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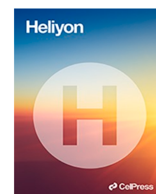

Research article

Fatty acid composition and anti-cancer activity of essential oil from *Tenebrio molitor* larvae in combination with zoledronic acid on prostate cancer

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ABSTRACT

The essential oil extracted from *Tenebrio molitor* larvae (EOTM) is a natural product containing trace elements with potential therapeutic properties. This study aimed to assess the anticancer effects of EOTM and its synergistic interactions with zoledronic acid, a bisphosphonate drug, on prostate cancer cell lines. The chemical composition of EOTM was analyzed using GC-MS revealing a high concentration of fatty acids. The cytotoxicity of EOTM, both as a standalone treatment and in combination with zoledronic acid, was evaluated on prostate cancer cell lines (LNCaP, PC3) and normal hSKM using MTT assays. Results demonstrated that EOTM exhibited selective toxicity, inhibiting the growth of cancer cells in a dose-dependent manner while sparing normal cells. Morphological assessments and gene expression analyses of BCL2 and BAX were conducted through microscopy, Western blotting, and real-time RT-qPCR. These analyses indicated that EOTM induced apoptosis in cancer cells, as evidenced by cellular shrinkage, membrane blebbing, and nuclear fragmentation. Western blot results showed that EOTM downregulated the anti-apoptotic protein BCL2 and upregulated the proapoptotic protein BAX, suggesting activation of apoptosis pathways. Additionally, the combination of EOTM with zoledronic acid amplified these effects. Hoechst 33258 staining further confirmed the purity of cells following treatment. In conclusion, EOTM exhibits strong anticancer properties by inducing apoptosis in prostate cancer cells and demonstrates synergistic potential when combined with zoledronic acid. These findings warrant further investigation of EOTM as a natural and effective cancer treatment option.

1. Introduction

Cancer remains a significant global health challenge, and there is growing interest in exploring natural compounds as potential anticancer treatments [1]. In recent years, insect larvae have emerged as a novel source of bioactive compounds with therapeutic potential [2]. Among these, *Tenebrio molitor* (TM), known as the mealworm, has garnered attention due to its traditional medicinal use and documented anticancer properties [3].

Insects, which comprise the majority of animal species on Earth, contain a diverse array of bioactive compounds that are often

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overlooked. These compounds, including anticancer agents, may have originally derived from plants and undergone transformative processes within insects. In some regions, TM larvae, cultivated for human consumption, have been traditionally used in Asian medicine to treat various diseases, including cancer [4].

Mealworms, the larvae of TM, are not only a nutritional food source but also a potential reservoir of bioactive compounds [5]. Previous studies suggested the presence of anticancer agents within TM, with its fatty acid composition potentially responsible for its anticancer activity [5–8]. In oncology, searching for novel and less harmful cancer treatments is unrelenting. Prostate cancer, one of the most common male malignancies, has seen significant advancements in both diagnosis and treatment. The treatment landscape ranges from traditional surgery and radiation therapy to immunotherapies and advanced hormonal. However, there remains a need to enhance treatments at different stages of prostate cancer, often by combining radiotherapy with hormonal or drug therapies for improved outcomes [9]. The emerging field of precision oncology, which leverages genetic profiling to tailor treatments, shows great promise. Additionally, understanding the role of the gut microbiome in treatment resistance and the development of new immunotherapies, such as CAR-T cell therapy, opens new avenues in patient care. Advanced diagnostic imaging technologies, mainly 18F-DCFPyL PET/CT, combined with machine learning, are revolutionizing tumor monitoring by providing non-invasive and highly accurate diagnostic capabilities. Moreover, artificial intelligence-driven identification of prostate cancer subtypes is paving the way for more personalized treatment strategies. The development of PSMA-targeting drugs and radiotheranostics represents a new frontier in managing advanced prostate cancer, offering greater precision in both detection and treatment. These innovations, along with ongoing clinical trials exploring the therapeutic potential of compounds such as tocotrienols, signal a new era in cancer treatment focused on efficacy and patient safety [10–12]. Investigating the anticancer potential of oil from TM larvae, which is rich in fatty acids such as palmitoleic, oleic, and linoleic acids, aligns with this innovative approach. These unsaturated fatty acids have been linked to transforming various cancer cell types into less aggressive forms, suggesting a new strategy for differentiation therapy in cancer treatment [13–15]. Despite the promising effects of these fatty acids on cancer cells, our understanding of their biological mechanisms remains incomplete. The exact processes by which ω-fatty acids influence cancer cell behavior are not fully understood, and personalized fatty acid profiling in clinical trials, critical for validating the efficacy of fatty acid-based treatments, has not yet become standard practice. Our study aims to address these knowledge gaps by elucidating the pathways involved and evaluating the effects of customized fatty acid interventions on cancer cell behavior. The therapeutic potential of insects, including TM, represents an emerging area of research. The growing interest in insect-based foods and bioactive compounds indicates that insects could play a significant role in sustainable food production and medical applications in the future. This study provides an early exploration of TM oil as a natural antitumor agent, focusing on prostate cancer, with the ultimate goal of developing insect-derived chemotherapeutic agents that offer safe and effective cancer treatments. In addition, zoledronic acid, a bisphosphonate drug, has shown promise in treating bone complications in men with prostate cancer that has metastasized to the bones. It also exhibits potential anticancer effects in prostate cancer cells by inducing apoptosis and inhibiting cell proliferation [16]. Recent studies emphasize the importance of specific proteins and natural compounds in cancer prognosis and treatment. Proteomics research is critical in understanding prostate cancer and identifying biomarkers for better disease management. Natural compounds like tocotrienols target specific cancer pathways and offer promising treatment options. For example, Anwaier highlighted the significance of biomarkers such as RAD50 and SMAD4 in predicting prostate cancer outcomes, underscoring the role of proteomics and natural compounds in advancing cancer therapy [17]. Similarly, Younes reviewed the potential benefits of tocotrienols, a form of vitamin E found in sources like rice bran and palm oil, in slowing the progression of various cancers, including prostate cancer, by targeting vital cellular processes such as proliferation and apoptosis [18].

