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Anti-cancer activity of phytochemicals extracted from *Urtica pilulifera* on Hela cells

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Abstract: *Urtica pilulifera* is effective against cancer cell growth but its bioactive compounds and the mechanism of action behind its effect are still to be clarified. This study evaluated the anticancer activity of compounds extracted from *Urtica pilulifera* leaves against cervical cancer cells. The cytotoxic effect of water, methanol and hexane extracts of *Urtica pilulifera* leaves was assessed by MTT assay. The most potent extract was fractionated by column chromatography and its anticancer activity was evaluated. GC-MS was used to identify the chemical components of the different fractions. Western blotting analysis was used to determine the level of specific apoptotic and cell cycle protein markers. Data showed that the hexanic extract exhibits the most potent cytotoxicity against Hela cells. Fraction 4 of the hexanic extract showed the most potent antiproliferative effect against Hela cells. GC-MS analysis showed that the most prominent compound in fraction 4 is phytol. Fraction 4 treatment induced cell cycle arrest and intrinsic apoptosis in Hela cells as evident by the increasing levels of p21, p53, PARP cleavage and active caspase 9. These findings reveal the ability of *U. pilulifera* hexanic extract to induce cell cycle arrest and apoptosis in cervical cancer cells.

Keywords: Cervical cancer, *Urtica pilulifera*, GC-MS, cell cycle arrest, apoptosis.

INTRODUCTION

Cancer is a leading cause of death worldwide (Sung *et al.*, 2021). Although there are several strategies to treat cancer, most cancer treatments cause adverse side effects to the patients. For example, chemotherapy is the most common strategy for cancer treatment causing different side effects ranging from mild effects such as weight loss and nausea to severe effects like harming normal body cells and immunodeficiency. In addition, several types of cancer develop resistance against chemotherapeutic agents, making them ineffective over time (Rapoport, 2017; Nurgali *et al.*, 2018).

Phytochemicals are effective bioactive compounds against several diseases including cancer. Phytochemicals are secondary metabolites produced by plants and are widely available in vegetables, fruits, legumes, grains and nuts. Many of these compounds are known to play an anti-cancer role in terms of treatment and prevention. Furthermore, phytochemicals have been shown to activate the immune system of cancer patients (Barbieri *et al.*, 2017).

The family of *Urticaceae* is one of the most plant families recognized for its therapeutic bioactivity against many diseases. *Urticaceae* family (nettle) includes many common herbs known as dioecy plants with stinging hairs and dark green leaves (Abdel-Kader *et al.*, 2007). The main types of Urtica genus are *Urtica pilulifera* L., *Urtica dioica* L., *Urtica urens* L., *Urtica kiovensis* Rogoff., *Urtica cannabina* L. and *Urtica membranacea* Poiret (Özen *et al.*, 2010). *U. pilulifera* is described as a non-toxic, non-mutagenic and non-embryogenic agent (Abo-elmatty *et al.*, 2013). Traditionally, *U. pilulifera* tea has been used as a tonic, hemostatic, blood purifier and hemoglobin enhancer (Chrubasik *et al.*, 2007; Khatib *et al.*, 2014). Several studies have confirmed that *U. pilulifera* is widely used as the treatment or complementary treatment for diabetes mellitus by the general public (Ali-Shtayeh *et al.*, 2000; Khatib *et al.*, 2014). A study conducted to evaluate the potential effects of *U. pilulifera* extracts in diabetic albino rats showed marked hypoglycemia related to the anti-inflammatory and antioxidant effects of *U. pilulifera* (Alkhatib and Alhassan, 2014). Researchers studied the effect of *U. pilulifera* on kidney function of diabetic mice, compared to metformin and found that *U. pilulifera* serves as metformin in restoring normal levels of renal parameters as creatinine, urea and uric acid (Alkhatib and Alhassan, 2014). It was also reported that *U. pilulifera* exhibits many medical aspects such as hemostatic, hypoglycaemic, diuretic, antidandruff, anti-inflammatory and anti-asthmatic effects (Abudoleh *et al.*, 2011; Zamani and Razavi, 2021). Furthermore, several studies have confirmed that *U. pilulifera* components showed strong antioxidant activity against free radicals which are a closely related cause of cancer (Hofseth and Wargovich, 2007; Özen *et al.*, 2010; Amawi and Alkhatib, 2020). Flavonoids containing C-7 and C-4 hydroxyl groups displayed anti-malignant and anti-mutagen activities (Kopustinskiene *et al.*, 2020). Another study revealed that methanolic extract of *U. pilulifera* exhibited antitumor activities in Ehrlich ascites carcinoma (EAC)- bearing mice, bioactive components that induced these activities

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were known to be phenolic acids and flavonoids (Abdel-Kader *et al.*, 2007). On the other hand, some research attributed the antiproliferative effect of *U. pilulifera* on Ehrlich's carcinoma to the bioactive phytochemicals specifically polysaccharides (Lichius *et al.*, 1999; Abu-Darwish and Efferth, 2018), which decrease sialic acid and phospholipids amount in cancer cells membranes (Tong *et al.*, 1994). Other researchers attributed apoptosis and antiproliferative activity induced by *U. pilulifera* to its content of isoflavone as genisteinglycoside and tyrosine kinase inhibitor (Yu *et al.*, 2003). Previously, we have shown that *U. pilulifera* can inhibit colorectal cancer *In vivo* and this was mainly by inducing cell cycle arrest in cancer cells (Aliwaini and Lubbad, 2016; Kichaoui *et al.*, 2016). In this regard, the current study explored the cytotoxic effect of phytochemical(s) isolated from *Urtica pilulifera* on the Hela human cervical cancer cell line. The results demonstrated that hexanic extract and its antioxidant compounds including phytol exert a potent anticancer effect against Hela cells by inducing cell cycle arrest and intrinsic apoptosis (Alqatati *et al.*, 2020).

