Apoptotic and anti-migratory effects of *Achillea millefolium* dichloromethane extract on MDA-MB-231 human breast cancer cells

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ABSTRACT. Several previous studies have reported the beneficial effects of *Achillea millefolium*, including its anti-cancer properties. The main objectives of this study were to explore the apoptotic and anti-migratory properties of *A. millefolium* extract against the highly aggressive MDA-MB-231 breast cancer cell line. *A. millefolium* was subjected to sequential extraction using various solvents, and these fractions were screened for their anti-proliferative effects using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Hoechst staining, reverse transcription-quantitative polymerase chain reaction, and scratch migration assays were employed to monitor the apoptotic and anti-migration activity of the most promising active extract, *A. millefolium* dichloromethane fraction (AMDF). Moreover, the chemical composition of AMDF was determined using gas chromatography-mass spectrometry (GC-MS). The results of this study demonstrated that AMDF exerted a significant cytotoxic effect on MDA-MB-231 breast cancer cells, accompanied by the induction of apoptosis through the upregulation of pro-apoptotic genes, including tumour protein p53, BCL2 associated X, caspase 3 and caspase 9. AMDF also demonstrated antimigratory properties in MDA-MB-231 cells. Additionally, GC-MS profiling revealed 22 constituents, with M-malein-imido-acetophenone, longifolenaldehyde, azuleno[4,5-b] furan-2,7-dione, and 1,8-cineole identified as major components. In conclusion, this study provided further evidence supporting the potential utility of *A. millefolium* as a source for novel treatments targeting breast cancer.

Introduction

Breast cancer remains a significant global health concern affecting women all over the world, with approximately 685 000 deaths and 2.3 million new cases recorded only in 2020. Furthermore, projections indicate that the burden of breast cancer will escalate to over 3 million cases by 2040 (Arnold et al., 2022). The MDA-MB-231 breast cancer cell line is the most studied cell line representing the triple-negative breast cancer (TNBC) subtype characterised by the absence of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2/Neu) (Yin et al., 2020). Given the lack of these markers, TNBC is considered the most aggressive form that does not respond to available therapies. Consequently, there is an urgent need for the development of innovative, safe, and effective agents against this disease to combat its deleterious effects.

Historically, plants have played a crucial role in the treatment and prevention of various

diseases, with nearly 80% of developed nations employing medicinal plants for health purposes (Sen and Samanta, 2015). One such therapeutic herb commonly utilised in pharmaceutical fields is *Achillea millefolium*, belonging to the family Asteraceae that is widely distributed throughout the world (Bashir et al., 2022). With a wide distribution, *A. millefolium* has long been employed in traditional medicine to treat various ailments such as jaundice, hepatitis, malaria, as well as respiratory and digestive conditions (Jangjoo et al., 2023). The medicinal properties of *A. millefolium* have also been explored for its potential in cancer treatment, inflammation reduction, and wound healing (Ali et al., 2017; Farasati Far et al., 2023).

These beneficial properties of *A. millefolium* can be attributed to its rich array of phytoconstituents, such as sesquiterpenoids, phenols, hydrocarbons, oxygenated monoterpenes, flavonoids, or essential oils (Ali et al., 2017; Barda et al., 2021). Additionally, the plant has also been reported to contain immunomodulatory components and anti-inflammatory compounds such as alkamides, making it a potential source of medicine components in the future (Saeidnia et al., 2011; Salehi et al., 2020).

The cytotoxicity and anti-cancer properties of various species within the genus *Achillea* have been previously described (Papakosta et al., 2020), and regarding *A. millefolium*, its anti-cancer effects have also been documented in several *in vitro* and *in vivo* studies (see review by Ali et al., 2017 and works therein). Phytocomponents such as kaempferol, camphene, linalool, and apigenin, isolated from *A. millefolium*, have demonstrated anti-cancer activity (Farasati Far et al., 2023).

The present study aimed to elucidate the therapeutic potential of *A. millefolium* against breast cancer. While previous studies have highlighted the cytotoxicity and anti-cancer properties of various species within the genus Achillea, including *A. millefolium*, we focused on investigating its underlying mechanism, including pro-apoptotic and anti-migratory effects. Additionally, we aimed to identify the phytocomponents present in *A. millefolium*.