Together, these studies emphasize the potential of integrating molecular and natural compound research to enhance cancer treatments. Our research investigates the antiproliferative and apoptotic effects of essential oil derived from TM larvae on prostate cancer cells, compared to normal cells, and its synergistic effect when combined with zoledronic acid. Given the high prevalence of prostate cancer among men and the need for treatments with fewer side effects, our work aims to contribute to the development of complementary or alternative treatments alongside traditional therapies.

2. Material and method

2.1. Preparation of Tenebrio molitor essential oil

The extraction and purification process of *Tenebrio molitor* oil is a detailed and precise operation designed to yield oil of the utmost purity. The process begins with the careful selection of *Tenebrio molitor* larvae, which are then fed a diet of wheat bran to ensure they are well-nourished and primed for oil production. Once they have reached optimal growth, the larvae undergo a dehydration process at a controlled temperature of 60 °C. This is a critical step as it ensures that the larvae are sufficiently dry and brittle, making them ideal for subsequent grinding into a fine powder. This powder is then placed into a Soxhlet extractor, where hexane (Merck, Germany), a non-polar solvent known for its ability to dissolve lipophilic substances effectively, is used to extract the oil. The Soxhlet extraction process is thorough, allowing the hexane to repeatedly wash over the powdered larvae to dissolve the oil completely. This cycle is repeated until the hexane solvent no longer takes on any color from the oil, indicating that the extraction is complete [19].

Following extraction, the hexane is carefully removed using a rotary evaporator. This step is crucial as it ensures that all solvent residues are eliminated, leaving behind the pure, yellowish oil of *Tenebrio molitor*. To verify the purity and determine the composition of the extracted oil, it is analyzed using gas chromatography-mass spectrometry (GC-MS). This analytical technique provides a comprehensive profile of the oil's physicochemical properties and its fatty acid composition, confirming the oil's suitability for research and potential applications. The meticulous nature of this extraction process underscores the commitment to quality and the importance of precision in obtaining a high-purity product for study and application.

2.2. GC/MS analysis

An Agilent Technologies GC-MS (GC 7890 and MSD 5975C) (Agilent Technologies, Santa Clara, CA, USA) used to examine the essential oil of *Tenebrio molitor*. 1 μL of the sample injected into a HP-5MS column (30 m × 0.25 mm × 0.25 μm) with a 10:1 split ratio. The carrier gas was helium at 1 mL/min. The temperature program was: 60 °C for 2 min, then 3 °C/min to 240 °C, and hold for 10 min. The injector and detector were both at 250 °C. The mass spectrometer used electron ionization at 70 eV and scanned from 50 to 550 *m/z*. We identified the compounds by comparing their retention times and mass spectra with standards and the NIST library. The relative percentage of each compound calculated using the normalization method [20].

2.3. Cell culture

The LNCaP and PC3 prostate cell lines and the hSKM fibroblast cell line got from Pasteur Institute of Iran. The prostate cell lines cultured in RPMI-1640 medium (Thermo Fisher Scientific Inc, USA) with 20 % FBS (Thermo Fisher Scientific, USA), 100 U/mL streptomycin (Sigma-Aldrich, USA), and 100 U/mL penicillin (Sigma-Aldrich, USA). The hSKM cell line cultured in DMEM (Thermo Fisher Scientific Inc, USA) medium with 10 % FBS, 100 U/mL streptomycin, and 100 U/mL penicillin.

The cells kept in flasks in an incubator at 37 °C with 5 % CO2 and humidity. When it was needed to collect the cells for experiments or analysis, trypsin-EDTA used to detach them from the flasks. Then, the cells washed with PBS to remove the trypsin-EDTA and get them ready for further steps.

2.4. MTT assay

To assess the cytotoxic and synergistic effects of EOTM on PC3 and LNCaP cell lines, a triplicate MTT assay was conducted. The assay was carried out using the following steps.

- Cell seeding: PC3 and LNCaP cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h at 37 °C with 5 % CO2.
- Treatment: The cells were treated with different concentrations (0, 0.5, 0.75, and 1 %) of EOTM [19]. The treatments included EOTM alone or in combination with ZA at concentrations of 0, 5, 10, and 20 μM [21]. The concentrations of TM oil and zoledronic acid used in our treatment assays were carefully selected based on a thorough review of the literature and preliminary dose-response experiments. For TM oil, we considered the bioactive fatty acid content and their solubility, ensuring that the chosen concentration is effective in inducing the desired cellular response without cytotoxicity. Zoledronic acid's concentration was informed by its established pharmacological profile, with adjustments made to suit our specific cell models and experimental conditions. This meticulous selection process is supported by data from pilot studies, which confirmed the therapeutic potential of these concentrations while maintaining cellular viability in comparison to the normal cells. The cells were incubated with the treatments for an additional 24 h.
- MTT assay: After the treatment period, 20 μL of MTT solution (CinaColon, Tehran, Iran) (5 mg/mL) was added to each well, and the plates were incubated for 4 h.
- Formazan crystal dissolution: The supernatant was carefully removed, and 150 μL of dimethyl sulfoxide (DMSO) (CinaColon, Tehran, Iran) was added to dissolve the formazan crystals formed by viable cells.
- Absorbance measurement: The absorbance of the formazan solution was measured at 570 nm using a microplate reader (BioTech, USA).
- Calculation of cell viability: The cell viability was calculated as the percentage of absorbance relative to the control group.
- Determination of IC50: The percentage of inhibition of cell viability was determined, and the concentration of EOTM that inhibited 50 % of cell viability (IC50) was calculated.

The results of the MTT assay provided information on the cytotoxic effects of EOTM on PC3 and LNCaP cell lines, both alone and in combination with zoledronic acid. The IC50 value indicated the concentration of EOTM required achieving a 50 % reduction in cell viability.

Table 1
The sequence of the primer used in the present study.