MATERIALS AND METHODS

Plant material

Samples of *U. pilulifera* were collected at three phenological stages before flowering, [(II) during flowering and (III) after flowering] in 2019 from different habitats in Al-Zawaidah village in the central governorate of Gaza strip, Palestine. Plant material was identified by using usual keys and iconographies with the support of the Department of Biology, Faculty of Science, Islamic University of Gaza. The plant was washed under running water, then dried with a piece of cloth. The dried leaves were then picked up and packed in bags empty of air and kept in a freezer (-80°C) until use (Jaradat, 2015).

Preparation of the crude extract from Urtica pilulifera leaves

U. pilulifera leaves were dried by heat drying oven at 37°C for 72 hours. Oven-dried leaves are then ground into a fine powder using an electric mill. 20gm of dried powder of *U. pilulifera* were extracted in a soxhlet apparatus with 200ml of different solvents (distilled water, methanol, or hexane) for 8 hours. The temperature of the system was controlled according to the boiling point of the solvent (68°C for hexane- 64.7°C for methanol- 100°C for distilled water). The solvents were evaporated using an incubator at 37°C to yield concentrated extracts. The extracts then were placed in a plastic bottle coated with tinfoil and stored in a refrigerator (-20°C) (Ali Hasan, 2012).

Cell culture

Human cervical carcinoma (Hela) cells were obtained from Dr. Johnny Stiban from Birzeit University, Palestine. Hela cells were maintained as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%), penicillin (100U/mL) and streptomycin (100µg/ml). Cells were grown to confluency in a 95% air- humidified incubator with 37°C & 5% CO2 in a polystyrene culture flask. The media was changed every 2-3 days.

Preparation of plant extract- media & working concentrations

Stock solutions of the hexane and methanol extracts were prepared by dissolving 1mg of each crude extract in 100µl dimethyl sulfoxide (DMSO), then vortexed and completed up to 1ml by adding DMEM media. Distilled water (dH_2O) extract- media prepared the same way except dH_2O extract was dissolved in 100µl dH_2O, not DMSO. For each type of extraction, the same concentration of the vehicle was used to treat control cells. The desired extract- media concentrations (0, 10, 20, 30, 40, 50, 60, 70µl/ml) were prepared by sequential dilutions of stock solutions with the proper volume of media.

Cytotoxicity determination by MTT assay

Hela cells were seeded in 96 well plates at a density of 5000 cells/well. Cells were incubated at 37˚C for 24 hours to reach 60-70% confluence on the day of treatment with the extract. The examined concentrations for hexanic extract were (0,5,10,15,20,25,30,35,40,45µg mL^{-1}) while for methanolic extract were (0,10,20,30,40,50,60,70 µg mL^{-1}) and for aqueous extract were (0,5,10,15,20,25,30, 35,40,45,50µg mL^{-1}).

MTT assay was used to assess the proliferation activity (Roche Diagnostics GmbH, Mannheim, Germany) (Aliwaini *et al.*, 2013). For each well, 10µl of the yellow MTT was added and incubated for 4 hours at 37°C. Then 100 µl of the solubilizing buffer was added to each well and incubated at 37˚C overnight. Finally, the absorbance at 585nm determined by Eliza reader and viability's mean was considered as a percentage of the vehicle controls.

Gas chromatography-mass spectrometry (GC-MS) analysis of the hexanic crude extract of U. pilulifera

For chemical analysis of crude extracts and the six fractions of the hexanic extract, GC-MS was used in which gas chromatograph interfaced to a mass spectrometer. 2µl of the extract was injected manually into the GC/MS employing the following conditions for analysis: silica capillary column (30m x 250µm x 025µm) composed of 100% dimethyl poly siloxane as a stationary phase while carrier gas (99.999% helium) act as mobile phase with a constant flow of 1 ml/min. An ion-source temperature is 280°C and an injector temperature is 250°C (Abdel-Lateef *et al.*, 2016). Oven temperature parameters were modulated 38 times to get optimum ones that give the best possible chromatogram (table 1).

Table 1: Programmed oven temperature

	Rate (°C/min)	Value (°C)	Hold time (min)	Run time (min)
initial		42	2	2
$ramp_1$	50	150	2	6.16
$ramp_2$	10	200	2	13.16
$ramp_3$	5	250	37	60.16

Identification of phytocomponents by GC-MS

Identification of chemical components of hexane extract and all six fractions was based on computer matching with the database of the National Institute Standard and Technology (NIST) library which have more than 62,000 patterns (Abdel-Lateef *et al.*, 2016). The spectrum of the unknown tested components was compared with the spectrum of the known components stored in the library of the NIST to ascertain the name, structure and molecular weight of the components of the tested sample (Sermakkani and Thangapandian, 2012).