Material and methods

Plant collection and extraction

A. millefolium was purchased at a market in Riyadh, Saudi Arabia. The plant was identified and verified (KSU voucher number No. 2100) by a taxonomist at the Herbarium unit of the Botany Department, King Saud University. After drying, the plant was ground using an electric mill, and 100 g of the powdered plant material was subjected to Soxhlet extraction for 24 h with hexane, dichloromethane, ethyl acetate, and methanol solvents (0.45 l each). The extract was subsequently concentrated at 45 °C using a rotary evaporator (Heidolph, Schwabach, Germany).

Cell culture

The MDA-MB-231 breast cancer cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Leibniz Institute DSMZ, Braunschweig, Germany). It is derived from a pleural effusion of a patient with invasive ductal carcinoma and serves as a ubiquitous model for late-stage breast cancer.

MTT Proliferation assay

The standard colorimetric 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was utilised to determine the anti-proliferative activity, as previously described (Nasr et al., 2020). MTT is a pale-yellow compound that enters living cells and is subsequently cleaved by dehydrogenase enzymes in the mitochondria of viable cells to produce an insoluble dark blue formazan. However, this compound is soluble in organic solvents and can be measured spectrophotometrically. Briefly, 5×10^4 breast adenocarcinoma cells (MDA-MB-231) were plated in a 24-well plate for 24 h. Next, the cells were exposed to various concentrations (12.5, 25, 50, and 100 µg/ml) of the extract and dimethyl sulfoxide (DMSO) (final concentration of 0.1% DMSO) as a vehicle. After the incubation period (48 h), 100 µl of MTT reagent (Invitrogen, Carlsbad, CA, USA) was added to each well to a final concentration of 5 mg/ml and further incubated for 2–4 h at 37 °C. Thereafter, the media was removed, and 1 ml of acidified isopropanol was added to each well in order to dissolve the formazan. Using an ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA), the absorbance was measured at 540 nm, and cell viability was calculated using the following formula:

% inhibition = (mean absorbance in treated wells / mean absorbance in control wells) × 100.

The half maximal inhibitory concentration (IC_{50} value) was calculated using OriginPro 8.5 Software (Origin Corporation, Northampton, MA, USA).

Hoechst 33258 nuclear staining

In healthy cells, nuclei are typically spherical in shape, with DNA evenly distributed within them. However, during apoptosis, DNA undergoes

condensation and fragmentation, which serves as a distinguishing feature between apoptotic, healthy, and necrotic cells. Fluorescent dyes, such as Hoechst dyes, which specifically bind to DNA, are commonly employed to visualise nuclear condensation. Herein, Hoechst 33258 nuclear staining was employed to examine the apoptotic morphologic features induced by *A. millefolium* dichloromethane fraction (AMDF) in MDA-MB-231 cells. Briefly, 5×10^4 cells were plated in a 12-well plate

using the cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The Applied Biosystems 7500 Fast Real-Time PCR System was employed to amplify the studied genes using the SYBR Green Master Mix, according to the manufacturer's instructions (Molecular Probes, Invitrogen, Carlsbad, CA, USA). RT-qPCR thermal cycling conditions were as previously described (Semlali et al., 2015). The primers utilised to amplify the target genes in this study are detailed in Table 1.

Table 1. List of primer sequences used in quantitative polymerase chain reaction

GenBank acc. no.	Gene	Amplicon size, bp	Forward/Reverse	Sequence 5'→3'
NM_001289745.3	*GAPDH*	188	Forward	GGTATCGTGGAAGGACTCATGAC
			Reverse	ATGCCAGTGAGCTTCCCGTTCAGC
NM_001407269.1	*TP53*	234	Forward	CCCAGCCAAAGAAGAAACCA
			Reverse	TTCCAAGGCCTCATTCAGCT
NM_001354779.2	*CASP3*	555	Forward	CTGGTTTTCGGTGGGTGTG
			Reverse	ACGGCAGGCCTGAATAATGAA
NM_001278054.2	*CASP9*	194	Forward	CAGGCCCCATATGATCGAGG
			Reverse	TCGACAACTTTGCTGCTTGC
NM_001291429.2	*BAX*	79	Forward	GGATGCGTCCACCAAGAAG
			Reverse	CCTCTGCAGCTCCATGTTAC