Primer	Tm	sequence	Product size (bp)
β- ACTIN	62	F:GGACATCCGCAAAGACCTGTA R:ACATCTGCTGGAAGGTGGACA	189
BCL2	61	F:GTGGATGACTGAGTACCTGA R:AGCCAGGAGAAATCAAACAGA	119
BAX	61	F:TTTGCTTCAGGGTTTCATCC R:CAGCTCCATGTTACTGTCCA	154

2.5. Real Time RT-qPCR

To analyze the expression levels of the *BAX* and *BCL2* genes, real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed. Total RNA extraction was carried out using RNX-plus™ Reagent (Cinnaclon, Iran), followed by cDNA synthesis from 1 μg of total RNA using M-MuLV-RT and random hexamer primers (Thermo Fisher Scientifc Inc.USA). To verify the absence of contamination, we included a negative control without cDNA. The reaction setup involved combining the Master Mix, primers, and water in a PCR tube, then adding the cDNA. Amplification was performed using primer sequences from Table 1 and the SYBR Green Takara Master Kit (Japan). We normalized the initial sample concentration to β-actin and adhered to precise thermal cycling conditions: 95 °C for 30 s for initial denaturation, followed by 45 cycles of 95 °C for 5 s, annealing at 61–62 °C for 30 s, and extension at 72 °C for 30 s. The SYBR Green PCR Master Mix, which includes SYBR Green I Dye, DNA Polymerase, dNTPs, Passive Reference, and optimized buffer components, made up 12.5 μL of the 25 μL reaction volume. Primer concentrations were carefully set between 0.3 and 0.5 μM. The cDNA template was added in volumes ranging from 1 to 5 μL, based on the sample and gene of interest, with Nuclease-Free Water used to adjust the total volume. After mixing and centrifugation, the tubes were placed in the Rotor-Gene 3000 (Corbett Research, Australia) to proceed with the specified cycling conditions, thus ensuring the accuracy of our gene expression analysis.

The obtained Ct values for *BAX* and *BCL2* were then used to determine the relative expression levels of these genes. This was achieved by normalizing the Ct values of the target genes to an internal control gene or a reference sample. Mathematical algorithms such as the comparative Ct method ($2^{-\Delta\Delta Ct}$) can be employed to calculate the relative expression levels.

By comparing the relative expression levels of *BAX* and *BCL2*, valuable insights can be gained regarding the balance between pro-apoptotic and anti-apoptotic pathways. This information contributes to the understanding of cell death and survival mechanisms in the studied system.

2.6. Hoechst 33258 nuclear staining assay

Cells stained with Hoechst 33258 (Sigma-Aldrich, USA) to see changes in their nuclei and apoptotic bodies. We followed the method by Kasibhatla et al. (2006) with some changes [22]. 1×10^4 cells/well put in 96-well plates and left them for 24 h. TM oil added at 0 %, 0.5 %, 0.75 %, and 1 % to the cells for 24, 48, and 72 h. The plates spun at 1000 rpm for 5 min and added 1 μL of Hoechst 33258 (1 mg/mL in PBS) to each well. We waited for 10 min and looked at the cells under a fluorescent microscope (Zeiss, Oberkochen, Germany) to find any contamination, nuclear changes, and apoptotic bodies.

2.7. Protein extraction and western blot analysis

Protein extraction was performed on ice using TNE buffer (10 mM Tris-HCl, pH 7.8, 1 % NP-40, 0.1 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml aprotinin, leupeptin, pepstatin, 1 mM Na3VO4, and NaF) with a protease inhibitor cocktail. Each 30 μl protein sample was heat-denatured at 100 °C for 5 min in loading buffer (6.25 mM Tris, pH 6.8, 12.5 % glycerol, 2.5 % SDS, 0.025 % bromophenol blue, 5 % β-mercaptoethanol), then separated via 15 % SDS-PAGE, loading 10 mg of protein per lane. Proteins were transferred to a nitrocellulose membrane at 4 °C using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA). The membrane was treated with TBST buffer (50 mM Tris, 200 mM NaCl, 0.1 % Tween-20, pH 7.4), blocked with TBST and 5 % nonfat dry milk for 30 min at room temperature, and incubated overnight at 4 °C with BAX and BCL2 antibodies (Sigma-Aldrich, USA) at a 1:100 dilution. After three washes with TBST, the membrane was incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies at a 1:500 dilution. Finally, the membrane was treated with enhanced chemiluminescence solutions (Santa Cruz Biotechnology, USA) and exposed to Kodak X-ray film (Kodak, USA) for protein band detection. All reagents and chemicals used in the Western blot were obtained from Sigma-Aldrich (USA).

2.8. Statistical analysis

Uniformity was ensured in the statistical analysis by conducting all experiments in triplicate, except for specified instances. Findings were reported as the mean ± standard deviation. General Linear Model (GLM) analysis, followed by Duncan's multiple range test, was applied for mean comparison, with a significance level set a $p < 0.05$. SAS version 9.4 served as the statistical software for these analyses. Additionally, Western blot band intensities were quantified through densitometry and normalized against β-actin protein levels. The data are presented as means ± SD from triplicate experiments, with significance indicated by $p < 0.05$. Relative gene expression was calculated using the ΔΔCT method, defined as ΔCT of the target sample minus ΔCT of the reference sample, and expressed as fold change normalized to a reference gene. This methodical approach facilitated a thorough analysis of TM oil and zoledronic acid's effects on cell growth, enabling a clear interpretation and discussion of the outcomes.

3. Results

3.1. GC/MS analysis

The GC-MS analysis of EOTM larvae identified 46 compounds (Table 2). The most abundant compounds, based on relative area, include palmitic acid (PA) (13.79 %), oleic acid (OA) (9.70 %), linoleic acid (LA) (8.24 %), and octanoic acid decyl ester (ODE) (8.56

%). PA is a saturated fatty acid (SFA), while OA and LA are unsaturated fatty acids (UFAs) with different carbon chain lengths and double bond positions. These fatty acids contribute to energy production, membrane structure, cardiovascular health, cholesterol regulation, skin health, and immune function. ODE is an ester formed by the reaction of octanoic acid and decyl alcohol. The peak results from the GC-MS analysis provide valuable information about the composition of EOTM (Fig. 1).