Chromatographic fractionation of hexane crude extract

Hexane crude extract of *U. pilulifera* was subjected to chromatography using solid silica gel as a stationary phase, while different solvents such as dichloromethane (CH_2Cl_2) and methanol (CH_3OH) were employed as a mobile phase (Ode *et al.*, 2011). The first five fractions were eluted by dichloromethane, while the sixth fraction was eluted by methanol. Six fractions were eluted and collected, overlapped parts of fractions were discarded and fractions were concentrated to be screened in cell culture.

Western blotting

Protein extract was prepared and separated as described previously (Al-qatati and Aliwaini, 2017). After blotting, different primary antibodies were used including: anti-PARP1/2 (sc-7150), anti-p53 (sc-126) and anti-p21 (sc-756), caspase-9 (SC- 56077), BCl-2 (SC-7382) (Santa Cruz, California, USA) and p38 (# 622401, BioLegend). Horseradish peroxidase-conjugated secondary antibodies (1:5,000; Bio-Rad Laboratories, Inc.,) and antibody-reactive proteins were visualized as previously described (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (Al-qatati and Aliwaini, 2017).

STATISTICAL ANALYSIS

Results are presented as mean ± SEM (Standard error of the means) of three independent tests and a value of $P<0.05$ was considered statistically significant. Statistical analysis of data was performed using the two-sample t-test in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Cytotoxic effects of different Urtica pilulifera crude extracts on Hela cells

The antiproliferative effects of three extracts of *U. pilulifera* using three solvents (hexane, methanol and distilled water) were examined against Hela cervical carcinoma cell line. A Range of concentrations (0-70 µg/ml) of different extracts was used to treat cancer cells. Cytotoxicity exerted on Hela by different extracts was investigated by MTT assay and the concentrations that inhibit cell growth by 50% (IC_{50}) were determined. After 48 hours of treatment, the hexanic extract showed a significant ability to inhibit the growth of Hela cells in a dose-dependent manner (fig. 1a). The inhibitory concentration 50% (IC_{50}) value obtained was 45µg/ml of the hexanic extract. Low concentrations such as 10µg/ml of the extract inhibited Hela cell proliferation significantly. Furthermore, hexanic extract treatment led to severe morphological changes including cell shrinkage and increasing of floating dead cells (fig. 1b & c). On the other hand, low levels of cytotoxicity with IC50s of 120.5 and 80.0µg/ml were observed for both methanolic and water extracts, respectively. Altogether, the hexanic crude extract of *U. pilulifera* exhibited the most cytotoxic effect among the three extracts. Therefore, this extract was chosen for further chemical analysis by GC-MS to identify its phytochemical components.

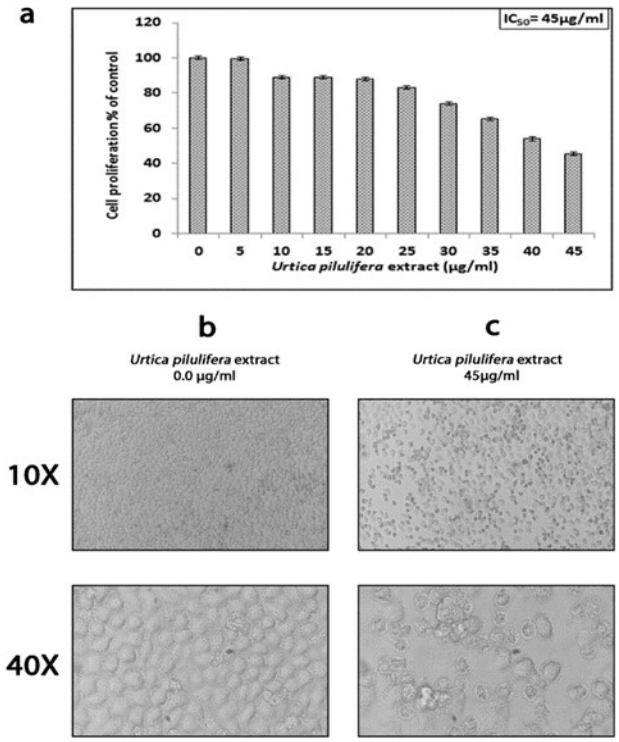

Fig. 1: Cytotoxic activity of hexanic extract of *Urtica pilulifera* leaves against Hela. The chart illustrates the antiproliferative effect of the extract on Hela which was assessed by performing an MTT assay. These results

represent the mean percentage ± SD of control cells (treated with a vehicle) of three trials conducted in quadruplicate (a). Morphological profile of untreated Hela cells has been taken with 10X and 40X magnification (b), and morphological changes of 45µg/ml- treated cells have been monitored with 10X and 40X magnification too (c).

