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Screening of anticancer potential of leaf aqueous extract from *Pulicaria incisa* growing wild in Palestine

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The anticancer potential of *Pulicaria incisa* aqueous extract was estimated by measuring its cytotoxic and cytostatic effect on human cervical cancer (Hela), human colorectal adenocarcinoma (HT-29), and skin melanoma cancer (B16–F1) cell lines. This effect was investigated by the exposure of cancer cell lines to the aqueous extract of *P. incisa* at different concentrations (5, 2.5, 1.25, 0.625, 0.3215 mg/mL) for 24 and 72 hours. The results showed that the studied extract inhibited the growth of the various cell lines, eventually leading to cell death, as shown by both MTT and LDH assays. The extract of *P. incisa* displayed the strongest cytotoxic effect against HT-29 cells at high concentrations at which viability was nearly zero %, while the strongest cytostatic effect of the prepared extract was on the B16–F1 cells at high concentrations. Nonetheless, *P. incisa* extract gave exciting results on Hela cells with a cytotoxic effect at high concentrations and a cytostatic effect at low concentrations. In conclusion, the cytotoxic and cytostatic effect of *P. incisa* aqueous extract against the three cell lines is due to the presence of several phytochemicals. So, using this plant species as a source for antitumor agents alone or in combination with other agents for cancer treatment is proposed.

Keywords: Anticancer, Cytostatic, Cytotoxic, Extract, *Pulicaria incisa*

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Introduction

The second leading cause of morbidity and mortality worldwide is cancer, particularly in low and middle-income populations[1]. The outcomes of cancer patients have greatly improved in recent years due to the development of surgery, radiotherapy, and chemotherapy[2]. The currently used anticancer agents exhibit serious side effects and combat increasing resistance from the target cancer cells. Therefore, it is necessary to find out and develop other innovative anticancer remedies[3]. Recently, it has been proved that natural products significantly played a role in creating novel curative agents. Several mechanisms utilizing natural bioactive compounds proved to reduce cancer outcomes. It includes endoplasmic reticulum stress, epigenetic modification, and modulation of oxidative stress[2]. Around 60% of the current anticancer drugs were derived from nature, specifically from the plant kingdom. This Kingdom contains several diverse phytochemicals that are essential in the treatment and prevention of various diseases[3]. In this regard, herbal treatment is found to be a safe method for cancer treatment and prevention[1].

Plants can synthesize secondary metabolites, including aromatic substances such as phenols or their oxygen-substituted derivatives[4]. Plants use these substances as a defence mechanism against predation by microorganisms, insects, and herbivores. In this aspect, plants produce enormous and highly diverse phenolic metabolites that contain one or more acidic hydroxyl residues attached to an aromatic (phenyl) ring[5]. On the other hand, naturally occurring phenolic compounds, in addition to others like ascorbic acid and vitamin E, possess an antioxidant power to reduce the oxidative damage associated with many diseases[6]. According to that, many researchers have focused on plant natural antioxidants, including their crude or raw extracts and pure natural compounds.

P. incisa (Lam.) DC., also known as Tamayout or Ameo from the Asteraceae family, is an endemic aromatic and medicinal plant with golden yellow flowers[7]. This plant species is traditionally used to treat human pains such as flu, fever, coughs, and diabetes[8]. In addition to carminative and anti-inflammatory issues[9]. *P. incisa* is also used as a substitute for tonic and tea, as well as an antispasmodic, hypoglycemic,

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and as one of the local perfume ingredients[10]. The pharmacological properties of isolated phytochemical metabolites from *P. incisa* justify the traditional use of this plant species. Among these phytochemicals are monoterpenes, flavonoids, acetylenes, isocomene, sesquiterpene lactones, and derivatives of caryophyllene[11-13].

Based on all previously mentioned information, the idea of the present study emerged to evaluate the *in vitro* anticancer activity of *P. incisa*, which is growing wild in Palestine. The anticancer potential of the *P. incisa* aqueous extract was evaluated by measuring its cytotoxic and cytostatic effect on (Hela), (HT-29), and (B16–F1) cell lines.

