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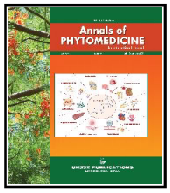

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The potential cytotoxic and apoptotic effects of *Pulicaria undulata* (L.) C.A. Mey. chloroform fraction on human lung adenocarcinoma A549 cells

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Abstract

Pulicaria undulata (L.) C.A. Mey. is a herbal plant distributed in variable regions around the globe. Emerging research showed that *P. undulata* extracts exhibit different biological activities including antitumor properties. However, the anticancer properties of *P. undulata* against different cancer cells remain uncertain. Herein, the antiproliferative effect of *P. undulata* extracts were explored against the adenocarcinoma lung cancer cells A549. The antiproliferative effect of *P. undulata* extracts were tested by MTT while DAPI staining and FACS were used to qualitatively and quantitatively determine the apoptosis initiation in treated cells. Further, apoptosis-related markers were estimated by RT-PCR and western blot analysis. GC-MS was utilised to determine the chemical components present in a promising fraction. The MTT viability assay revealed that the *P. undulata* chloroform fraction (PUCF) diminished the A549 cells' growth. DAPI staining, along with FACS analysis revealed the presence of apoptotic cells in PUCF-treated samples. Moreover, the PUCF apoptotic effects were validated by upregulation of Bax and caspase-3, alongside a decrement in the levels of the antiapoptotic Bcl-2 proteins. Additionally, GC-MS analysis identified palmitic acid (13.29%), parthenolide (6.35%), cedran-diol, 8S,13- (6.22%) and triacontane (5.07%) as major constituents. Taken together, *P. undulata* showed a promising anticancer effect affirming further exploration for its efficacy in lung carcinoma treatment.

1. Introduction

Lung cancer is the primary reason of cancer death globally, with around 2 million cases and 1.8 million deaths reported each year. It ranks as the second most common type of cancer diagnosis among both men and women (Thandra *et al*., 2021). Throughout history, natural products have played a pivotal role in the development of new drugs. Recent studies highlight this significance with a substantial increase in the number of newly approved drugs from natural products (Newman and Cragg, 2020).

Several plant constituents with activity against lung cancer have been previously documented. These bioactive compounds inhibit lung cancer cell proliferation through diverse actions such as arresting cell cycle, prompting apoptosis, restraining cell migration and adhesion, as well as modulating the immune system and enhancing cancer cells' sensitivity to antitumour medications (Cao *et al*., 2019; Yang *et al*., 2021).

Pulicaria genus ranks as the third largest genus within the Asteraceae family, encompassing 85 species of shrubs, small shrubs, and herbs. These species are found across Asia, Arabian lands, Europe and North Africa (Chah *et al*., 2006). *Pulicaria* species have a history of use in folk medicine for addressing various ailments including coughs, excessive sweating colds, as well as colic conditions (Al Zain *et al*., 2023). Moreover, *Pulicaria* species have demonstrated a variety of bioactivities, including antioxidant, antispasmodic, cytotoxic, antipyretic, antimicrobial, and anti-inflammatory properties (Chan *et al*., 2010; Yusufoglu, 2014; Foudah *et al*., 2016). Additionally, several classes of compounds such as sesquiterpenes, flavonoids, triterpenes and monoterpenes have been isolated from *Pulicaria* species and displayed cytotoxic effects against several cancer cell lines (Zekry *et al*., 2021; Alamdary and Baharfar, 2023).

Pulicaria undulata (L.) C.A. Mey. is a perennial aromatic herb, found in various regions including the Arabian Peninsula, Egypt, Iran, India, and tropical parts of Africa (Boulos, 2002). In these regions, it has been utilized for several purposes such as herbal tea, and insect repellents as well as for treating bruises, addressing skin infections and gastrointestinal disturbances (Zoghet and Al Alsheikh, 1999; Stavri *et al*., 2008; El-Hassan, 2015). Continuous research has been conducted on the pharmacological properties of *P. undulata*, particularly in the anticancer field. For instance, the antitumor impact of *P. undulata* was examined against numerous cancer cells such as HepG2 (Emam *et al*., 2019), HT29 and HCT116 (Mohammed *et al*., 2020), and MCF-7 cancer cells (Mohammed *et al*., 2021). Moreover, *P. undulata* essential oil has shown antiproliferative activity in numerous cancer cells (Ali *et al*., 2012; Mustafa *et al*., 2020).

Despite the documented pharmacological benefits of *P. undulata*, particularly its cytotoxic effects against various cancer cell lines, there remains a significant gap in understanding its specific anticancer properties and mechanisms of action against A549 lung carcinoma cells. Previous studies have primarily focused on its effects in other

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cancer types, leaving a lack of comprehensive research addressing its potential in lung cancer treatment. Therefore, this study aimed to explore the antitumor and proapoptotic effects of *P. undulata* using human lung carcinoma A549 cells as a model. The chemical composition of *P. undulata* chloroform extract was also determined.