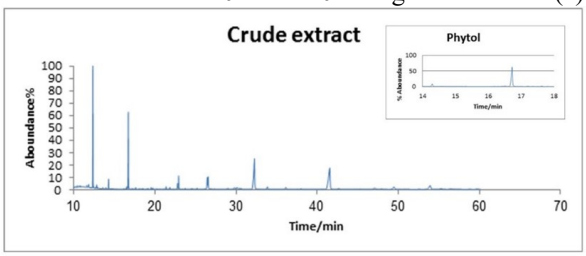

Fig. 2: GC-MS profile of the hexanic crude extract of *Urtica pilulifera* leaves.

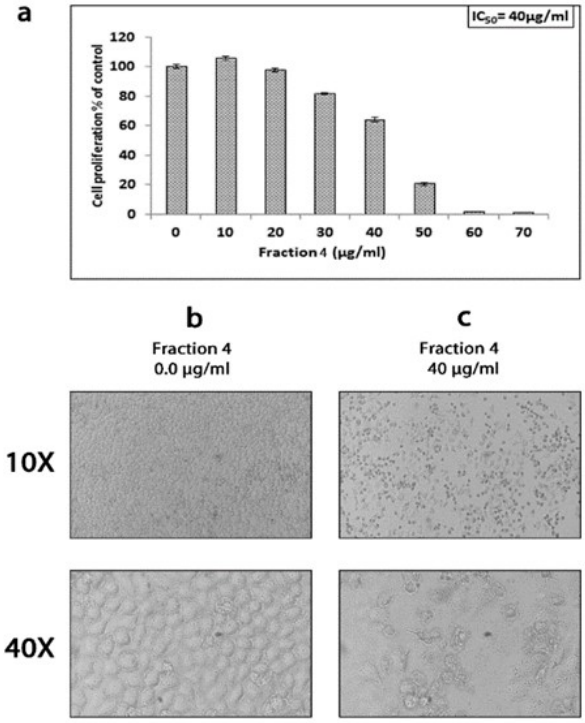

Fig. 3: Cytotoxic activity of Fraction 4 on Hela cells. Cells were incubated with different concentrations of fraction 4 (ranging from 0 to 70 µg/ml) for 48 hours. The chart illustrates the antiproliferative effect of the extract on Hela which was assessed by performing an MTT assay. These results represent the mean percentage ± SD of untreated cells in three trials conducted in quadruplicate (a). Morphological profile of untreated Hela cells has been taken with 10X and 40X magnification (b), and morphological changes of cells treated with 40µg/ml- of fraction 4 have been monitored with 10X and 40X magnification too (c).

Determination of the total content of the hexanic extract of Urtica pilulifera

Using GC-MS, the qualitative analysis of the extract of *U. pilulifera* leaves revealed the presence of nineteen phytochemical compounds. The first compound that had been identified with the less retention time was pinane, whereas eicosane which took the longest retention time was the last one to be identified. The last two compounds couldn't be identified. All Compounds with their retention time (RT), molecular formula, molecular weight and percentage of peak area are presented (table S1). The major components identified in the hexanic extract of *U. pilulifera* leaves were eicosane, heptacosanol, pinane and phytol. Fig. 2 demonstrates GC-MS profile of *U. pilulifera* extract.

Six fractions of Urtica pilulifera extract exert different levels of toxicity against Hela cells.

To identify the phytochemicals that are directly responsible for the aforementioned anticancer activity, the fractionation process was carried out using the column chromatography technique. A total of six fractions of *U. pilulifera* extract were eluted and then identified. The first five fractions were eluted by dichloromethane while the sixth fraction was eluted by methanol. The obtained fractions differ in terms of color, texture and final mass after evaporating the solvent.

Six fractions obtained from the fractionation process were tested for their ability to exert toxicity on Hela cells. After 48 hours of incubating cells with mentioned fractions, an MTT assay was performed. The fractions were grouped according to their antiproliferative levels into three groups as detailed next. The first three fractions did not show any remarkable toxic activity against Hela cells (data not shown). But significant toxicity after 48 hours of the treatment with fraction 4 was observed. Fig. 3 shows that fraction 4 inhibits the growth of HeLa cells in a dose-dependent manner with an IC_{50} of 40µg/ml. While a concentration of 50µg/ml inhibited 80% of cell proliferation, a complete inhibition was observed at 60µg/ml of fraction 4. The cytotoxic effect of the fifth and the sixth fractions extracted from *U. pilulifera* leaves showed moderate toxicity against Hela cells, with IC_{50} of 80.4µg/ml and 50.3µg/ml for the fifth and the sixth fractions respectively. Altogether, these results show that fraction 4 has the most potent cytotoxic effect and is therefore used for further chemical and biological analysis to identify its bioactive ingredients and how it exerts its biological effects.