Materıals and Methods

Plant material

The wild plant species (*P. incisa* (Lam.) DC) was collected from Jrico/West Bank, Palestine. The plant under study was identified and classified based on Flora Palaestina[14,15] by a plant taxonomist, Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University, Palestine. A plant specimen with voucher number (1474) was deposited at An-Najah National University herbarium. The plant material for the anticancer study was washed, air–dried, ground, and stored in a dry place at room temperature.

Extract preparation

For aqueous extract, five grams of crushed plant sample was soaked in 100 mL of sterile warm distilled water at room temperature for one week with continuous rotary shaking. Then, the mixture was centrifuged for 10 min at 4500 rpm, and the obtained supernatant was dried by lyophilization[16]. The lyophilized powder was dissolved in RPMI media to a final concentration equal to 10 mg/mL. After that, the mixture was sterilized by microfiltration.

Cell lines and culture medium

Human cervical cancer (Hela), Human Colorectal Adenocarcinoma (HT-29), and skin melanoma cancer (B16–F1) cell lines under study were obtained from ATCC (American Type Culture Collection). The cells were cultured in liquid Roswell Park Memorial Institute (RPMI) media freshly supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% amphotericin, and 1% L-glutamine. Cells were incubated in a CO_2 incubator at 37 °C, 95% humidity, and 5% CO_2 at dark until cell confluence reached 90%.

MTT Assay

MTT assay is a colourimetric assay that is based on the reduction of a yellow tetrazolium salt (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide) (MTT) by NAD(P)H–dependent oxido–reductase enzymes in the viable cells to a deep purple coloured insoluble crystalline formazan[17]. According to the MTT kit (Sigma), 20,000 cells/100 µL total volume/well for the cytotoxic test and 5,000 cells/100 µL total volume/well for the cytostatic test were treated with 100 µL of 5–0.3125 mg/mL concentrations of the studied plant species. It was then incubated for 24 hours for the cytotoxic test and 72 hours for the cytostatic test. After incubation, the media was removed from each well and washed with PBS. Then, 100 µL of serum-free RPMI media was added to each well, followed by 10 µL MTT solution (0.5 mg/mL) and incubated for four hours. Following the removal of the medium and subsequent washing, cells were treated with 100 µL of acidic isopropanol (0.08N HCl) for 15 min to dissolve the formazan crystals. The absorbance of MTT formazan was determined at 570 nm in a microplate reader (Labtech, UK). Cell viability was calculated according to the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{Treatment Absorbance}}{\text{Control Absorbance}} \text{ X } 100\%$$

LDH Assay

LDH assay is a colourimetric assay for quantifying cell death and cell lysis based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The lactate dehydrogenase (LDH) test looks for signs of damaged cells[17]. According to the LDH kit (Roche), 20,000 cells/100 µL total volume/well for the cytotoxic test and 5,000 cells/100 µL total volume/well for the cytostatic test were applied in microplates. In the beginning, the high (maximum LDH release), low (spontaneous LDH release), and background (LDH activity in the assay medium) controls were constructed. Cells were treated with 100 µL of 5–0.3125 mg/mL concentrations of the studied plant species and incubated for 24 hours in cytotoxic analysis and for 72 hours in cytostatic ones. After incubation, 100 µL/well supernatant was carefully removed and transferred into corresponding wells of a new microplate. Then, 100 µL of the freshly prepared reaction mixtures (catalyst and dye solution) were added, followed by 30 min of incubation at room temperature in the dark. After that,

the absorbance of the samples at 490 nm was measured using a microplate reader (Labtech, UK). The resulting values are substituted in the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{A Treatment} - \text{A Low control}}{\text{A High control} - \text{A Low control}} \text{ X } 100\%$$