2. Materials and Methods

2.1 *P. undulata* collection and extraction

The *P. undulata* aerial parts were gathered from Riyadh region, Kingdom of Saudi Arabia. The *P. undulata* was authenticated by Professor Ramzi Mothana, College of Pharmacy, King Saud University (KSU), Riyadh, Saudi Arabia. A Voucher Specimen No. (PU 3/2023) was placed in the Herbarium unit. The collected plant materials were dried before being ground. Subsequently, the powder material (100 g) was sequentially extracted in the Soxhlet apparatus with n-hexane, chloroform, ethyl acetate as well as methanol. The collected extracts were then evaporated and stored at -80°C and utilized as required.

2.2 Antiproliferative MTT assay

The adenocarcinoma A549 lung cancer cells (ACC107) were cultured using DMEM media. The MTT assay was performed in accordance with the protocol detailed in previous work (Nasr *et al*., 2023). In brief, cells were seeded into a 96-well plate (50,000 cells/well) in DMEM medium (100 µl). Plates were then incubated at 37°C for 24 h to allow the cells to attach. Next, the *P. undulata* fractions were evaluated for their cytotoxic effects at 31.25, 62.5, 125, and 250 µg/ml with doxorubicin employed as a positive control. After a 24 h incubation period, MTT at 5 mg/ml (prepared in PBS) was applied, and incubated for 2-4 h at 37°C. Subsequently, the purple formazan was solubilized with isopropanol. A plate reader (BioTek, USA) was utilized to measure the optical density at 570 nm, and the IC_{50} values were calculated using Origin Pro 8.5 software.

2.3 DAPI staining

Qualitative detection of apoptosis was identified using the DAPI staining method (Al-Zharani *et al*., 2019). Briefly, A549 cell lines were seeded in a 12-well plate. After 24 h, PUCF at IC_{50} and $2 \times IC_{50}$ were added and further incubated for 24 h. After cell fixation, cells were stained with DAPI dye (1 µg/ml) and further incubated for half an hour in the dark. Nuclear alterations were examined using an inverted fluorescence microscope (Optika, Italy).

2.4 Annexin V FITC/PI assay

The FITC-V/PIkit from Biolegend company was utilized following the manufacturer's instructions. Briefly, PUCF at 75 and 150 µg/ml were added to A549 cells for 24 h. The collected cells were then resuspended in an appropriate buffer. Then, cells were stained with both dyes for 15 min and subjected to FACS analysis.

2.5 Reverse transcriptase-polymerase chain reaction (RT PCR)

Following treatment with PUCF (75 and 150 µg/ml), A549 cells were harvested in Trizol solution to prepare total RNA as described earlier (Rio *et al*., 2010). Afterwards, 1 µg of purified RNA was utilized for cDNA synthesis with a commercial cDNA synthesis kit. Subsequently, the levels of Bcl-2, Bax and caspase-3 were estimated, with normalization to the mRNA expression of β-actin.

2.6 Western blot analysis

Immunoblotting was achieved to determine the apoptosis-related markers of A549-treated cells as previously described (Alqahtani *et al*., 2022). The isolated protein c was quantified with the Bradford method and subsequently separated on 12% SDS-PAGE. Next, samples were shifted onto a nitrocellulose membrane, blocked with 5% non-fat milk, and incubated overnight at 4°C with primary antibodies of target proteins. Membranes were then incubated with secondary antibodies dilutedin BSA for 1 h. Following each step, the blots were washed three times with TBST buffer. Next, membranes were incubated with ECL Western blotting detection reagents, and the bands were visualized by exposing the membranes to X-ray films.

2.7 GC-MS analysis of phytochemical constituents

The phytoconstituents of PUCF were analyzed using the GC-MS instrument as the previously designated protocol (Nasr *et al*., 2020). In summary, the program initiated at 40°C, then raised to 200°C at a rate of 5°C per min after 2 min of hold, and kept for another 2 min. Subsequently, the temperature was further raised from 200°C to 300°C at a rate of 5°C per min and held for an additional 2 min. The components present in the extract were identified by comparing the obtained mass spectra with referenced libraries (Adams, 2007; McLafferty *et al*., 1989).