3.2. Effect of EOTM on cells growth

The effects of EOTM on tumor cell growth were investigated. It was found that EOTM significantly inhibited the growth of LNCAP and PC3 tumor cells ($P < 0.01$), in a concentration- and time-dependent manner, while having no effect on normal L929 cells. The maximum inhibition rate on LNCaP cells was 60 ± 0.98 % after 48 h of treatment with 1 % EOTM, and 45 ± 1.05 % after 24 h. On the other hand, the inhibition rate on LNCaP cells was 60 ± 2.17 % after 48 h of treatment with 1 % EOTM, which was lower compared to the inhibition rate on PC3 cells, which was 59 ± 0.65 % after 48 h of treatment with 1 % EOTM. (Table-3).

The IC50 values of EOTM on LNCAP and PC3 cells after 48 h of treatment were 63 % and 79 %, respectively. These findings suggest that EOTM exhibits selective inhibitory effects on tumor cell growth, particularly on LNCAP cells (Fig. 2) (Fig. 3).

The concentration of EOTM required to affect SKM cells is higher than 100 %, indicating a low toxicity profile for non-cancerous cells. The lower IC50 value for LNCaP cells suggests that they are more sensitive to EOTM treatment compared to PC3 cells. The fact

Table 2
Chemical composition of *Tenebrio molitor* essential oil.

No.	Peak Name	Retention Time	Area	Height	Relative Area	Relative Height	Plates (EP)
		Min	pA*min	pA	%	%	
1	Acetamide, TMS derivative	10.072	0.619	14.084	1.25	1.22	387536
2	Ethanamine, 2TMS derivative	10.809	0.452	12.288	0.91	1.07	531611
3	4-Methylethcathinone, N-trimethylsilyl-	10.952	0.833	20.805	1.68	1.81	488695
4	Propylene glycol, 2TMS derivative	11.782	0.083	1.997	0.17	0.17	488221
5	Propanoic acid, 2,3-dihydroxy-	11.993	0.077	1.960	0.15	0.17	608353
6	Tris(trimethylsilyl)carbamate	12.420	0.069	2.029	0.14	0.18	807180
7	1,3-Propanediol, 2TMS derivative	12.842	0.061	1.570	0.12	0.14	693853
8	Lactic Acid, 2TMS derivative	12.946	0.561	16.176	1.13	1.41	888738
9	L-Alanine, 2TMS derivative	13.750	0.751	22.746	1.51	1.98	1128951
10	Formamide, TMS derivative	14.038	0.054	1.566	0.11	0.14	1183703
11	Hydracrylic acid, 2TMS derivative	14.476	0.058	1.450	0.12	0.13	956338
12	L-Isoleucine, TMS derivative	15.112	0.079	1.383	0.16	0.12	510429
13	1-Propanol, 3-(octadecyloxy)-	15.424	0.063	1.939	0.13	0.17	1404792
14	L-Valine, 2TMS derivative	15.812	0.578	18.172	1.16	1.58	1589914
15	Octanoic acid, TMS derivative	16.521	0.542	16.559	1.09	1.44	1666891
16	L-Leucine, 2TMS derivative	16.789	1.632	33.165	3.29	2.88	594936
17	Silanol, trimethyl-, phosphate	16.895	0.812	26.145	1.63	2.27	1975198
18	L-Isoleucine, 2TMS derivative	17.141	0.364	11.290	0.73	0.98	1893481
19	L-Proline, 2TMS derivative	17.233	1.426	42.773	2.87	3.72	1746519
20	Butanedioic acid, 2TMS derivative	17.378	0.098	3.162	0.20	0.28	2020772
21	L-Threonine, 3TMS derivative	18.664	0.101	3.363	0.20	0.29	2234634
22	L-Threonine, 3TMS derivative	18.709	0.098	3.029	0.20	0.26	1775169
23	Decanoic acid, TMS derivative	19.594	0.129	4.037	0.26	0.35	2556921
24	L-5-Oxoproline, 2TMS derivative	20.751	0.254	7.572	0.51	0.66	2570457
25	2-Hexadecanol	22.858	0.189	5.782	0.38	0.50	3326844
26	a-D-Glucopyranoside, methyl 2-(acetylamino)-	23.782	0.156	3.323	0.31	0.29	2380479
27	9,12-Octadecadienoyl chloride, (Z,Z)-	24.981	0.327	9.877	0.66	0.86	3660160
28	E−2-Octadecadecen-1-ol	25.049	0.522	15.128	1.05	1.32	3728702
n.a.	2,2-Dimethyl-6-methylene-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
29	Palmitic acid	26.379	6.848	92.297	13.79	8.03	832949
30	Octanoic acid, decyl ester	26.497	4.253	116.494	8.56	10.13	3734778
31	Isopropyl palmitate	26.666	0.630	19.500	1.27	1.70	4589748
32	Hexadecanoic acid, trimethylsilyl ester	27.284	3.597	104.000	7.24	9.04	4192711
33	Cyclopropaneoctanoic acid, 2	27.803	0.402	13.380	0.81	1.16	5032894
34	Linoleic acid	28.381	4.093	50.403	8.24	4.38	708091
35	Linoleic acid, TMS	29.151	3.221	86.831	6.48	7.55	n.a.
36	Oleic Acid, (Z)-, TMS derivative	29.199	4.817	136.445	9.70	11.87	3712559
37	Stearic acid, TMS derivative	29.415	1.716	50.876	3.45	4.42	5029236
38	2-Bromotetradecanoic acid	30.726	0.165	4.047	0.33	0.35	4272026
39	Pentacosane	31.945	0.718	23.109	1.45	2.01	6687723
40	Bis(2-ethylhexyl) phthalate	32.578	1.399	39.876	2.82	3.47	5364268
41	1-Monopalmitin, 2TMS derivative	32.945	0.640	16.184	1.29	1.41	4585507
42	Eicosane	34.110	0.754	18.247	1.52	1.59	4268380
43	monoolein TMS	35.096	2.616	29.019	5.27	2.52	870905
44	Glycerol monostearate, 2TMS derivative	35.338	0.164	3.339	0.33	0.29	3469547
45	Glycerol tricaprylate	38.018	1.776	30.766	3.58	2.68	n.a.
46		38.135	0.877	11.658	1.77	1.01	2090491