GAPDH – glyceraldehyde-3-phosphate dehydrogenase, *TP53* – tumour protein p53, *CASP3* – caspase 3, *CASP9* – caspase 9, *BAX* – BCL2 associated X, apoptosis regulator

and exposed to AMDF at 25 µg/ml and DMSO at 0.1% for 48 h. Next, the cells were stained using Hoechst 33258 (Sigma, St. Louis, MO, USA), following the protocol described in our previous study (Al-Zharani et al., 2019). A digital camera attached to a Zeiss fluorescent microscope (Zeiss, Jena, Germany) was used to capture images of the stained cells within 30 min.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

By detecting changes in the expression of genes associated with apoptosis, RT-qPCR provides a sensitive and specific method for studying molecular mechanisms underlying apoptosis regulation in the cells examined. In the present study, changes in the expression of selected apoptosis-related genes after treatment with AMDF were assessed using RT-qPCR. Briefly, MDA-MB-231 cells (5×10^4) were seeded in a 6-well plate and incubated for 24 h. Subsequently, the cells were exposed to DMSO (0.1%) as a vehicle control and AMDF ($IC_{50} = 25$ µg/ml). Total RNA was extracted from both untreated and treated cells after 48 h of incubation using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To synthesise cDNA, 1 µg of mRNA was reverse-transcribed

Scratch migration assay

The *in vitro* wound healing assay is an efficient and economical experiment to study cell migration *in vitro*. The method involves making a scratch on a confluent cell monolayer, prompting the cells at the scratch to migrate towards the gap to close it. During this process, images are captured at the beginning and then at regular intervals to calculate the rate of cell migration. MDA-MB-231 cells were grown to approximately 70–80% confluency in a 12-well plate. A sterile pipette tip (10 µl) was then used to slowly and gently scrape the monolayer across the centre of the well. The cells were then washed with phosphate buffered saline (PBS) and treated with AMDF. Images were taken over a period of 0, 24, and 48 h to monitor the closure or migration of cells to fill the scraped area using an inverted microscope coupled to a Leica MC-170 HD camera (Leica, Wetzlar, Germany). To analyse the images, ImageJ software (NIH Image, Bethesda, MD, USA) was used. The relative migration ratio was determined using the following equation (Luanpitpong et al., 2010):

relative migration ratio = (distance at 0 h − distance at 48 h) / distance at 0 h.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was conducted to identify the chemical constituents of AMDF using a Perkin Elmer Clarus 600 GC-MS (Turbomass, PerkinElmer, Inc., Waltham, MA, USA) as previously described (Nasr et al., 2023). For analysis, the extract was injected and the column temperature was increased from 40 °C to 200 °C at a rate of 5 °C per min. After holding for 2 min, the temperature was increased to 300 °C at a rate of 5 °C per min. and maintained for an additional 2 min. Helium was used as the carrier gas at a flow rate of 1.00 ml per min. Peak areas were calculated to determine the quantitative amount of extracted components by comparing the data obtained with the WILEY and NIST mass spectral libraries (McLafferty and Stauffer, 1989; Adams, 2017).

Statistical analysis

The results are presented as means ± standard deviation (SD) derived from three independent experiments, each comprising 2 or 3 replicates per experiment. Statistical comparison of data sets was performed using Student's t-test, with differences considered statistically significant at $P < 0.05$.

Results

Percentage yields of extracts

A. millefolium powder (100 g) was extracted successively in n-hexane, dichloromethane, ethyl acetate, and methanol (solvents with increasing polarity) using a Soxhlet apparatus. The extraction efficiency is summarised in Table 2. The yield was defined as the weight percentage of the extract relative to the weight of the dried powder. The highest percentage yield was obtained for methanol extract, whereas the lowest for ethyl acetate.

Table 2. Weight and percentage yield of individual *Achillea millefolium* fractions

Weight of plant powder, g	Weight of extract, g Yield, %			
	n-hexane	Dichloromethane	Ethyl acetate	Methanol
100	2.3 (2.3%)	3.3 (3.3%)	0.76 (0.76%)	16 (16%)

Cytotoxicity evaluation

Using the MTT assay, the anti-proliferative effect of *A. millefolium* fractions (n-hexane, dichloromethane, ethyl acetate, and methanol) against
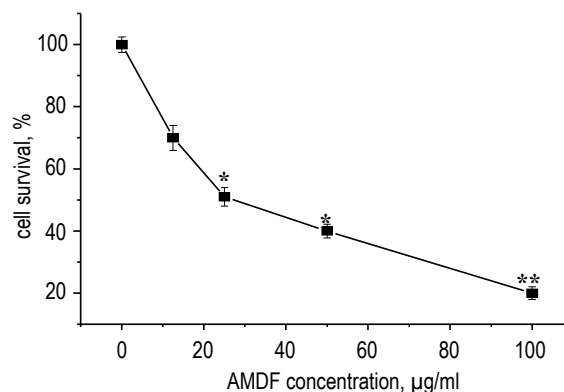