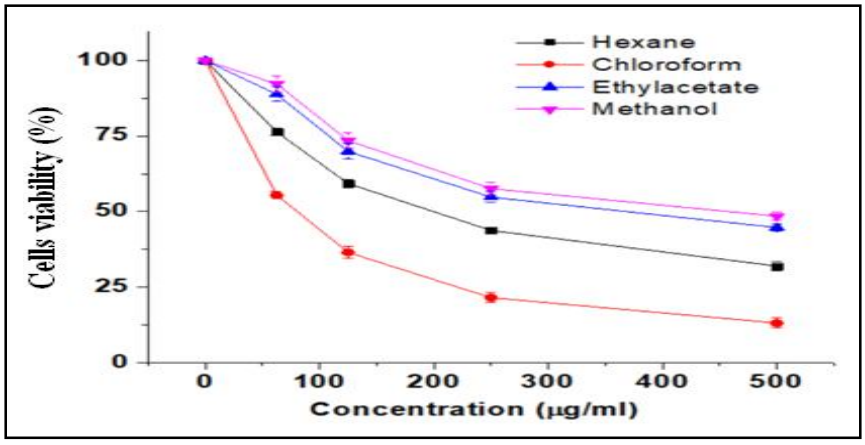

Figure 1: The inhibitory effects of *P. undulata* fractions on A549 cell growth. Cells were treated with 0.1% DMSO as a vehicle and indicated concentrations for 24 h. The data are presented as the mean values ± standard deviation (SD) of triplicates.

2.8 Statistical analysis

Assessing the statistical significance of the collected triplicate data was done by Student's t-test. The IC_{50} values were computed using Origin Pro 8.5.NIH Image J software was employed to quantify band intensities.

3. Results

3.1 *P. undulata* chloroform fraction inhibits A549 cell proliferation

A549 cell growth was measured using the MTT assay to assess the antiproliferative potential of *P. undulata* fractions**.** All fractions exhibited a dose-dependent inhibition of proliferation in tested cell lines (Figure 1). Notably, the *P. undulata* chloroform fraction (PUCF) demonstrates the potent activity against A549 cells with IC_{50} 75.5 µg/ml. The remaining fractions display a weak cytotoxic activity in terms of IC_{50} (Table 1). As a result, this fraction was selected for further investigation.

3.2 Nuclear staining with DAPI fluorescent dye

To determine if the cytotoxic impact of the extract was a consequence of apoptosis initiation, A549 treated was examined under the fluorescence microscope. Untreated A549 cells exhibited a normal nuclei shape and uniform staining with DAPI (Figure 2). Conversely, the cells treated with PUFC displayed distinctively stained nuclei, demonstrating chromatin condensation and the presence of apoptotic bodies characteristic of both early and late stages of apoptosis (Figure 2).

3.3 PUCF induce apoptosis in A549 cells

To elucidate whether A549 cell proliferation inhibition was correlated with apoptosis induction, the quantitative analysis of apoptotic cell death induced by the PUCF was conducted. This analysis focused on the externalization of phosphatidylserine, a characteristic marker of apoptotic cells. As illustrated in Figure 3, treatment A549 cells treated with doses of 75 and 150 µg/ml for 24 h led to a significant dose-dependent increase in the percentage of apoptotic cells. Specifically, early apoptotic cell percentage was increased to 12.8 ± 0.4 % and 21.1 ± 1 %, respectively. Similarly, a remarkable increase in the proportion of late-stage apoptotic cells following PUCF treatment (increased to 20.7 ± 0.9 % and 42.1 ± 0.5 %, in relation to 1.1 ± 0.2% in the untreated group). A notable increment in the necrotic cell death was also observed with high dose (Figure 3).

Table 1: IC_{50} values for *P. undulata* fractions determined by MTT assay

Fraction	A549 IC_{50} (µg/ml)
Hexane	202.1 ± 2.6
Chloroform	75.5 ± 1.9
Ethyl acetate	362.6 ± 3.5
Methanol	444.5 ± 4.9
Doxorubicin	1.5 ± 0.05