Fraction 4 induces cell cycle arrest and intrinsic apoptosis

To determine the mechanism of action that fraction 4 exerts to induce its effect on Hela cells, we detected the levels of cell cycle and apoptosis proteins in treated and control cells. Proteins assessed in this study are (P53, P21, PARP-1, BCL-2, Caspase 9 and P38). The cells were treated with different concentrations (0, 20, 40 and 60µg/ml) of fraction 4 for 48 hours. The levels of these proteins were then detected by western blotting. Results demonstrate that P53 and its downstream target p21

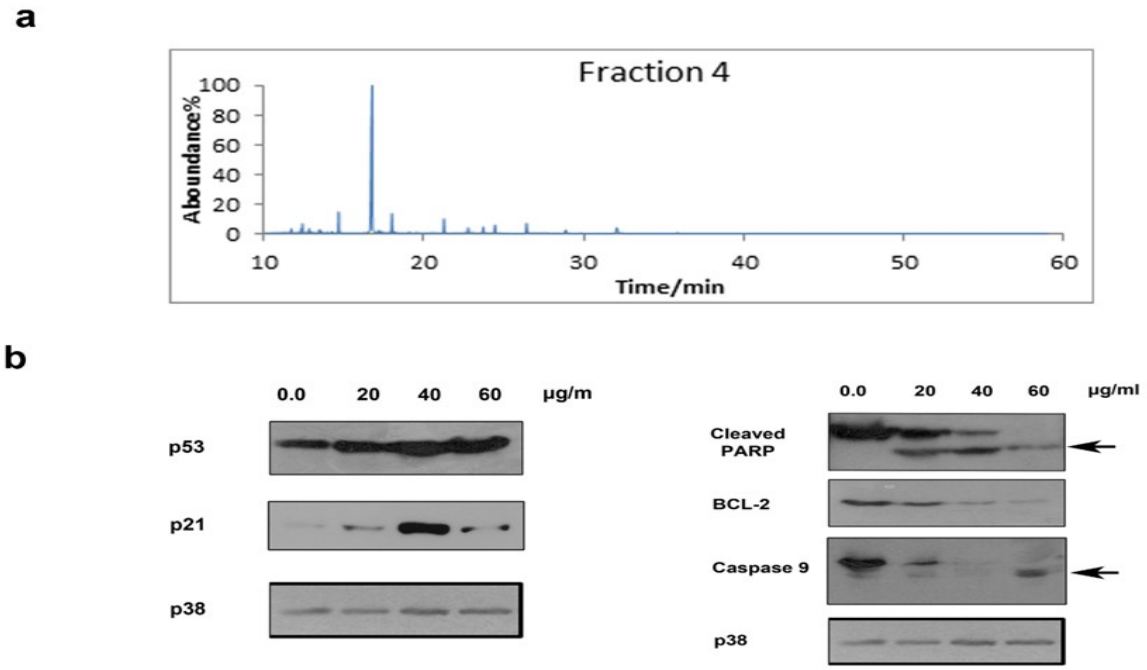

Fig. 4: (a) Chromatogram profile for fraction 4. (b) Alterations in cell cycle and apoptosis proteins in response to fraction 4 treatment. Cells were treated with increasing concentrations (0, 20, 40 and 60 µg/ml) of fraction 4 and incubated for 48 hours. Western blotting was performed to detect any alterations in P53, P21, cleaved PARP-1, BCL-2, Caspase 9 and P38 levels.

protein increased remarkably in the cells treated with high concentrations of fraction 4 (fig. 4b). Furthermore results show that fraction 4 treatment induced intrinsic apoptosis as evident by the increasing levels of cleaved PARP-1 and active caspase 9 (fig. 4b). Importantly, the anti-apoptotic protein BCL-2 decreased significantly in fraction 4 treated cells. Altogether, these findings show that fraction 4 treatment mainly induces cell cycle arrest and intrinsic apoptosis in Hela cells.

Gas Chromatographic- Mass Spectrometric analysis of the fractions

All fractions were subjected to GC-MS to identify their chemical components. The same conditions applied in the analysis of crude extract have been set here. Phytochemicals of all fractions were identified and matched with phytochemical constituents of the crude extract (table S2). The chromatogram of each fraction was obtained and the chromatogram of fraction 4 is represented (fig. 4a).

Bioassay-guided analysis of fraction 4

Fraction 4 has been further analyzed qualitatively using GC-MS to identify its bioactive components. Results revealed the presence of nine phytochemical compounds. The first component that has been identified with the less retention time was 6, 10, 14-trimethyl-2-Pentadecanone, whereas heptacosanol which took the longest retention time was the last one to be identified. These compounds with their retention time (RT), percentage of matching (qual), molecular formula and percentage of peak area are presented in table S3. The chemical structure and mass spectrum of fraction 4 components are shown in fig. 5.

DISCUSSION

Common cancer treatments are ineffective in many cases because of their high toxicity and side effects on normal cells. Hence the interest was directed to discovering plants' properties and their therapeutic capabilities. Many plants proved to be effective in treating several serious diseases. For example, *U. pilulifera* was reported as a popular treatment used by the Palestinians in the West Bank, Palestine to treat cancer and other diseases (Ali-Shtayeh *et al.*, 2000). It was also reported that people swallow a mixture of *U. pilulifera* seeds with honey to treat several types of cancer, most notably leukaemia (Wetherilt, 1992). Based on the above data we explored the anticancer effect of different extracts of *Urtica pilulifera* and identified the bioactive compounds in these extracts. This study provides several lines of evidence that the hexanic extract of *Urtica pilulifera* holds promise as an anticancer natural extract.

