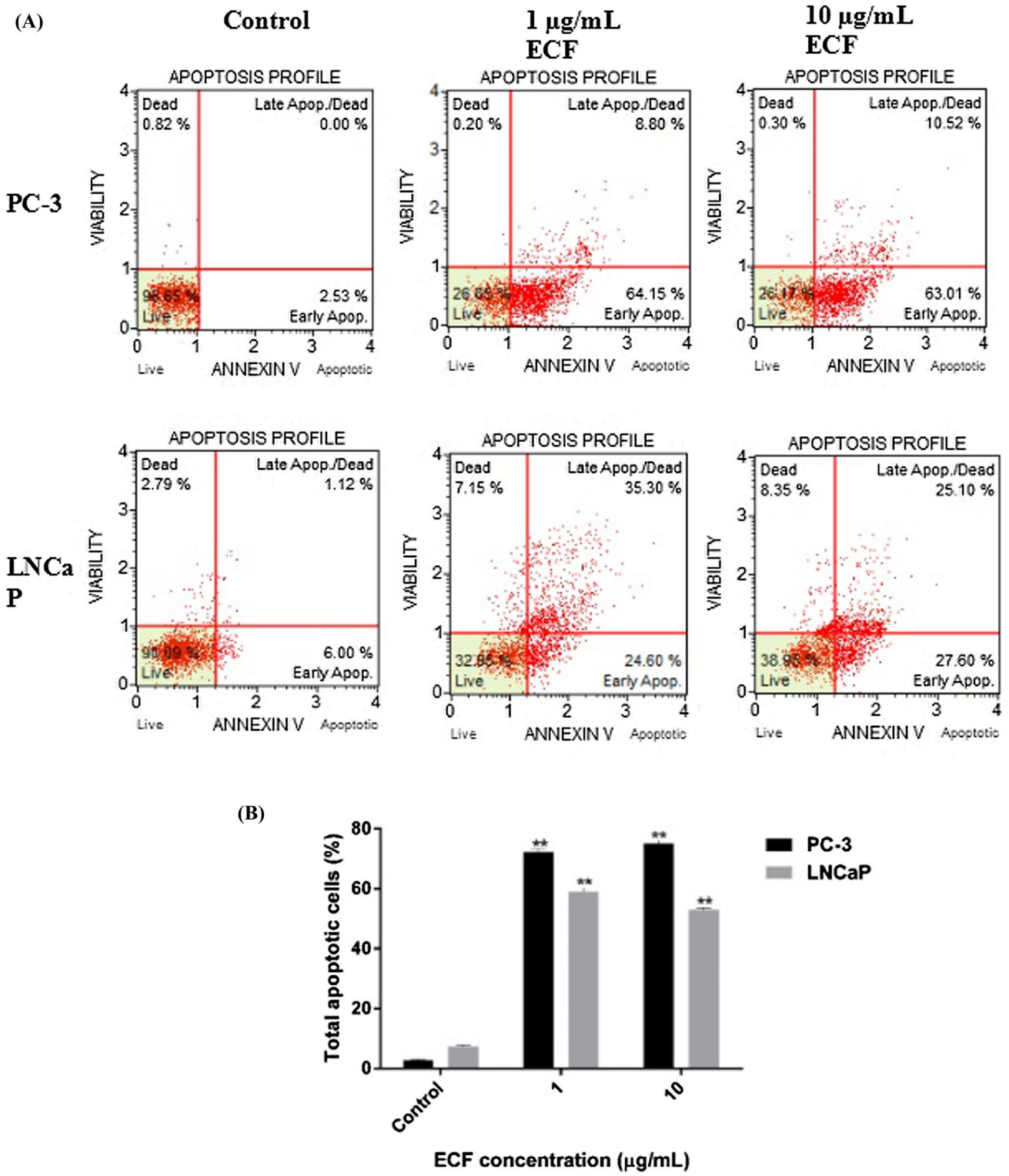


Fig. 3 Determination of the apoptotic effect of ECF in PC-3 and LNCaP cells. **A** The apoptotic effect of ECF (1 and 10 µg) was determined by Annexin V analysis. **B** The total apoptotic effect of ECF was compared with the control group and statistically analyzed (*$p < 0.05$, **$p < 0.01$)