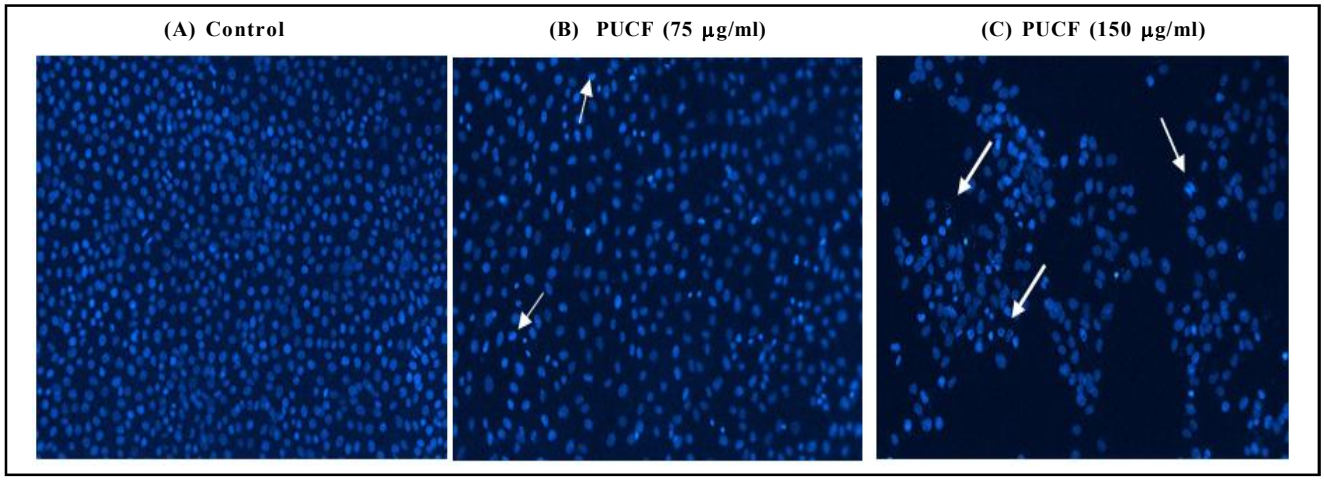

Figure 2: Nuclear morphological change assessment in A549 cells that were treated with PUCF 75 and 150 µg/ml. (A) Control untreated cells (B) after treatment with PUCF IC_{50} (75 µg/ml) and (C) PUCF IC_{50}(150 µg/ml). Arrows point to nuclear fragmentation observed under an inverted fluorescence microscope at 20 × magnification.

3.4 The induction of apoptosis by PUCF was linked with the downregulation of Bcl 2 and the upregulation of Bax and caspases-3

To explore whether the apoptotic induction by PUCF was correlated with the alteration of key apoptosis regulatory genes, RT-PCR was carried out. A dose-dependent reduction in Bcl-2 (anti-apoptotic) gene was detected. In contrast, the alteration of pro-apoptotic Bax gene was elevated along with the elevation of caspase-3 levels in response to the treatment (Figure 4).

3.5 Alteration of apoptotic-related protein expression by PUCF

Western blot was also utilized to confirm the effect of PUCF on the expression of apoptotic proteins.A similar trend was observed in the protein expression levels following treatment with 75 and 150 µg/ml of PUCF. As shown in (Figure 5), Bax and Caspase-3 were dose-dependent increases compared to untreated cells. Similarly, the levels of antiapoptotic Bcl-2 protein were reduced over 24 h compared to untreated cells (Figure 5).