Total Phenol Content (TPC) Determination

The TPC of *P. incisa* extract was determined according to the Folin–Ciocalteau method of Slinkard and Singleton with some modifications as the assay was constructed to be applied through microplate[18]. Triplicate from *P. incisa* extract sample and gallic acid standards (100–0.195 µg/mL) were tested. The total phenolic content of the prepared extract was calculated according to the method of Singleton and Rossi[19]. The obtained results were compared with a gallic acid standard curve, and the total phenolic content of the sample was expressed as mg of gallic acid equivalents per gram of extract using the following formula:

$$C = C1\frac{V}{m}$$

where C = total phenolic content in mg/g in gallic acid equivalent, C1 = concentration of gallic acid established from the standard curve in mg/mL, V = volume of extract in mL, and m = the weight of the plant extract in g.

DPPH Antioxidant assay

The free radical assay was carried out in a microplate using 2,2–diphenyl–1–picryl–hydrazyl–hydrate (DPPH)[20]. The assay started with the addition of 100 µL of various concentrations of extract in methanol (50–0.195 µg/mL) and 100 µL of different concentrations (50–0.195 µg/mL) of the standard (ascorbic acid) to 100 µLof 0.01% DPPH dissolved in methanol. After a 30-minute incubation period at room temperature in the dark, the absorbance for all examined wells was measured using a microplate reader (Labtech, UK) at 540 nm. The DPPH radical scavenging activity (%) was calculated as the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \text{ X } 100\%$$

where the absorbance of control [DPPH + Methanol without sample] and the absorbance of the sample [DPPH + Sample (extract/standard)].

The dose-response curve was obtained using Microsoft Excel by plotting the percentage inhibition versus concentration. Then, the concentration that inhibited 50% of the DPPH radical scavenging activity (IC50) was determined by non–linear regression.

Results

Effect of plant extract on cancer cell line activity

P. incisa extract anticancer bioactivity effect was tested against three cell lines, (Hela), (HT-29) and (B16–F1) cell lines. This effect was examined by the exposure of cancer cell lines to the aqueous extract of *P. incisa* at different concentrations (5, 2.5, 1.25, 0.625, and 0.3215 mg/mL) for 24 and 72 hours. The results showed that the studied extract inhibited the growth of the various cell lines, eventually leading to cell death, as shown by both MTT and LDH assay.

MTT Assay results

The treatment of HT-29 cancer cells by *P. incisa* aqueous extract led to a almost similar cytotoxic and cytostatic effect at 5 mg/mL concentration. On the other hand, the lowest examined concentration (0.3125 mg/mL) of this extract gave up cyostatic behaviour rather than cytotoxic one (Fig. 1a). MTT

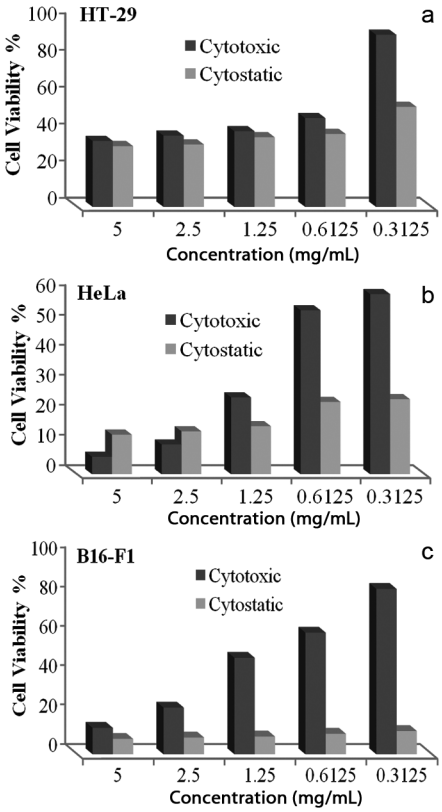

Fig. 1 — *P. incisa* aqueous extract cytotoxic and cytostatic effect on a) HT-29 cells; b) HeLa cells; and c) B16–F1 cells at different concentration by MTT assay.

results proved that *P. incisa* extract showed a cytostatic effect more than the cytotoxic effect on Hela cells (Fig.1b). Cytotoxic agents in the examined plant extract result in the killing of HeLa cells and promote 40% cell death at low concentration (0.3125 mg/mL).