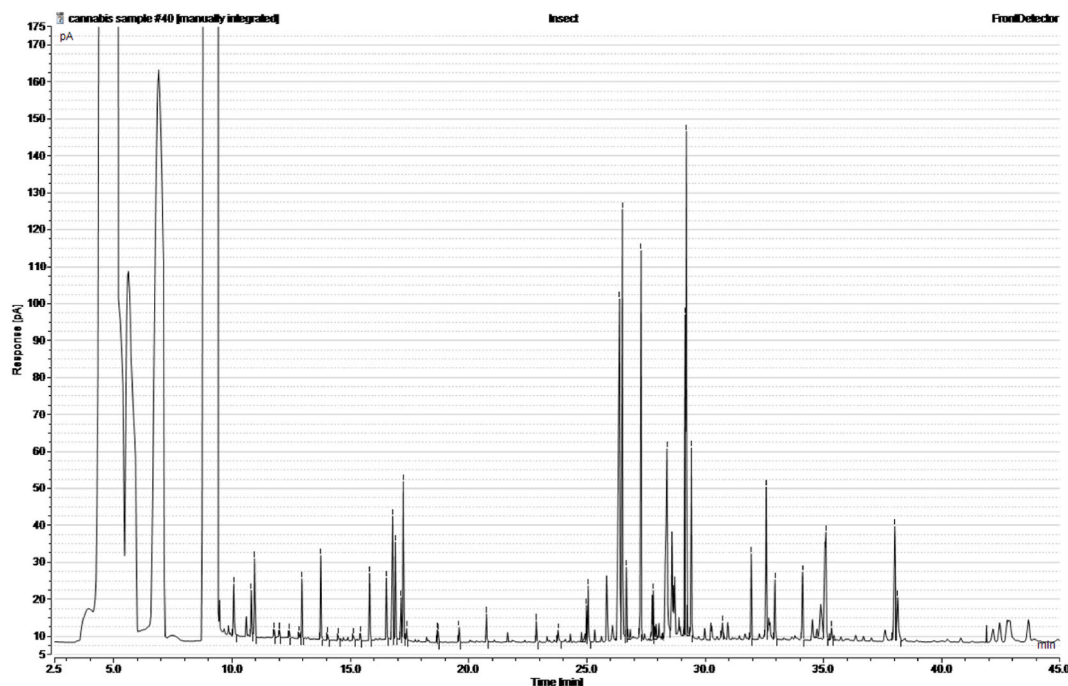

Fig. 1. GC-MS chromatogram of essential oil of *Tenebrio molitor*.

that EOTM did not affect SKM cells indicates a selective toxicity towards tumor cells, which is a desirable characteristic in cancer treatment. Based on the data provided, EOTM alone at a concentration of 1 % for 48 h shows a higher inhibition rate on LNCaP cells compared to PC3 cells. This suggests that EOTM has a slightly more pronounced mortality effect on LNCaP cells than on PC3 cells under these specific conditions.

3.3. Effect of EOTM on morphological changes in cells

The effect of EOTM on morphological changes in cells was investigated. EOTM contains various fatty acids and bioactive compounds that may exert antiproliferative and proapoptotic effects on cancer cells. Specifically, EOTM treatment induced morphological changes in LNCaP and PC3 prostate cancer cell lines, including cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and cytoplasmic vacuolization. These observations suggest that EOTM may trigger apoptotic and necrotic processes in these cancer cells. The exact mechanisms underlying these morphological changes are still being explored, but it is hypothesized that the fatty acids present in EOTM may interact with cellular pathways and metabolic processes, potentially influencing cell survival and proliferation. Further research is warranted to fully elucidate the impact of EOTM on cellular morphology and its potential implications for cancer treatment.

3.4. Western blot analysis

Western blot analysis substantiated alterations in BAX and BCL2 proteins post-treatment, showing an increase in BAX levels ($p < 0.05$) and a decrease in BCL2 levels ($p < 0.05$), leading to an elevated BAX/BCL2 ratio. The intensity of the bands was quantified using densitometry, with values normalized to *β-ACTIN*. Densitometric analysis was performed using ImageQuant TL software (GE Healthcare). Reflectance densitometry was used to assess the Western blot strips, and the antibody responses to BAX and BCL2 were quantified, based on the band intensities ($p < 0.05$). The data are presented as means ± standard error (SE), and the displayed Western blots represent three independent experiments (Figure-4).

3.5. Real Time RT-qPCR

This study also investigated the effects of EOTM and ZA, a bisphosphonate, on the expression of *BCL2* and *BAX* genes in prostate cancer cell lines. *BCL2* and *BAX* are critical regulators of apoptosis, determining whether cells survive or undergo programmed cell death. RNA samples were extracted from both treated and untreated cells, and their quality and quantity were assessed via spectrophotometry and agarose gel electrophoresis. Real-time RT-qPCR was conducted to measure the samples' *BCL2* and *BAX* gene expression levels.

The results of the study demonstrated that both EOTM and zoledronic acid led to a decrease in the expression of the *BCL2* gene,

Table 3
Comparative analysis of EOTM and ZA inhibition effects on tumor cell lines.