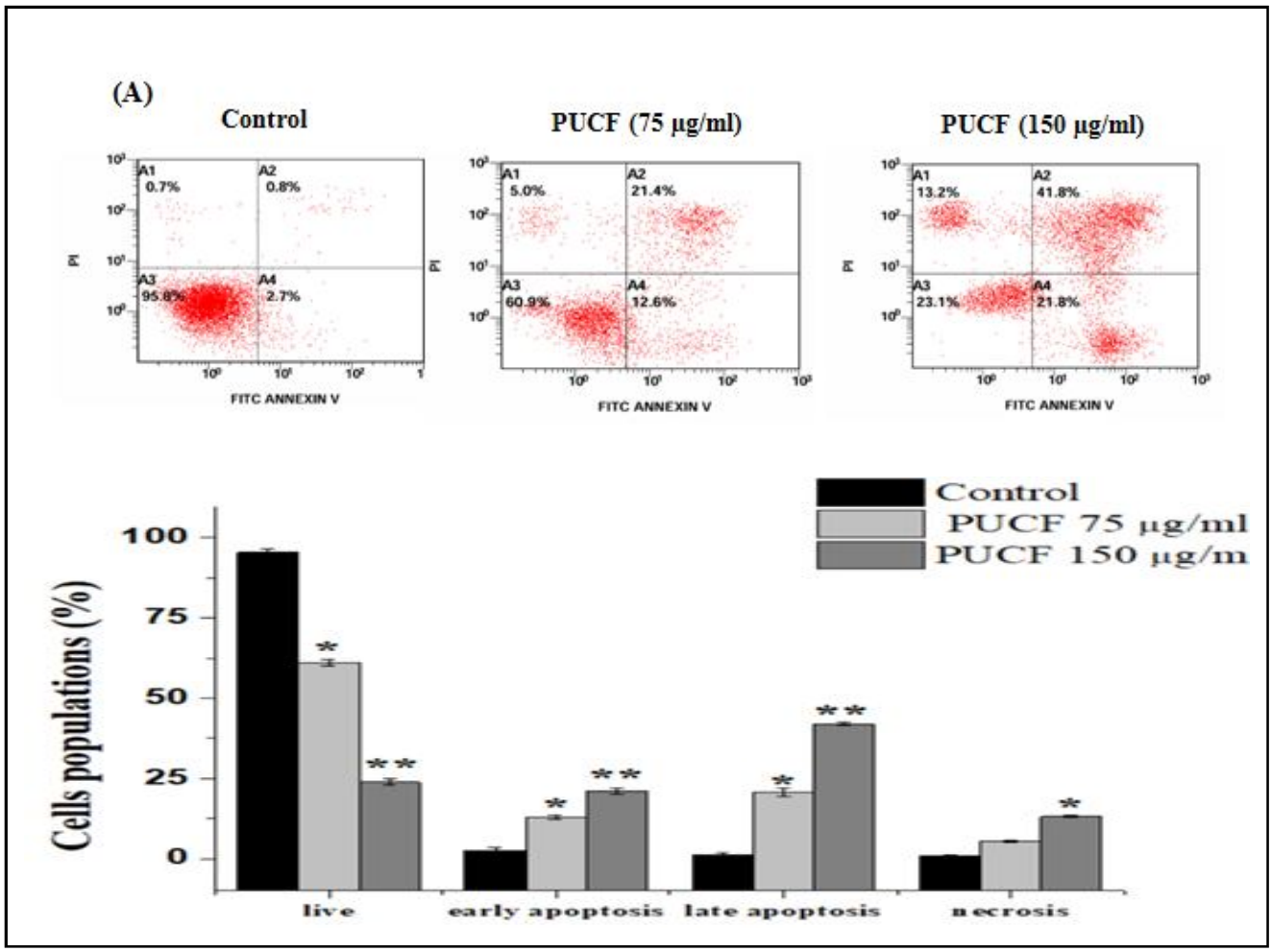

Figure 3: *P. undulata* chloroform fractions promote A549 cell apoptosis. Cells were treated with PUCF and subject to flow cytometry analysis, (A3) represents the percentage of viable cells, (A4) represents the percentage of early apoptotic cells (A2) represents late apoptotic and (A1) represents necrotic cells. (B) Column bars representingthe percentage of cells at various stages. Statistical significance compared to control is denoted with *p<0.05 and **p<0.01.

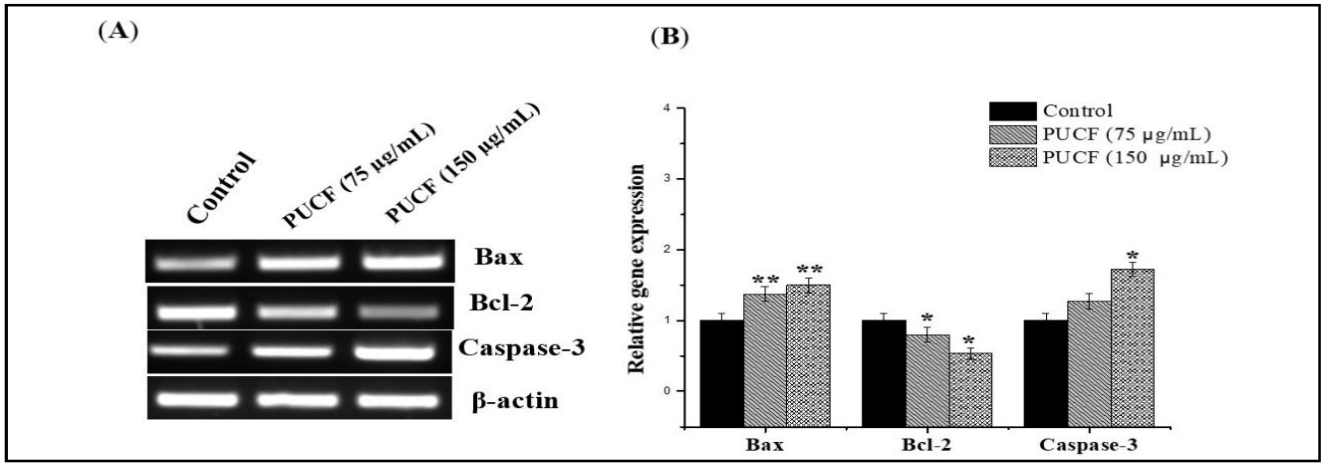

Figure 4: Effects of *P. undulata* chloroform fractions on the apoptosis-related genes. Them RNA levels were determined by RT-PCR for PUCF-treated and untreated cells. (A) The expression of target genes after indicated treatments: (B) Corresponding bands were utilized to estimate the relative expression which was measured using Image J software. Statistical significance compared to control is denoted with *p<0.05 and **p<0.01.