Thus, at a high concentration (5 mg/mL), the plant extract led to around 95% cytotoxic effect. It could still have a good cytotoxic effect if continued for a lower concentration. While cytostatic agents found in the same extract inhibited tumour growth without direct cytotoxicity at a low concentration (0.3125 mg/mL), causing 75% death of HeLa cells. *P. incisa* extract also showed a more cytostatic effect than the cytotoxic effect on B16–F1cells as provided by MTT results (Fig. 1c). The recorded data at low concentration of the examined extract (0.3125 mg/mL) indicated that B16–F1 cell viability reached11.8%. Moreover, at other plant extract concentrations (2.5, 1.25, and 0.625 mg/mL), the cell viability decreased to around 10%. However, the cytotoxic effect of high concentration (5 mg/mL) gave 84% cell death.

LDH Assay results

LDH is used to support and emphasize MTT assay results. Results of LDH clearly showed that the percentage of cell death in HT-29 cells at 5 mg/mL plant extract concentration reached 98.0% after 24 hours (Fig. 2a). However, the cytostatic effect of this extract at the same concentration (5 mg/mL) is equal to 74% cell death. The percentage of HeLa cell death at high extract concentrations was around 63.0% after 24 hours treatment (Fig. 2b). Compared to lower concentrations, the studied extract was not cytotoxic effective. Contrary to the cytotoxic activity of *P. incisa* extract, all screened concentrations revealed very low cytostatic activity. Fig. 2c showed the LDH results of *P. incisa* extract against B16–F1 cells. The obtained results illustrate that the strongest cytostatic effect of this plant extract at all studied concentrations was against B16–F1 cells. However, the extract of *P. incisa* displays the strongest cytotoxic effect against HT-29 cells at high concentrations at which viability is nearly 0% (Table 1). The strongest cytostatic effect of the prepared extract was on the B16–F1 cells at high concentrations. Nonetheless, *P. incisa* extract gave amazing results at low concentrations on Hela cells (Table 2).

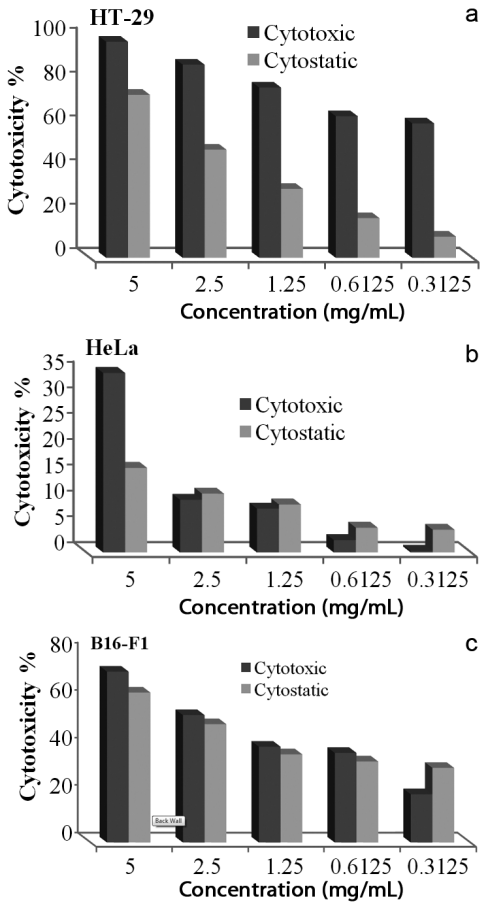

Fig. 2 — *P. incisa* aqueous extract cytotoxic and cytostatic effect on a) HT-29; b) HeLa cells; and c) B16–F1 cells at different concentrations by LDH assay.