Figure 1. *Achillea millefolium* dichloromethane fraction (AMDF) antiproliferative effects. MDA-MB-231 cells were exposed to different concentrations of AMDF for 48 h, and cell survival was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Student's t-test was used to analyse statistical differences. The data from three experiments are shown as mean ± SD; * $P < 0.05$, ** $P < 0.01$ indicate significant differences compared to the control (n = 3)

MDA-MB-231 breast cancer cells was assessed. Of all fractions, the *A. millefolium* dichloromethane fraction caused a significant reduction in the MDA-MB-231 cell viability (Figure 1), as evidenced by the lowest IC_{50} value (25 ± 0.8 μg/ml). Conversely, the other fractions showed very low activity (hexane and ethyl acetate), with IC_{50} values exceeding 100 μg/ml, while methanol showed no discernible activity (Table 3). Based on these results, the *A. millefolium* dichloromethane fraction (AMDF) was selected for further efficacy testing using the remaining methods.

Table 3. Half maximal inhibitory concentration (IC_{50}) values of *Achillea millefolium* fractions obtained by MTT assay (n = 3)

Fraction	IC_{50}, μg/ml
n-hexane	213 ± 2.6
Dichloromethane	25 ± 0.8
Ethyl acetate	557 ± 4.5
Methanol	no activity

Dichloromethane fraction of *Achillea millefolium* induces apoptosis in MDA-MB-231 breast cancer cells

To ascertain whether AMDF inhibits MDA-MB-231 cell growth by inducing apoptosis, the nuclei of treated and untreated cells were stained with Hoechst 33258 fluorescence dye. As displayed in Figure 2, MDA-MB-231 cells treated with AMDF (IC_{50} = 25 μg/ml) exhibited DNA fragmentation, a hallmark feature of apoptotic cells. In contrast, untreated cells had normal nuclear morphology.
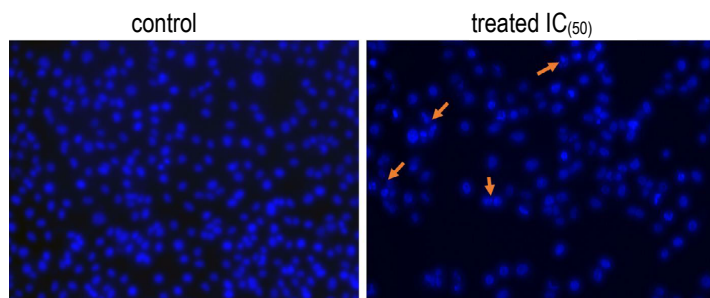
Figure 2. Effect of *Achillea millefolium* dichloromethane fraction (AMDF) treatment on MDA-MB-231 cells nuclear morphology. MDA-MB-231 cells were treated with a vehicle control (DMSO) or with AMDF at its IC50 for 48 h. Nuclear changes were detected using a fluorescence microscope following staining with Hoechst 33258. Apoptotic cells are indicated by arrows (n = 3)

AMDF treatment led to apoptosis initiation through upregulation of apoptosis-associated gene expression

Quantitative PCR was employed to evaluate the expression of pro-apoptotic genes in MDA-MB-231 cells. Following AMDF treatment, a significant increase in the expression of pro-apoptotic genes, including tumour protein p53 (*TP53*) and BCL2 associated X, apoptosis regulator (*BAX*), was observed compared to untreated cells (Figure 3). A significant increase in the expression of the caspase 9 (*CASP9*) and caspase 3 (*CASP3*) genes was also detected (Figure 3).
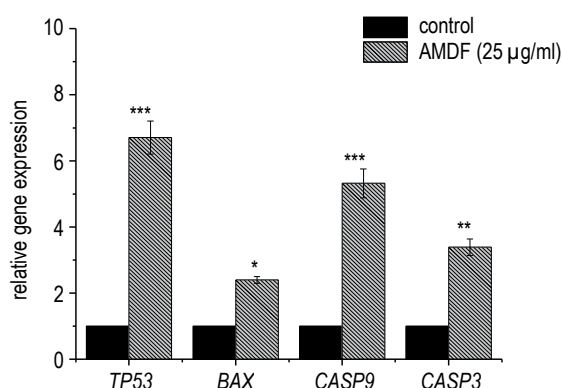