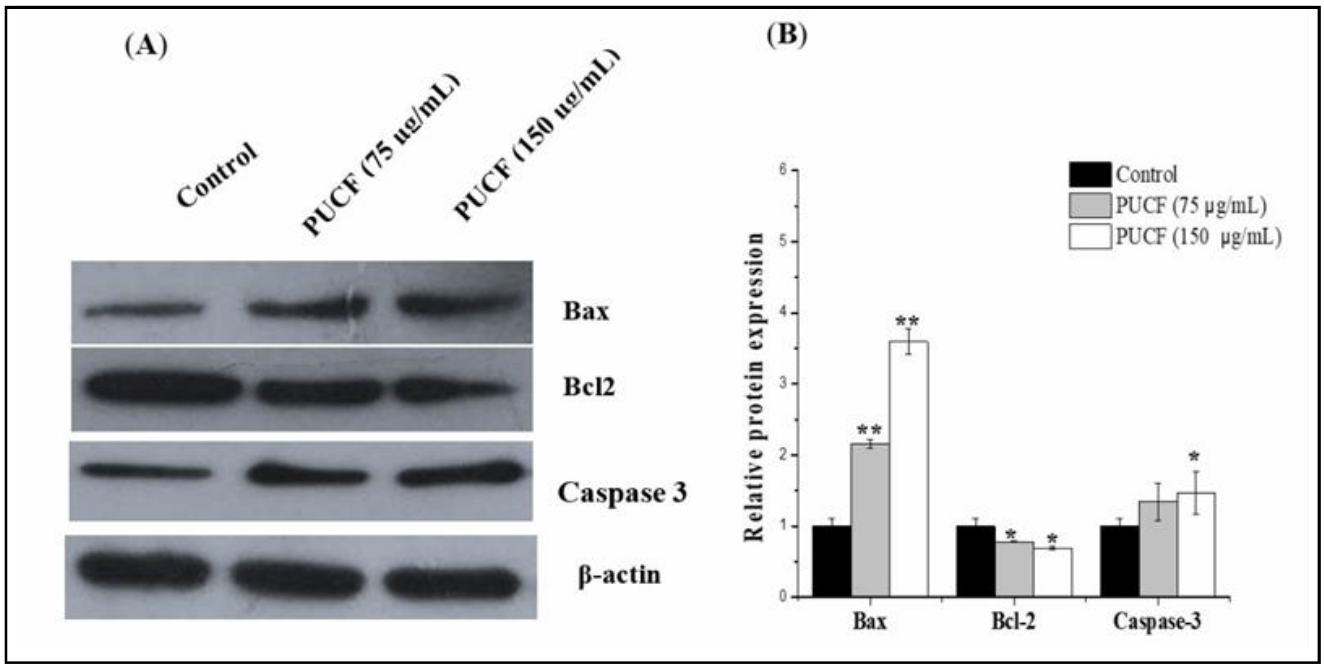

Figure 5: Influence of PUCF treatment on the protein expression of apoptosis related genes. A549 cells were treated with 75 and 150 µg/ml of PUCF for 24 h. (A) Bands of studied proteins where β-actin was used as internal control. (B) The numerical data of corresponding bands in compare to control. *p<0.05 and **p<0.01 vs. untreated cells.

3.6 Phytochemical analysis

To determine which compounds are responsible for the observed effect prompted by PUCF, a compositional analysis was conducted using GC-MS. This analysis identified 37 compounds within the PUCF. Table 2 displays the chemical profile of PUCF constituents according to their elution on the HP Innowax column. Around 50 components were identified with major constituents namely palmitic acid (13.29 %), parthenolide (6.35%), cedran-diol, 8S,13- (6.22%) and triacontane (5.07%). The other constituents are listed in Table 2.

Table 2: GC-MS analysis of *P. undulata* chloroform fraction

Compound name	Chemical formula	MW (g/mol)	RT (min)	Area (%)
(2Z)-2,6-Dimethyl-2,7-octadiene-1,6-diol	$C_{10}H_{18}O_2$	170.25	12.453	0.4860
Dihydroactinidiolide	$C_{11}H_{16}O_2$	180.24	15.367	0.8221
Isocamphane	$C_{10}H_{18}$	138.25	15.608	0.6298
Caryophyllene oxide	$C_{15}H_{24}O$	220.35	16.016	0.6167
Epi-Bicyclosesquiphellandrene-1	$C_{15}H_{24}$	204.35	16.76	3.3200
Caryophyllene oxide	$C_{15}H_{24}O$	220.35	17.167	0.6781
Methyl 12-methyltridecanoate	$C_{15}H_{30}O_2$	242.40	17.587	0.4763
Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo-	$C_{14}H_{18}O_2$	218.29	18.051	0.4492
Spiro[2,4,5,6,7,7a-hexahydro-2-oxo-4,4,7a-trimethylbenzofuran]-7,2'-(oxirane)	$C_{12}H_{16}O_3$	208.25	18.586	1.583
Pinane	$C_{10}H_{18}$	138.25	18.898	2.6647
6,10,14-Trimethylpentadecan-2-one	$C_{18}H_{36}O$	268.5	18.993	1.7006
9-Octadecyne	$C_{18}H_{34}$	250.5	19.165	0.5512
1,4-Eicosadiene	$C_{20}H_{38}$	278.5	19.362	0.7754
Methyl palmitate	$C_{17}H_{34}O_2$	270.5	19.871	12.8521
1-Methylnaphthalene	$C_{11}H_{10}$	142.20	20.195	4.0294
Palmitic acid	$C_{16}H_{32}O_2$	256.42	20.501	13.2997