The current results displayed that the hexanic crude extract of *U. pilulifera* leaves has a potent antiproliferative effect on Hela cells superior to methanolic and distilled water extracts. The calculated IC_{50} at 48 hours for hexanic extract was 45µg/ml, while the methanolic and distilled water extracts had IC50s of

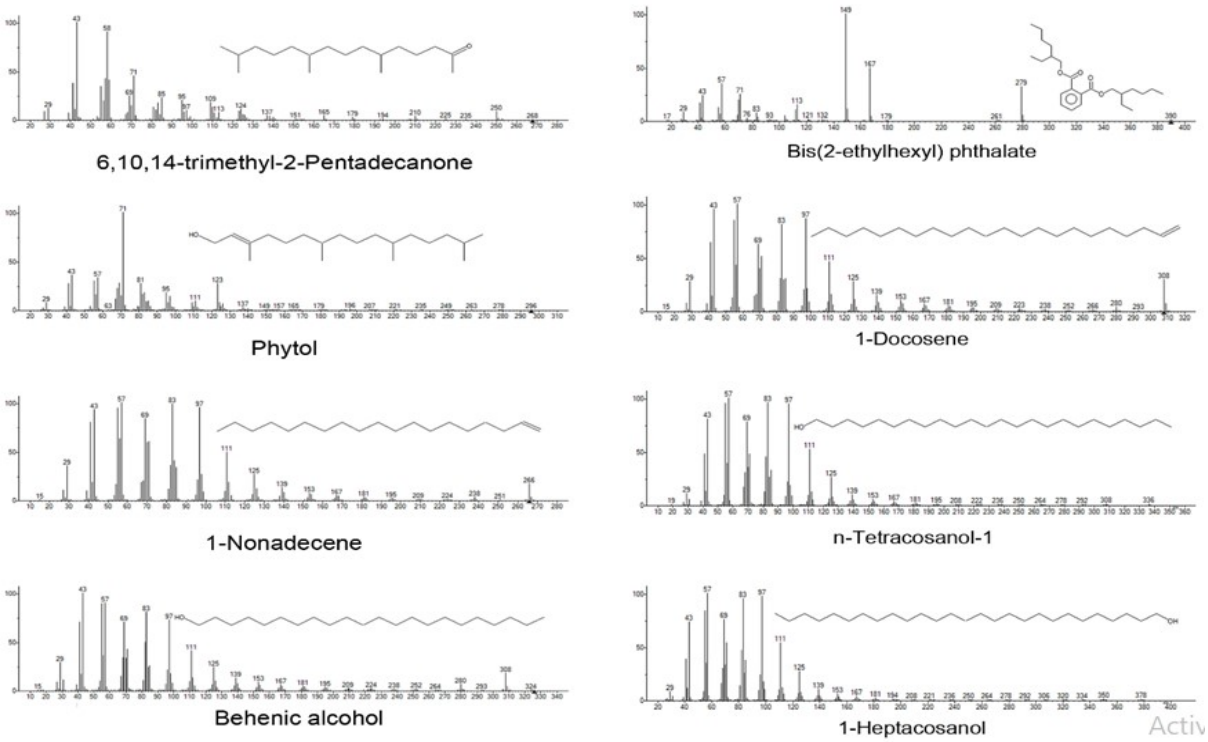

Fig. 5: Chemical structure and mass spectrum of fraction (4) components.

120.5µg/ml and 80µg/ml, respectively. These results are consistent with another study that showed that *U. pilulifera* extract induces significant toxicity and antiproliferative activity in different cancer cell lines (Kichaoui *et al.*, 2016).

Phytochemical constituents differ from one plant to another, as well as phytochemicals obtained from any specific plant differ drastically based on where they were cultivated, when they were collected, or even from one year to another in the same region (Li-Weber, 2013). The solvents used also contribute to the nature of the chemical composition extracted from the plant. Solvents used in this study (water, methanol and hexane) were selected as they have very low toxicity to human cells. The current study revealed the presence of many biologically active phytochemicals extracted from *U. pilulifera*. GC-MS analysis showed that the major class of obtained compounds is hydrocarbons such as eicosane and pinane.

Additionally, there were fatty alcohol compounds such as behenic alcohol and n-tetracosanol-1(lignoceric alcohol). Furthermore, fatty acid compounds such as linolenic acid were also detected. The volatile nature of the obtained compounds is due to the action of hexane as a solvent. The presence of linolenic acid, phytol and 6,10,14-trimethyl-2-pentadecanone in our analysis of the crude extract is consistent with their presence by another analysis performed on the *U. dioica* a species belongs to the same Urticaceae family (Ilies *et al.*, 2004). Previous studies have reported that some of the identified phytochemicals as phytol (J. P. Costa *et al.*, 2016) and squalene (Ravi Kumar *et al.*, 2016) possess antioxidant properties. Linolenic acid was reported to have cancer preventive, anti-inflammatory, hepatoprotective, antieczemic, insectifuge, nematicide and hypocholesterolemic activities (Sermakkani and Thangapandian, 2012). The presence of these biologically active components justifies the use of *U. pilulifera* for various therapeutic purposes in traditional medicine.