Table 1 —*Pulicaria incisa* aqueous extract cytotoxic and cytostatic effect on HT-29, HeLa and B16–F1 cell lines at different concentration by MTT assay

	HT-29		HeLa		B16–F1	
Concentration (mg/mL)	Cytotoxic* (%)	Cytostatic (%)	Cytotoxic (%)	Cytostatic (%)	Cytotoxic (%)	Cytostatic (%)
5	64.3	67.1	94.2	86.8	86.6	92.2
2.5	61.4	66.2	90.1	85.7	76.3	91.5
1.25	59	62.3	74.4	84	51	91
0.6125	51.9	60.5	45.4	75.9	38.2	89.6
0.3125	6.7	45.8	40	75	16	88.2

*Percentage of dead cells

Table 2 —*Pulicaria incisa* aqueous extract cytotoxic and cytostatic effect on HT-29, HeLa and B16–F1 cell lines at different concentrations by LDH assay

	HT-29		HeLa		B16–F1	
Concentration (mg/mL)	Cytotoxic * (%)	Cytostatic (%)	Cytotoxic (%)	Cytostatic	Cytotoxic (%)	Cytostatic (%)
5	98.1	74	63	16.4	71.9	93.1
2.5	87.7	49.1	10.2	11.4	53.6	49.8
1.25	77.3	31.3	8.5	9.3	40.3	37.1
0.6125	64.3	18	2.4	4.8	37.7	34.1
0.3125	61	9.6	0.3	4.4	20.3	31.4

*Percentage of dead cells

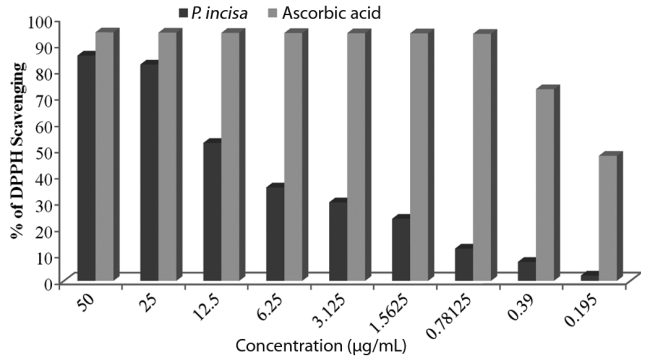

Fig. 3 —Percentage of DPPH inhibition activity of the aqueous extracts from *P. incisa* and ascorbic acid.

Total phenol content and antioxidant results

The current research estimated the TPC of *P. incisa* leaf aqueous extract. The TPC was calculated from the regression equation of the calibration curve: ($y = 0.0069x + 0.0696$), $R^2 = 0.9994$, and expressed in gallic acid equivalents per gram dry extract weight. The amount of total phenolic content in this aqueous extract is equal to 155 mg/g in GA equivalent. In addition, the prepared extract showed a high DPPH scavenging power that resembles the scavenging power of ascorbic acid mainly at high concentration, as it reaches around 80% at 50 µg/mL concentration (Fig. 3). Given that, the IC50 value for this aqueous extract was 20.9274 µg/mL.