Treatment	Concentration of EOTM (%)	Concentration of ZA (μM)	Time (hours)	Effect on SKM Cells (%)	Effect on PC3 Cells (%)	Effect on LNCaP Cells (%)
EOTM Alone	0.5	0	24	5	25	22
EOTM Alone	0.5	0	48	10	40	37
EOTM Alone	0.75	0	24	8	31	24
EOTM Alone	0.75	0	48	15	45	40
EOTM Alone	1	0	24	12	50	45
EOTM Alone	1	0	48	20	59	60
ZA Alone	0	5	24	3	20	16
ZA Alone	0	5	48	5	35	30
ZA Alone	0	10	24	6	41	35
ZA Alone	0	11	48	10	51	51
ZA Alone	0	20	24	8	45	39
ZA Alone	0	22	48	15	54	56
EOTM + ZA	0.5	5	24	6	43	41
EOTM + ZA	0.5	5	48	12	57	54
EOTM + ZA	0.5	10	24	7	43	43
EOTM + ZA	0.5	10	48	13	58	55
EOTM + ZA	0.5	20	24	7	45	43
EOTM + ZA	0.5	20	48	15	59	57
EOTM + ZA	0.75	5	24	9	45	46
EOTM + ZA	0.75	5	48	16	59	60
EOTM + ZA	0.75	11	24	11	47	49
EOTM + ZA	0.75	10	48	18	60	64
EOTM + ZA	0.75	21	24	11	48	51
EOTM + ZA	0.75	20	48	19	60	66
EOTM + ZA	1	6	24	14	48	56
EOTM + ZA	1	6	48	21	61	67
EOTM + ZA	1	11	24	16	50	61
EOTM + ZA	1	10	48	24	62	73
EOTM + ZA	1	21	24	18	61	70
EOTM + ZA	1	22	48	27	63	81

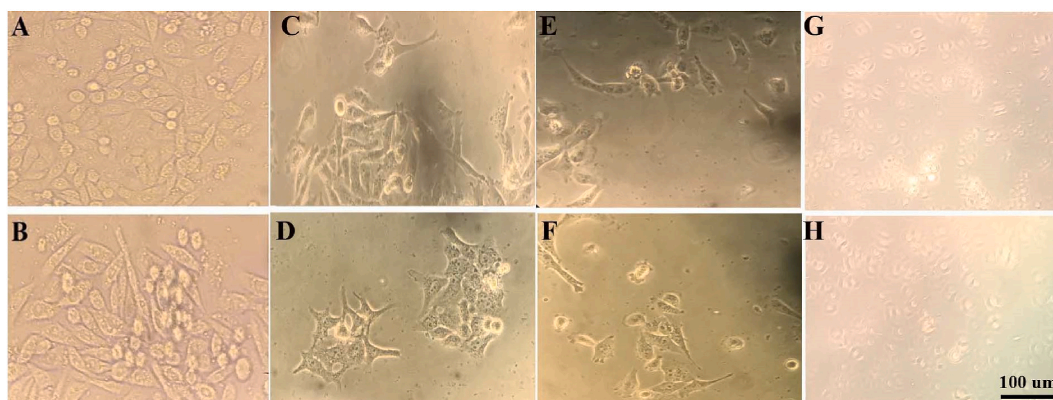

Fig. 2. Effect of EOTM and zoledronic acid on PC3 (First line) and LNCAP (Second line) cell lines **A, B** control (No Treat) **C, D** Cells were treated with 20 μM zoledronic acid, **E, F** cells were treated with 1 % EOTM, G, H Cells were treated with 20 μM zoledronic acid in combination with 1 % EOTM.

which inhibits apoptosis, and an increase in the expression of the *BAX* gene, which promotes apoptosis. Interestingly, the combination of EOTM and zoledronic acid displayed more effective than zoledronic acid alone. Consequently, the researchers concluded that both EOTM and zoledronic acid possess anti-cancer properties by modulating the *BCL2/BAX* ratio in favor of apoptosis ($p < 0.05$). These findings highlight the potential of EOTM as a therapeutic agent in prostate cancer treatment, potentially enhancing the efficacy of existing treatments such as zoledronic acid (Figure- 5).

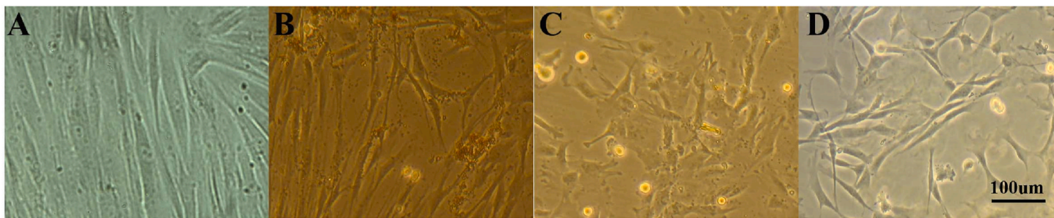

Fig. 3. Effect of EOTM and zoledronic acid on hSKM cell line **A** control (No Treat), **B:** Cells were treated with 20 μM zoledronic acid, **C:** cells were treated with 1 % EOTM, **D:** Cells were treated with 20 μM zoledronic acid in combination with 1 % EOTM.

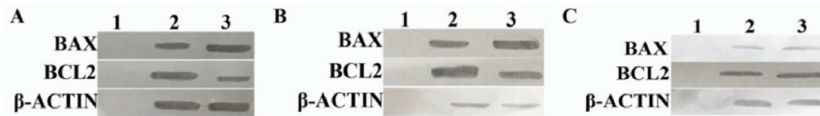

Fig. 4. Comparative Western blot analysis demonstrating the expression levels of BAX and BCL2 proteins in cells subjected to treatment versus untreated control cells.

3.6. Hoechst 33258 nuclear staining assay

Hoechst 33258 nuclear staining was performed to assess the presence of contamination in cells treated with EOTM. This assay explicitly targets cell nuclei, allowing for the visualization and detection of potential contaminants.

The results of the Hoechst 33258 nuclear staining assay indicated that there was no contamination observed in the cells treated with EOTM. The staining of the nuclei appeared normal and consistent with the expected characteristics of the treated cells. This finding suggests that the cells used in the study remained free from any external contaminants during the treatment process with EOTM (Fig. 6).

The absence of contamination in the treated cells is critical to ensure the reliability and validity of the study results. This confirms that the observed effects or changes in the cells can be attributed directly to EOTM treatment without interference from external factors. This strengthens the confidence in the study's conclusions regarding the effects of EOTM on the cells.