Figure 3. Effect of *Achillea millefolium* dichloromethane fraction (AMDF) on the expression of pro-apoptotic markers. MDA-MB-231 cells were incubated with a vehicle and AMDF (25 µg/ml). Following the incubation period, apoptosis-associated genes were quantified by RT-qPCR. Relative mRNA levels were normalised to GAPDH mRNA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ treatment group vs control group (n = 3)

GAPDH – glyceraldehyde-3-phosphate dehydrogenase, *TP53* – tumour protein p53, *CASP3* – caspase 3, *CASP9* – caspase 9, *BAX* – BCL2 associated X, apoptosis regulator

Dichloromethane fraction of *Achillea millefolium* suppresses breast cancer migration

A scratch assay was conducted to test the anti-migratory potential of AMDF on the MDA-MB-231 breast cancer cell line. Treatment with a half maximal inhibitory concentration (IC_{50}) of AMDF led to a decrease in cell migration into the scratched region compared to untreated cells (Figure 4).
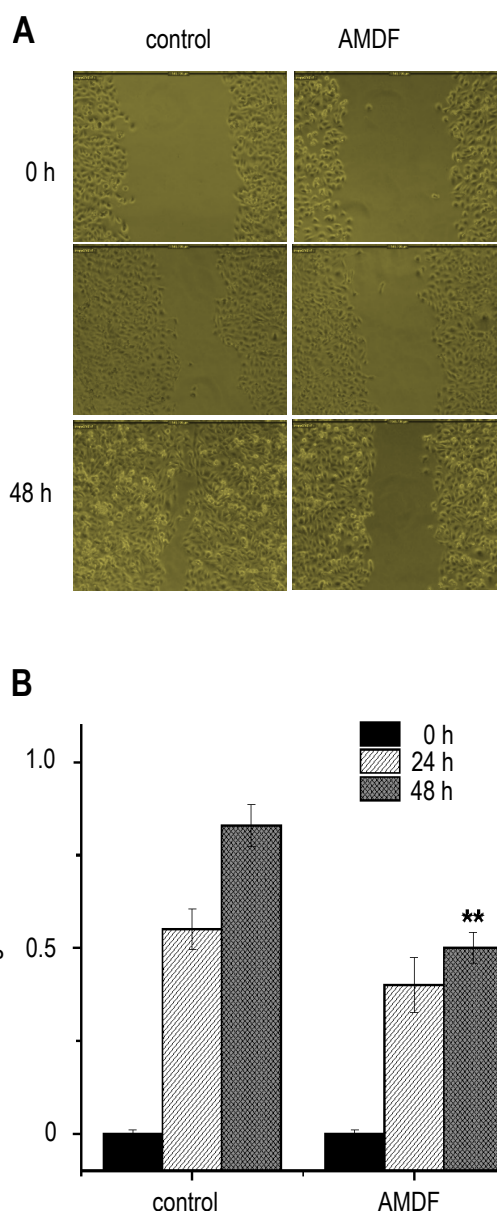

Figure 4. Effects of *Achillea millefolium* dichloromethane fraction (AMDF) on MDA-MB-231 cell migration. (A) MDA-MB-231 cells were treated with a vehicle dimethyl sulfoide (DMSO), 0.1%) or AMDF (25 µg/ml) and imaged 0, 24, and 48 h after scratching. (B) The migration rate of MDA-MB-231 into the gap area was calculated as shown in the methods section. Experiments were conducted in triplicate (** $P < 0.01$; n = 3)

GC-MS analysis of *Achillea millefolium* dichloromethane fraction

The recognised compounds present in the *A. millefolium* dichloromethane fraction, along with their chemical formulae, retention time (RT), and area percentage, are shown in Table 4. The main detected compounds in AMDF were M-maleinimido-acetophenone (26.31%), longifolenaldehyde (19.42%), azuleno[4,5-b] furan-2,7-dione (11.82%), and 1,8-cineole (6.64%). Additionally, several other compounds were identified, albeit with less prominent peaks, as illustrated in Figure 5.