Discussion

The search for new chemopreventive agents against cancer has shed light on the field of phytochemicals. From the down of the area of medicine, the plant kingdom has been playing a vital role in providing diversity in medications used for cancer treatment[21]. Combination therapy is a smart strategy that enhances the activities of different drugs and reduces both the dose as well as side effects. *P. incisa*, the studied plant species in this research, was reported to be subjected to a different phytochemical screening. The phytochemical analysis of *P. incisa* showed an intermediate amount of total tannins, alkaloids, and saponins reported to have medicinal activity and exhibit physiological activity[22]. High concentrations of carvotanacetone and chrysanthenone were found in both leaves and flower oils[23]. Also, El–Shahaby *et al.* quantified the active secondary ingredients in *P. incisa*, including alkaloids, phenolics, flavonoids, and tannins[24]. Furthermore, *P. incisa* contains large quantities of total flavonoids and total phenolics, especially in the flower[7,25]. Consequently, *P. incisa* total aerial part can be used as an antitumor agent. According to the literature, flavonoids have been shown to have anticancer activities[26]. They intervene with many signal transduction pathways, leading to proliferation, angiogenesis, and metastasis limitation or apoptosis enhancement. Moreover, phenols have antibacterial and anti-inflammatory activities[27]. Also, they are responsible for an antioxidant and free radical scavenging effect of plant materials[28]. In addition to that, phenols possess a wide range of biological activities, most of which are correlated to the control of carcinogenesis and have been identified to affect all stages of cancer progression[29]. In this aspect, phenolic extracts were shown to inhibit the growth of HT-29, and HCT–116 tumour cell line[30]. Likewise, they showed a strong anti-skin cancer effect[31]. Similarly, tannins have been found to have powerful anticancer activities against various cancer cells. Generally, proliferation inhibition, apoptosis induction, invasion suppression, and angiogenesis inhibition are predominantly influenced by tannins[32]. Moreover, several alkaloids isolated from natural herbs have demonstrated both *in vitro* and *in vivo* anti-proliferation and anti-metastasis effects against many types of cancers. Camptothecin and vinblastine are examples of alkaloids that have been successfully formulated to be used as anticancer medicines[33]. In this regard, other compounds such as camptothecin, a topoisomerase I inhibitor, and vinblastine, which interacts with tubulin, have already been successfully

developed into chemotherapeutic drugs[34]. In addition to the mentioned phytochemicals, plant saponins have been already reported to possess a wide range of biological activities[35]. These activities include the anticancer cytotoxic activity of saponins, as they have a significant cytotoxic effect on HeLa cells[36]. Moreover, saponins reveal significant anticancer activities by targeting various cancer-related proteins and pathways, such as cell cycle arrest, apoptosis induction, ER stress activation, migration inhibition, invasion inhibition, and MDR reversal[37]. According to the previous information, the results in the current research indicated that *P. incisa* has significant cytotoxic and cytostatic activity against (Hela), (HT-29), and (B16–F1) cell lines. These activities may be explained by the fact that the prepared extract could be affecting the cell cycle by stimulating cell death through the activation of caspases, in addition to the induction of cell cycle arrest and apoptosis. However, the finding in this research is in accordance with El–Naggar *et al.*, study as they also found that the *P. incisa* extract was one of their tested plants that showed high anticarcinogenic impact on HepG–2 and/or MCF7 cell lines[38]. *P. incisa* provides a high cytotoxic effect against MCF-7 cell lines (less than 30 ug/mL). The presence of secondary metabolites like phenolics and flavonoids that offer antioxidant capacity may be the possible causative agents for such cytotoxic activities. It was shown that the leaf and flower oils of *P. incisa* have cytotoxic activity against liver cell carcinoma HEPG–2 which was determined using an MTT assay. That suggested the efficient cytotoxic activity of leaf oil may be attributed to its high content of carvotanacetone[24]. Also, previous reports have shown that *P. jaubertii* has anticarcinogenic and chemopreventive activity[39,40].

Conclusion

The cytotoxic and cytostatic effect of *P. incisa* against the three cell lines is due to the presence of several phytochemicals. So, it is proposed to use this plant species as a source for antitumor agents either alone or in combination with other agents for cancer treatment. Furthermore, elaborated *in vivo* studies are required to understand the medicinal values against cervical, colorectal, and melanoma cancers. Taken into account, the anticancer effect of plant extracts depends on the plant species, the solvent used, and the used concentrations. In conclusion, traditional medicinal plants in Palestine are a valuable source for the discovery and formulation of new anticancer agents. However, there is a need for further studies that fully characterize their activity in order to exclude or include other compounds that may be found in *P. incisa*.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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