From a synthetic viewpoint, the main difference between effective fractions of hexanic extract (4, 5 & 6) and the ineffective ones (1, 2 & 3) is the phytol content of the effective fractions. Of these active fractions, fraction 4 is the most effective one and this might be attributed to the high amount of phytol in this fraction. Of the ten compounds identified in fraction 4, the most prevalent compound was phytol (68.6266%) followed by 6, 10, 14-trimethyl-2-pentadecanone (4.5058%) and n-tetracosanol-1 (3.9588), followed by 1-heptacosanol (3.721) and 1-nonadecene (3.3658). Among these ten compounds just two compounds were reported to have antioxidant activity: phytol and 1-nonadecene (Wei *et al.*, 2011; Renukadevi *et al.*, 2011; Raman *et al.*, 2012; Costa *et al.*, 2016). Phytol is a major member that belongs to terpenoids, especially the diterpenes group that is well known to have antioxidant properties (Baccouri and Rajhi, 2021). Phytol is a basic component of chlorophyll and commonly serves as a precursor for manufacturing

Table S1: Chemical components identified in Urtica pilulifera crude extract

No.	RT	Name of the compound	Molecular Formula	Molecular weight	Peak area %
1	12.364	Pinane	$C_{10}H_{18}$	138.2499	19.788
2	12.433	6,10,14-trimethyl-2-Pentadecanone	$C_{18}H_{36}O$	268.4778	0.268
3	12.832	Diisobutyl phthalate	$C_{16}H_{22}O_4$	278.3435	0.525
4	13.975	Isophytol	$C_{20}H_{40}O$	296.5310	0.217
5	14.293	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.3435	1.512
6	16.511	Methyl linolenate	$C_{19}H_{32}O_2$	292.4562	0.429
7	16.731	Phytol	$C_{20}H_{40}O$	296.5310	14.868
8	17.626	Linolenic acid	$C_{18}H_{30}O_2$	278.4296	0.3155
9	19.55	[E]-5-Eicosene	$C_{20}H_{40}$	280.5316	0.338
10	21.3749	1-Nonadecene	$C_{19}H_{38}$	266.5050	0.5119
11	21.837	Tetradecanal	$C_{14}H_{28}O$	212.3715	0.419
12	22.7901	Behenic alcohol	$C_{22}H_{46}O$	326.6000	1.097
13	23.7254	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.5561	0.212
14	25.187	1,19-Eicosadiene	$C_{20}H_{38}$	278.5157	0.283
15	26.562	n-Tetracosanol-1	$C_{24}H_{50}O$	354.6532	3.715
16	28.919	1-Docosene	$C_{22}H_{44}$	308.5848	0.2853
17	29.895	Squalene	$C_{30}H_{50}$	410.7180	0.4152
18	32.2692	Heptacosanol	$C_{27}H_{56}O$	396.7329	19.2677
19	41.546	Eicosane	$C_{20}H_{42}$	282.5475	20.4072
20	49.4712	Undefined			
21	53.8959	Undefined			

Table S2: Matching crude extract Components with fraction components

Pk	Rt	Name of the compound	F1	F2	F3	F4	F5	F6
1	12.364	Pinane	√					
2	12.433	6,10,14-trimethyl-2-Pentadecanone				√	√	
3	12.832	Diisobutyl phthalate				√	√	
4	13.975	Isophytol			√			
5	14.293	Dibutyl phthalate		√				
6	16.511	Methyl linolenate						√
7	16.731	Phytol				√	√	√
8	17.626	Linolenic acid		√				
9	19.55	[E]-5-Eicosene			√			
10	21.3749	1-Nonadecene				√		
11	21.837	Tetradecanal	√					
12	22.7901	Behenic alcohol				√	√	
13	25.187	1,19-Eicosadiene	√					
14	26.562	n-Tetracosanol-1				√		
15	28.919	1-Docosene				√	√	
16	29.895	Squalene			√			
17	32.2692	Heptacosanol				√	√	
18	41.546	Eicosane	√					

synthetic forms of vitamin E and vitamin K1 (Li *et al.*, 2016).

Several studies have documented the activity of phytol as an antioxidant and anticancer compound (Islam *et al.*, 2015; de Alencar *et al.*, 2019).

A similar result to ours was achieved by a study designed to examine the antioxidant and anticancer activity of 5 different extracts of *Thymus serpyllum* on MCF-7, MDA-MB-231, HepG2, A549, PC3 and HCT-116 cell lines. Findings revealed that only hexanic extract showed the best anticancer effect against all cell lines. The GC-MS profile of this hexanic extract demonstrated the presence of major compounds that are well known as antioxidants including phytol (Salma Baig *et al.,* 2014).