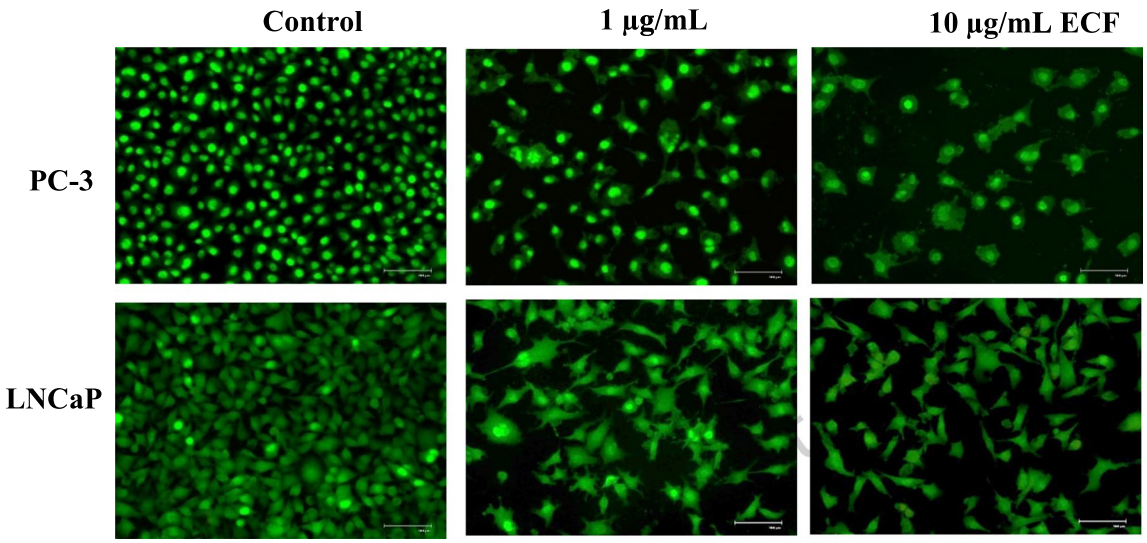
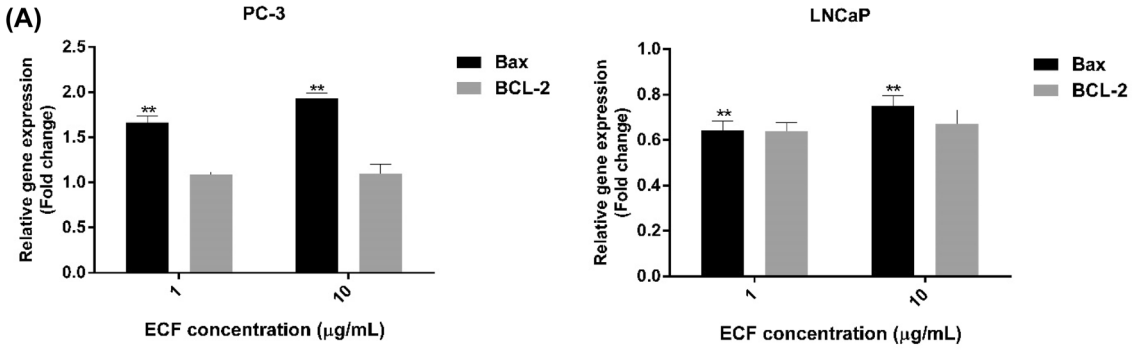


Fig. 4 Imaging the apoptotic effect of ECF in PC-3 and LNCaP cells by acridine orange (AO) staining. Morphological changes in PCa cells treated with ECF (1 and 10 µg) were visualized



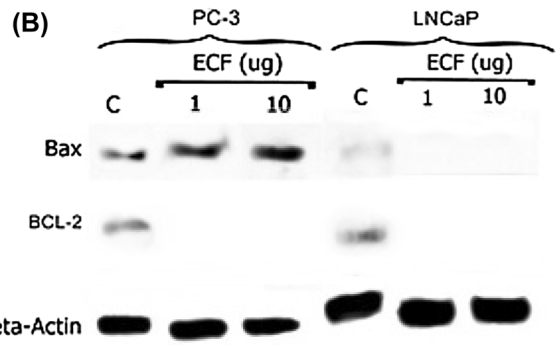


Fig. 5 The change in Bax and Bcl-2 gene and protein expression levels in PCa cells after ECF treatment at different concentrations was determined by RT-PCR and Western Blot analysis. **A** The fold change in gene expression levels of Bax and Bcl-2 for 24 h ECF treatment in PC-3 and LNCaP cells were statistically analyzed. **B** The fold change in protein expression levels of Bax and Bcl-2 was statistically analyzed (*$p < 0.05$, **$p < 0.01$)

Conclusions

ECF has a potential anticancer effect on PCa cells, especially AR-insensitive cells. However, the molecular effects of ECF on cancer cells and its effects on signalling pathways are largely unknown and need to be investigated. In addition, the anti-metastatic effect of ECF in different tumours will evaluate due to its content. In this context, further studies should be carried out to investigate the anti-metastatic effect, especially based on the proteins found in ECF.

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Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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