Discussion

Breast cancer remains one of the most serious threats affecting women globally. Despite significant advancements in cancer treatment strategies, there are still many risks associated with their side effects (Anand et al., 2023). Medicinal plants continue to serve as valuable source of natural products used to treat various ailments. Whether extracted in their crude form or as purified compounds, they contribute substantially to the exploration of innovative drug therapies (Aware et al., 2022).

Table 4. Phytocomponents present in the dichloromethane fraction of *Achillea millefolium* identified by GC-MS analysis

Compound	Chemical formula	Molecular weight, g/mol	RT, min	Area	Area%
1,8-Cineole	$C_{10}H_{18}O$	154.25	5.20	296115	6.640
4,4,5-Trimethyl-2-hexene	C_9H_{18}	126.24	5.31	157425	3.530
Bicyclo[3.1.1]heptan-3-ol	$C_7H_{12}O$	112.17	6.05	26283	0.590
Pinocarvone	$C_{10}H_{14}O$	150.22	6.15	26739	0.600
3,4 Dimethylcyclohexanol	$C_8H_{16}O$	128.21	6.23	59332	1.330
1,2,4-Trihydroxy-p-menthane	$C_{10}H_{20}O_3$	188.26	9.21	75657	1.700
2,2-Dibromocholestanone	$C_{27}H_{44}Br_2O$	544.40	9.67	57632	1.290
Spathulenol	$C_{15}H_{24}O$	220.35	9.88	91002	2.040
Oplopenone	$C_{15}H_{24}O$	220.35	10.05	58605	1.310
9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.50	11.80	139489	3.130
Azuleno[5,6-c]furan-3(1h)-one	$C_{15}H_{22}O_4$	266.33	12.55	136880	3.070
9-Methyl-z-10-tetradecen-1-ol	$C_{17}H_{32}O_2$	268.40	12.59	107619	2.410
1-Undecyne	$C_{11}H_{20}$	152.28	12.69	29570	0.660
Azuleno[4,5-b]furan-2,7-dione	$C_{15}H_{18}O_3$	246.30	12.78	527163	11.820
2-Octyldodecan-1-ol	$C_{20}H_{42}O$	298.50	13.08	159092	4.61
Curcuquinone	$C_{15}H_{20}$	232.32	13.15	40468	0.910
M-maleinimido-acetophenone	$C_{12}H_9O_3N$	215	13.47	1173524	26.310
2-Octyldodecan-1-ol	$C_{20}H_{42}O$	298.5	13.77	134869	3.020
Pseduosarsasapogenin-5,20-diene	$C_{28}H_{44}O_3$	428.6	13.97	181221	4.060
Palustrol	$C_{15}H_{26}O$	222.37	14.67	18571	0.420
Longifolenaldehyde	$C_{15}H_{24}O$	220.35	15.03	866116	19.420

GC-MS – gas chromatography-mass spectrometry, RT – retention time
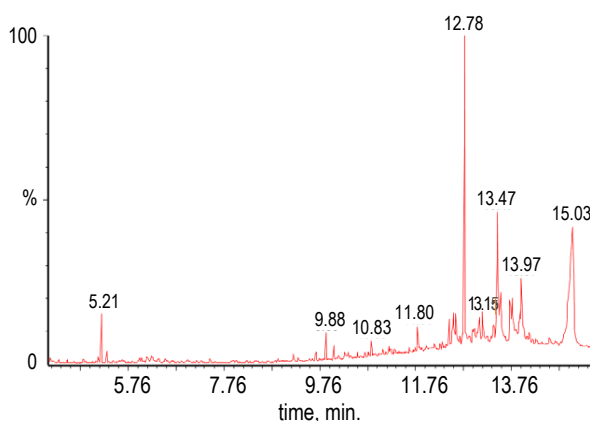

Figure 5. Gas chromatography-mass spectrometry of the dichloromethane fraction of *Achillea millefolium* (n = 3)

In this study, *A. millefolium* was examined for its potential to trigger cytotoxicity, apoptosis, and anti-migratory effects in the triple-negative MDA-MB-231 breast cancer cell line. The cytotoxicity assay revealed that the *A. millefolium* dichloromethane fraction exhibited potent cytotoxicity against treated MDA-MB-231 cells. The National Cancer Institute (NCI) defines a crude extract as promising for further exploration when its IC_{50} value against cancer cells is less than 30 μg/ml (Itharat et al., 2004). In the present study, the IC_{50} value of the AMDF was found to be within this specified range, categorising it as a promising extract. Consistent with our findings,