Methyl 10-methyl-hexadecanoate	$C_{18}H_{36}O_2$	284.5	20.851	0.4712
Hexadecanoic acid, trimethylsilyl ester	$C_{19}H_{40}O_2Si$	328.6	21.067	1.3934
3-Cyclohexylthiolane 1,1-dioxide	$C_{10}H_{18}O_2S$	202.32	21.391	1.6615
Preg-4-en-3-one, 17.alpha.-hydroxy-17.beta.-cyano-	$C_{20}H_{27}NO_2$	313.4	21.589	2.2658
1,5-Dimethyl-7-oxabicyclo[4.1.0]heptane	$C_8H_{14}O$	126.20	21.735	3.766
Tetradecanal	$C_{14}H_{28}O$	212.37	22.015	1.5865
6-(3,5-Dimethyl-1H-pyrazol-1-yl)-3-methyl-1,2,4-triazolo[4,3-b][1,2,5,6]tetrazine	$C_9H_{10}N_8$	230.23	23.217	4.505
Podocarp-13-en-8-ol	$C_{17}H_{28}O$	248.4	23.459	1.1587
Methyl arachidate	$C_{21}H_{42}O_2$	326.6	23.593	2.7903
E,E-6,11-Tridecadien-1-ol acetate	$C_{15}H_{26}O_2$	238.37	23.688	0.5144
2-Furancarboximidic acid, N-[[(5-methyl-3-phenyl-4-isoxazolyl)carbonyl]oxy]-	$C_{16}H_{12}N_2O_5$	312.28	23.873	3.0689
2-Pentadecen-4-yne, (Z)-	$C_{15}H_{26}$	206.37	24.324	1.6900
Xanthinin	$C_{17}H_{22}O_5$	306.4	24.731	4.8679
Cedran-diol, 8S,13-	$C_{15}H_{26}O_2$	238.37	24.961	6.2257
Parthenolide	$C_{15}H_{20}O_3$	248.32	25.088	6.3582
Methyl behenate	$C_{23}H_{46}O_2$	354.6	25.221	0.4829
Heneicosane, 11-pentyl-	$C_{26}H_{54}$	366.7	25.603	1.3028
Caryophyllene oxide	$C_{15}H_{24}O$	220.35	25.978	0.8698
9-Octylheptadecane	$C_{25}H_{52}$	352.7	26.468	1.7594
Heneicosane	$C_{21}H_{44}$	296.6	27.442	3.2479
Triacontane	$C_{30}H_{62}$	422.8	27.887	5.0763

4. Discussion

Lung cancer is considered as one of the most life-threatening illnesses worldwide (Long *et al.,* 2024). The increasing global burden of cancer demands the exploration of alternative treatment options. Among all natural products, traditional pharmaceutical plants serve as a vital source of novel compounds with anticancer properties (Khan *et al.,* 2019). Among these, the medicinal species from the Asteraceae family have shown to possess a promising anticancer effect in both *in vitro* and *in vivo* models (Bessada *et al.,* 2015; Baile *et al.,* 2021; Butala *et al.,* 2021). Numerous studies have reported that the genus Pulicaria, within the Asteraceae family, contains various chemical constituents with a diverse range of carbon skeletons, and many of these compounds demonstrate noteworthy bioactivities (Liu *et al.,* 2010; Zekry *et al.,* 2021).