At the molecular level, we investigated p21 protein (cyclin-dependent kinases inhibitor) expression to assess cell cycle progression status under the effect of fraction 4. Western blotting analysis showed a markedly increased level of P21 protein compared to control cells suggesting that fraction 4 inhibited the cell cycle progression (Wang *et al.*, 2021). Results also showed a noticeable augmented level of the major anti-tumor protein (p53), which regulates both cell cycle arrest and apoptosis

Table S2: Matching crude extract Components with fraction components

Pk	Rt	Name of the compound	F1	F2	F3	F4	F5	F6
1	12.364	Pinane	√					
2	12.433	6,10,14-trimethyl-2-Pentadecanone				√	√	
3	12.832	Diisobutyl phthalate				√	√	
4	13.975	Isophytol			√			
5	14.293	Dibutyl phthalate		√				
6	16.511	Methyl linolenate						√
7	16.731	Phytol				√	√	√
8	17.626	Linolenic acid		√				
9	19.55	[E]-5-Eicosene			√			
10	21.3749	1-Nonadecene				√		
11	21.837	Tetradecanal	√					
12	22.7901	Behenic alcohol				√	√	
13	25.187	1,19-Eicosadiene	√					
14	26.562	n-Tetracosanol-1				√		
15	28.919	1-Docosene				√	√	
16	29.895	Squalene			√			
17	32.2692	Heptacosanol				√	√	
18	41.546	Eicosane	√					

Table S3: Components identified in fraction 4.

PK	RT	Name of the compound	Qual	Formula	Peak area %
1	12.4213	6,10,14-trimethyl-2-Pentadecanone	91	$C_{18}H_{36}O$	1.6883
2	16.7883	Phytol	93	$C_{20}H_{40}O$	68.6266
3	18.0129	Pentadecyl trifluoroacetate	94	$C_{16}H_{34}$	4.5058
4	21.2649	1-Nonadecene	91	$C_{19}H_{38}$	3.3658
5	22.7784	Behenic alcohol	90	$C_{22}H_{46}O$	1.4384
6	23.7199	Bis (2-ethylhexyl) phthalate	91	$C_{24}H_{38}O_4$	1.8193
7	24.4535	1-Docosene	91	$C_{22}H_{44}$	2.5289
8	26.4348	n-Tetracosanol-1	94	$C_{24}H_{50}O$	3.9588
9	28.8724	1-Docosene	91	$C_{22}H_{44}$	1.4506
10	32.0783	Heptacosanol	91	$C_{27}H_{56}O$	3.7210

(Androutsopoulos and Spandidos, 2018). Our results demonstrated both cell cycle arrest and a clear inverse relationship between Bcl-2 and caspase-9 in a way proves the induction of apoptosis (Thakor *et al.*, 2017a). Similar to our results, researchers attributed the anti-carcinogenic activity presented by *U. pilulifera* to apoptosis induction through the intrinsic pathway in addition to cell cycle arrest (Aliwaini and Lubbad, 2016).

Phytol showed a wide variety of anticancer mechanisms and pathways as reported by *In Vitro* studies. For example, phytol succeeded in targeting hepatocellular cancer cells Huh7 and HepG2 by inducing intrinsic apoptosis through activating caspase-9/3and cleaving (PARP) (Kim *et al.*, 2015). The same study showed the ability of phytol to inhibit epithelial-mesenchymal transition (EMT) via loss of E-cadherin. Another similar study attributed phytol anti-proliferation effect on human lymphoid leukemia Molt 4B cells to the induction of apoptosis based on forming apoptotic bodies and fragmentation of DNA (Hibasami *et al.*, 2002). Phytol exhibited a strong anti-proliferative effect against human lung adenocarcinoma cell line A549 through mitochondrial membrane depolarization, increasing the number of cells in a sub-G0 phase, Bax overexpression, Bcl-2 down-regulation, inhibition of vascular growth and activation of caspases 3 and 9 (Sakthivel *et al.*, 2018). Phytol also activated FAS, TNF-α and TRAIL receptors, in addition to its binding with glucose-6-phosphate dehydrogenase (G6PD) which has a role in tumor development and tumorigenesis in the A549 cell line (Thakor *et al.*, 2017).

Importantly, several studies conducted on animal models showed that *U. pilulifera* is not toxic for normal tissues, therefore we didn't test it in normal cells. For example, a study conducted to test the toxicity of fixed and volatile oils extracted from leaves and seeds of *U. pilulifera* on Swiss Albino mice showed that *U. pilulifera* is not toxic even at high doses up to12.8 ml/kg (Özbek *et al.*, 2004).

In addition to this, another study found that exposure of mice to the hexane extract of *U. pilulifera* at doses up to 2 g/kg does not affect the physiological and pathological parameters (Abo-elmatty *et al.*, 2013). This belief was additionally supported by our previous study to assess the preventive activity of *U. pilulifera* leaves against 1, 2-dimethylhydrazine (DMH) - induced colon cancer. Results showed a 40% reduction in tumor weight in response to *U. pilulifera* treatments. Interestingly, *U. pilulifera* did not show any side effects either at the visible or histological level as opposed to the garlic that induced necrotic lesions and inflammatory reactions in the colon wall (Aliwaini and Lubbad, 2016).

CONCLUSION

Altogether, this study showed that *Urtica pilulifera* hexanic extract and especially fraction 4 and its main component, phytol, possess a strong anti-cervical cancer effect. Fraction 4 of *Urtica pilulifera* hexanic extract displays its bioactivity through the recruitment of two major mechanisms; cell cycle arrest and apoptosis. This study highlights the ability of *Urtica pilulifera* and its ingredients to influence anticancer therapeutic industries.

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