4. Discussion

Previous studies have demonstrated that the fatty acids present in EOTM may exert differential effects on LNCaP and PC3 cells based on their androgen receptor (AR) status and sensitivity to fatty acids [23]. For example, palmitic acid (PA), the most abundant fatty acid in EOTM, induced apoptosis in LNCaP cells but not in PC3 cells likely through the modulation of AR signaling and oxidative stress [24]. Oleic acid (OA), another major fatty acid in EOTM, inhibited proliferation and migration in LNCaP cells but not PC3 cells by downregulating cyclin D1 and MMP-9 expression. These findings suggest that EOTM may have more potent cytotoxic effects on LNCaP

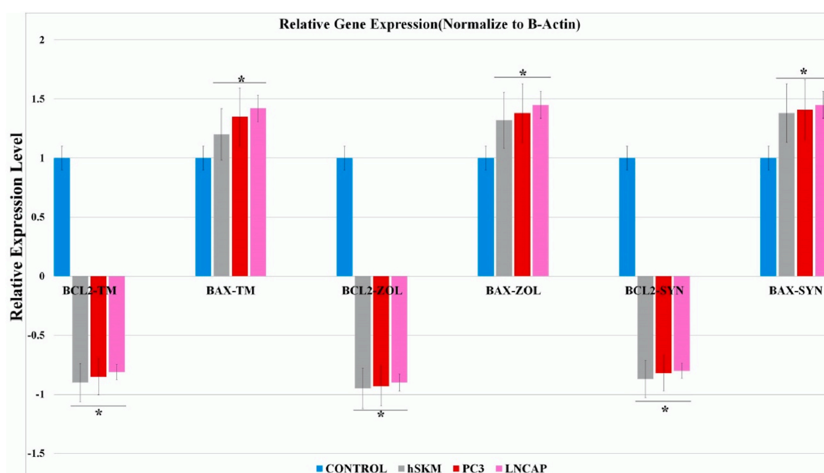

Fig. 5. Quantification of *BAX* and *BCL2* transcripts using Real Time-qPCR revealed that expression levels of *BAX* increased significantly ($p < 0.05$) compared to the control group, while expression levels of *BCL2* decreased significantly ($p < 0.05$) after treatment in comparison to controls.

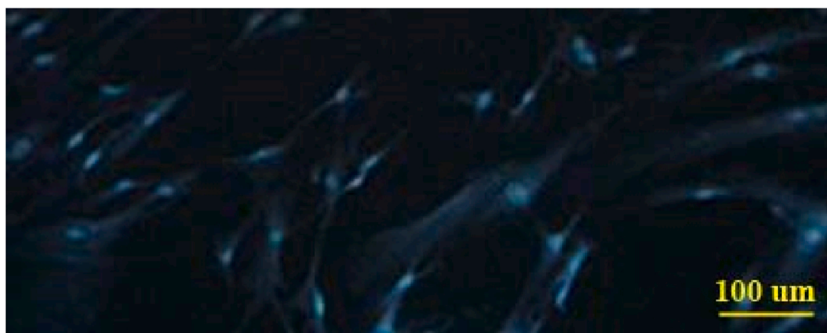

Fig. 6. Cells were stained with Hoechst 33258 and seen in an inverted fluorescent microscope (100 ×).

cells than PC3 cells by affecting AR-dependent pathways and metabolic processes [25,26].

In contrast, hSKM cells, which control cytotoxicity assays, appear more resistant to EOTM than LNCaP and PC3 cells. This may be due to their higher antioxidant capacity and lower lipid peroxidation levels. Additionally, hSKM cells likely exhibit distinct fatty acid metabolism and signaling compared to cancer cells, influencing their response to EOTM. Thus, EOTM may exert less cytotoxicity on hSKM cells, preserving their normal physiological functions.

The impact of EOTM on the growth of PC3 and LNCaP cells has been investigated, with results showing that EOTM selectively targets and inhibits the growth of cancer cells without affecting healthy cells. The inhibitory effect of EOTM on PC3 and LNCaP cells is both concentration and time-dependent. Notably, the cytotoxic effect of EOTM on these cancer cells is significantly higher than its effect on fibroblasts, while SKM cells remain unaffected.

Apoptosis is a critical mechanism for maintaining proper cell population, and previous studies have shown that EOTM and fatty acids derived from other sources exhibit anticancer effects through various mechanisms, including apoptosis, necrosis, and autophagy.

The composition of EOTM, particularly its high content of linoleic acid (LA) and other omega-3 polyunsaturated fatty acids (ω-3 PUFAs), has been linked to preventive effects against LNCaP and colorectal cancer cells. Additionally, EOTM contains significant amounts of OA and PA, which have been shown to induce apoptosis in colon tumor cells via caspase-3 activation.

EOTM inhibits PC3 and LNCaP prostate cancer cells. By inducing apoptosis by activating caspase-8, -9, and -3, leading to DNA damage and subsequent cell death. The presence of omega-3 fatty acids (ω-3 FAs), OA, and PA in EOTM likely contributes to these effects. However, further research is necessary to elucidate the underlying molecular mechanisms fully.

Our study is the first to investigate the combined use of EOTM and ZA in treating prostate cancer cell lines. We demonstrated that EOTM inhibited tumor cell growth, induced apoptosis, and modulated vital genes. Moreover, we found that ZA enhanced the effects of EOTM, highlighting a synergistic potential between these compounds. This approach differs from previous studies that utilized different extracts or products from *Tenebrio molitor* larvae for treating other cancer types.

For example, Darbemamieh et al. (2020) explored the antiproliferative and apoptotic properties of aqueous and ethanolic extracts from *T. molitor* larvae on breast cancer cells. They found that the ethanolic extract had a more pronounced effect on cytotoxicity and apoptosis than the aqueous extract [27].

In contrast, our study focused on the oil extract (EOTM), which, as revealed by GC-MS analysis, contains a complex array of bioactive compounds, with fatty acids such as PA, OA, and LA being the most prominent. These fatty acids are integral to various biological functions, including the modulation of cellular energy metabolism and membrane dynamics.