Herein, the *P. undulata* chloroform fraction (PUCF) demonstrated a remarkable cytotoxic activity compared to other fractions suggesting that PUCF may contain some potent agents with anticancer properties. Based on criteria founded by the U.S. National Cancer Institute (NCI) which defines $IC_{50} \leq 20$ µg/ml as highly cytotoxic and IC_{50} between 21 and 200 µg/ml as moderate cytotoxic (Niksic *et al.,* 2021). PUCF has a moderate cytotoxic effect while other fractions exhibited a weak cytotoxic efficacy. Previously, the essential oils of *P. undulata* grown in Sudan demonstrated variable effectiveness against different types of cells, with half-inhibitory concentration values ranging from 9.6 to more than 100 µg/ml (Mohammed *et al.,* 2020). Similarly, the *P. undulata* native to Egypt displayed moderate cytotoxicity against different cancer cells with an IC_{50} values ranging from 18.53 to 40.64 µg/ml (Mustafa *et al.,* 2020). In this study, the half-inhibitory concentration value of PUCF was within these values. In comparison to a 95% aqueous-ethanol extract of *P. undulata*, which exhibited IC_{50} values of 519.2 µg/ml for MCF-7 and 1212 µg/ml for K562 (Mohammed *et al.,* 2021), the chloroform extract in this study demonstrated a significant cytotoxic activity in terms of IC_{50} value (75 µg/ml). This discrepancy can be attributed to the differential solubility of bioactive compounds in polar versus non-polar solvents as well as the differential sensitivity of different cells to cytotoxic compounds.

Apoptosis is a widely recognized mechanism of cell death and evasion of this pathway is a prominent feature of cancer cells (Hanahan and Weinberg, 2011). Consequently, targeting apoptotic pathways is considered a promising strategy in the cancer therapy field. Screening medicinal herbs and derived products from them as apoptosis inducers has become a key strategy in anticancer drug research (Rajabi *et al.,* 2021). Herein, apoptotic cell death analysis was conducted using DAPI dye which can be used to visualize nuclear changes and assess apoptosis (Wallberg *et al*., 2016). Nuclear morphological analysis using the IC_{50} and double IC_{50} concentrations clearly showed the incident of apoptosis in treated cells which showed nuclear fragmentation in comparison to uniformly stained control cells. Moreover, one notable biochemical feature of apoptosis is the presence of phosphatidylserine on the outer side of the plasma membrane during apoptosis initiation (Nagata *et al.,* 2016).The Annexin-V-FITC which detects externalization of phosphatidylserine

as well as propidium iodide were employed for the detection of apoptosis and necrosis events in A549 cells treated with PUCF.The flow cytometry data suggested that the PUCF exhibited an apoptotic potential in A549-treated cells.The relative levels of apoptotic markers were also estimated to clarify the PUCF effects. The PUCF treatment resulted in a Bax/Bcl-2 ratio increment in A549 treated cells. Moreover, caspase-3 serves as the principal executioner of apoptosis initiation (Porter and Jänicke, 1999). Herein, RT-PCR and Western blot revealed that PUCF activates the caspase-3 expression. Additionally, these findings strongly suggest that PUCF induces cell death *via* apoptosis-dependent pathways.

Further, GC-MS was performed to define the possible compounds that may be responsible for the observed effect of PUCF. The analysis data discloses the presence of 37 constituents in the PUCF. Some of these 37 compounds have been documented to holdan antitumor effect. Palmitic acid was reported to possess antitumour efficacy against various malignancies *via* inducing apoptosis as well as enhancing sensitivity to chemotherapy (Wang *et al.,* 2023). Parthenolide was also reported to halt A549 cell growth and apoptosis induction *via* proapoptotic genes upregulation and down-regulating antiapoptotic genes *via* the p53-mediated apoptotic pathway (Talib and Al Kury, 2018). In addition, the *in vitro* cytotoxic activity has been reported for triacontane compound against murine melanoma cells. Triacontane compound also exhibited very promising antitumor protection against subcutaneous melanoma *in vivo* (Figueiredo *et al.,* 2014).

While this study provides valuable insights into the anticancer effects of *P. undulata* on A549 lung carcinoma cells, the findings are based on *in vitro* experiments, and further research is needed to validate these results through *in vivo* studies. Additionally, challenges may arise in translating the *in vitro* findings into clinical practice, as factors such as pharmacokinetics, dosage, and tumor microenvironment.

5. Conclusion

In summary, this study demonstrated that *P. undulata* chloroform fraction (PUCF) suppressed the A549 growth *via* apoptosis inductions. Molecular mechanisms underlying this effect showed that PUCF altered the ratio of proapoptotic (Bax) to antiapoptotic Bcl-2, and promoted the caspase 3 activation. Phytoconstituents analysis identified key bioactive compounds, including palmitic acid, parthenolide, cedran-diol, and triacontane, which may contribute to the observed anticancer activity. The combined modes of action of these bioactive compounds remain unclear, and elucidating their detailed mechanisms will be beneficial for their potential use in both cancer prevention and treatment. Moreover, *in vivo* studies are required to confirm the efficacy and safety of PUCF in animal models, which will help bridge the gap between *in vitro* findings and clinical applications.

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Conflict of interest

The author declare no conflicts of interest relevant to this article.

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