Alshuail et al. (2022) demonstrated that the dichloromethane fraction of *A. fragrantissima* was the most effective fraction against MDA-MB-231, as indicated by the half-inhibitory concentration values (Alshuail et al., 2022). Furthermore, a recent study by Mohammed et al. (2023) showed that the n-hexane fraction of *A. millefolium* exhibited moderate cytotoxicity ($IC_{50} > 100$ µg/ml) against MCF-7 breast cancer cells (Mohammed et al., 2023). It should also be noted that *A. millefolium* has previously demonstrated varying efficacy against different cell lines. According to Csupor-Löffler et al. (2009), the chloroform fraction showed strong activity against HeLa and MCF-7 cells, while only modest effects against the A431 epidermoid carcinoma cell line (Csupor-Löffler et al., 2009). These findings suggest that different components extracted using various solvents may act synergistically, leading to varying effectiveness and distinct responses in different cell lines.

Nuclear staining with Hoechst fluorescence dye was conducted to determine whether the growth inhibition of AMDF-treated MDA-MB-231 cells was correlated with apoptosis induction. The results indicated that AMDF induced DNA fragmentation, a prominent characteristic of apoptosis events (Majtnerová and Roušar, 2018). Previous studies have reported induction of apoptosis and cell cycle arrest in HeLa cancer cells by the ethyl acetate fraction of the same species (Abou Baker, 2020). Gene expression analysis further revealed that AMDF induced apoptosis by upregulating the mRNA expression levels of pro-apoptotic genes, including *TP53, BAX, CASP3,* and *CASP9* in MDA-MB-231 cells. Caspases, a subset of cysteine proteases, play a crucial role in apoptosis, and their activation serves as a distinctive marker of apoptosis (Shi, 2004). Among them, caspase-3 is recognised as the key executioner caspase. In this study, exposure of MDA-MB-231 cells to AMDF resulted in a significant upregulation of *BAX* and *CASP3* levels. These findings are in line with a previous study which demonstrated that a methanolic extract of *A. teretifolia* induced apoptosis by upregulating the levels of the *BAX* and *CASP3* genes in prostate cancer cells (Bali et al., 2015).

Preventing cancer cell migration is crucial for successful cancer treatment. Compounds that inhibit the migration of cancer cells play an important role in disease treatment and prognosis (Asma et al., 2022). The potential anti-cancer activity of AMDF is further highlighted by its observed anti-migratory effect on MDA-MB-231 cells, a highly invasive and aggressive cell line. To the best of our knowledge, there are no previous studies reporting the anti-migratory effects of *A. millefolium*.

The GC/MS analysis identified several compounds with varying pharmacological potential. Among these, 1,8-cineole, present in various plants extract, has been documented for its anti-cancer and apoptotic activity in different types of cancer cells (Murata et al., 2013; Sampath et al., 2017; Rodenak-Kladniew et al., 2020). Other studies have also revealed that plant extracts containing octadecenoic possess anti-tumour activity, which may involve disrupting membrane integrity and inducing apoptosis in tumour cells (Yu et al., 2008; Manivannan et al., 2017). Spathulenol is another compound identified in the present study, and although it has been relatively poorly studied, it may contribute to observed cytotoxic effects in plants with anticancer activity (Santos et al., 2020).

Conclusions

In summary, the present study demonstrated that the dichloromethane fraction from *Achillea millefolium* induced cell death in triple-negative breast cancer cells through apoptosis. Furthermore, we elucidate the mechanism by which apoptosis was induced and showed that the extract suppressed the migration of breast cancer cells. GC-MS analysis provided insights into the diverse bioactive compounds present in *A. millefolium* dichloromethane fraction (AMDF), and highlighted its pharmacological properties. The observed anti-proliferative effects, apoptosis induction, and cell migration inhibition collectively suggest a diverse mechanism of action that may address critical aspects of cancer progression. Therefore, the findings suggest that AMDF is a promising, natural potential therapeutic agent for the treatment of triple-negative breast cancer. Further research, including detailed studies on its mechanism of action and *in vivo* experiments, are required to validate the results obtained *in vitro* and fully elucidate the therapeutic potential of AMDF in breast cancer therapy.

Funding

Conflict of interest

The Authors declare that there is no conflict of interest.

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