In our oncological research, the impact of EOTM on cancer cells was significantly more pronounced than on normal cells, as demonstrated by proteomic and transcriptomic analyses. Western blot analysis indicated a significant upregulation of the proapoptotic protein BAX and a downregulation of the anti-apoptotic protein BCL2 in cancer cell lines following EOTM treatment. This shift in the BAX/BCL2 ratio suggests an activation of the apoptotic pathway, corroborated by morphological changes typical of apoptosis, such as cellular shrinkage and nuclear condensation. Additionally, Real-Time RT-qPCR analysis revealed that EOTM modulates gene expression, leading to a decrease in BCL2 mRNA and an increase in BAX mRNA, indicating transcriptional reprogramming that favors apoptosis.

Further research has also analyzed the nutritional composition and ACE inhibitory activity of *Tenebrio molitor* and *Galleria mellonella* larvae and pupae [28]. The findings showed that both insects possess high protein, fat, and mineral content and significant ACE inhibitory activity, suggesting potential health benefits beyond oncology [29].

Another relevant study by Ding et al. (2021) examined the effects of oil extract from *Tenebrio* molitor larvae on hepatocellular carcinoma and colorectal adenocarcinoma cells. They found that the oil extract inhibited cancer cell growth, induced apoptosis, and activated caspases, with the effects attributed to the fatty acid content of the oil extract. However, unlike our study, Ding et al. did not investigate combination therapies or compare the oil extract with other products derived from *T. molitor* larvae [30].

A related study demonstrated that insect meal affects hepatic apoptosis and autophagy in three fish species, with liver proteome changes showing species-specific responses. This highlights the need for strategic management [31].

When replacing fishmeal with insect meal in fish diets. For example, a study on mealworm meal and largemouth bass revealed that up to 25 % of fishmeal in the diet could be replaced by mealworm meal without adverse effects. However, higher substitution levels reduced growth and liver damage, characterized by inflammation and apoptosis [32]. In a comparative analysis, Abou-Hashem et al.

(2019) demonstrated that the chloroform fraction of Moringa peregrina seed extract (CFEE) exhibited potent antitumor properties, particularly inducing apoptosis in HELA and PC-3 cells [33]. Our study aligns with these findings, as EOTM showed significant inhibitory effects on LNCaP and PC3 cells, as indicated by the inhibition rates post-treatment. While Abou-Hashem et al. focused on apoptosis induction through cell cycle arrest and DNA fragmentation, our research further quantified cytotoxicity, directly comparing the mortality effects across different cancer cell lines.

Our methods parallel those used by Abou-Hashem et al., utilizing flow cytometry for cell cycle analysis and Western blotting for protein expression measurement. However, our study extends the molecular analysis by evaluating the effects of EOTM on mRNA expression levels of critical apoptotic markers, providing a more comprehensive view of the cellular mechanisms involved.

Both studies underscore the potential of natural extracts as effective anticancer agents. The selectivity and efficacy of CFEE in inducing apoptosis in HELA and PC-3 cells are mirrored by EOTM's effects on LNCaP and PC3 cells, with our data revealing a slightly higher mortality rate in LNCaP cells. This comparative analysis validates the antitumor effects of Moringa peregrina and highlights EOTM as a promising therapeutic agent, warranting further investigation into its mechanism of action and potential clinical applications.

In addition, a novel, synergistic approach combining EOTM and zoledronic acid (ZA) offers several advantages over monotherapy, including enhanced efficacy, reduced toxicity, and a broader therapeutic spectrum. We also employed gas chromatography-mass spectrometry (GC-MS) to determine the chemical composition of the oil, providing deeper insights into the active compounds and their potential interactions.

The scope of this study was limited to two prostate cancer cell lines, which constrains the breadth of our findings. The lack of a comparative analysis with other *Tenebrio molitor* extracts restricts a comprehensive understanding of EOTM's relative effectiveness and safety. Furthermore, the absence of in vivo experimentation leaves the pharmacological profile of EOTM unexplored. Despite these limitations, the study demonstrates EOTM's capacity to suppress prostate cancer cell proliferation, induce apoptosis, and modulate key gene expressions, potentially enhancing these effects with zoledronic acid (ZA). Acknowledging these constraints is crucial for contextualizing the study's contribution to cancer research.

While the research highlights the anticancer potential of EOTM and its cooperative action with ZA, the inherent variability of natural products like EOTM could affect results consistency. Additionally, the study's narrow focus on specific cell lines may not fully reflect the heterogeneity of prostate cancer, and the in vitro concentrations used may not correspond to clinically relevant doses. Moreover, the investigation does not comprehensively address long-term treatment outcomes, detailed molecular interactions, or the safety profile of EOTM—the observed enhancement of therapeutic efficacy with combination therapy warrants further exploration.

Future research should aim to expand the investigation to include a broader range of cell lines, in vivo trials, evaluations of long-term effects, and detailed mechanistic studies. This would deepen the understanding of EOTM as an anticancer agent and clarify its potential clinical applications.

5. Conclusion

In conclusion, this study underscores the therapeutic potential of EOTM in oncology, particularly for managing prostate cancer. The investigation outlines the inhibitory effects of EOTM on prostate cancer cell proliferation and its facilitation of apoptotic cell death, likely driven by the fatty acid constituents within EOTM. Integrating EOTM with ZA, a known anticancer agent, revealed synergistic interactions that may enhance therapeutic efficacy while reducing adverse effects. However, translating these findings from bench to bedside will require extensive research to identify the active molecular entities, elucidate the underlying mechanisms of action, optimize dosing strategies, and rigorously evaluate the long-term implications and safety profiles in clinical settings. This research contributes to the growing body of knowledge regarding insect-derived bioactive compounds and their potential as innovative cancer therapeutics, offering promise for improved patient outcomes and quality of life.

CRediT authorship contribution statement

Nahid Askari: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Seyed Mozaffar Mansouri:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation.

Data availability statement

Data will be made available on request.

Ethics statement

Ethical clearance was obtained from the Graduate University of Advanced Technology (KGUT), Institute of Sciences and High Technology and Environmental Sciences Committee under code: 01.3423, dated March 13, 2023.

Declaration of competing interest

Please check the following as appropriate.

o All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
oThis manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
oThe authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript
oThe following authors have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript

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