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In vitro Comparative Study for Anti-proliferative Activity of Some Plant Extracts, Fam. Apiaceae, on Human Cervical (HeLa) Cancer Cell Line

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ABSTRACT

In this research, the biological activities of five plant extracts from family Apiaceae; Italian Parsley (*Petroselinum neapolitanum*), Fennel (*Foeniculum vulgare),* Celery (*Apium graveolens*), Cilantro (*Coriandrum sativum*) and Dill (*Anethum graveolens*), were studied. Antiproliferative effect of eleven ethanol crude extracts was tested in Human Cervical (HeLa) cancer cells. Parsley leaves extract, cilantro leaves extract and cilantro stems extract showed no significant difference with the positive control (Actinomycin D). As for, fennel bulb extracts, fennel stalks extracts, celery stems gave better results than the positive control with no significant difference through the 24, 48 and 72h treatment. There were no significant difference between Fennel extracts and the positive control, which showed high effect on the cancer cells survival. There were no significant difference between both extracts of Cilantro leaves and stems through each time but the best result was after 72h of treatments. Regarding Dill leaves and stems, cell numbers recorded no significant difference between the both on time dependent manner. Further investigation for ethanolic extracts of parsley leaves, fennel bulb, fennel stalks, celery stems, cilantro leaves and cilantro stems which showed better results than using the commercial drug Actinomycin D (25µL/mL) for 24h treatment or less depending on concentrations manner. Also, further investigation on different types of cancer cell lines to avoid the toxic effect of chemotherapy.

Key words: Apiaceae - Anti-proliferative - Extracts - HeLa Cell Line

INTRODUCTION

Nowadays, worldwide patients prefer natural extracts and folk medicine to avoid chemical toxic effect introduction into their bodies under extensive use of synthetic drugs, especially when it involves tumor with the numerous side effects of radiation and/or chemotherapy (Sharma *et al.,* 2011; Taher *et al*., 2019). Between 2012 and 2018, countries with legal and regulatory framework for traditional and complementary medicine increased from 79 to 109 in number (WHO, 2019). On 2018, 124 countries (64% of WHO Member States) reported having laws/regulations on herbal medicines. Most countries reported that, partial coverage was available for traditional and complementary medicine. The 2018 Declaration of Astana on Primary Health Care acknowledges the need to include traditional medical knowledge and technologies in the delivery of primary health care (WHO, 2019).

Out of total 250.000 plant species existing on earth, approximately one thousand exhibited anticancer activities (Bibi *et al.,* 2012 and Artun *et*

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al., 2016). In 2018, World Health Organization reported that cancer is one of the main human diseases and responsible for 9.6 million deaths every year (WHO, 2019). Cancer can be initiated by viral infections, such as hepatitis and human papilloma virus; and cause cervical cancer; (HPV), which is responsible for up to 25% of cancer cases (Plummer *et al.,* 2012). Cervical cancer is one of the greatest threats to women's health. It is the second most common cancer in women living in less developed regions with an estimated 570.000 new cases, almost 84% of worldwide new cases every year (Ferlay *et al.,* 2018). In 2018, WHO reported that, approximately 311.000 women died from cervical cancer; more than 85% of these deaths took place in low- and middle-income countries. This also was confirmed by studies reporting that early treatments of cervical cancer were expensive (WHO, 2019). Chemotherapy and radiation are not more likely to be used for cancer treatment and better to be replaced by herbal treatments/therapy to avoid harming healthy tissue near the affected one (Korrapati *et al.,* 2017). Reducing cancer treatment side effect, also affects the productivity and patient mode which in return decreases fear swings and time consumed for treatment decision.

The aim of this research was to study anticancer effect of some natural herb extracts that is available globally with affordable cost and used in daily meals. These herbs are recommended as prophylactic to minimize cancer risk of those who have cancer history in their family records.

MATERIAL AND METHODS

Sample

This research was conducted at Pharmacology and Cancer biology Dept., Duke University School of Medicine, in collaboration with Keen Lab, Department of Molecular Genetics and Microbiology, Duke University Medical Center, NC, USA. Five plants of *Apiaceae* family were collected during August-September 2018 from Whole Foods local market of North Carolina, USA. Plants were grown, packed and distributed by Cal Organic Farms, division of Grimmway Enterprises, Inc. Bakersfield, CA 93380-1498, USA, Certified organic by CCOF (California Certified Organic Farmers), Non GMO project verified, product of USA. Plant materials were identified and Authenticated by Duke Biology Green houses, Duke University, NC, USA. (Appendix A)

Extraction

Whole plant samples were manually screened; bad ones were removed and then, separated into leaves and stems, dried at ovens of 40-50°C. After complete dryness, plant parts were grinded into fine powder using electric grinder, then, extracted by ethanol (El- Hallouty *et al.,* 2015).

Ethanol extraction were performed by addition of 20g of plant dried powder to 200mL ethanol (95%) and extracted for 48h twice on a shaker (New Brunswick Gyrotory Shaker M. G2, Netkraft Inc, Richmond, VA, USA) (Lall *et al.,* 2018). The extract was filtered using Whatman filter paper No. 2, and concentrated under vacuum using a rotary evaporator (Labconco Centrivap Vacuum Concentrator System, 8811 Prospect Avenue, Kansas City, MO, USA) at 45°C. The extract then freeze dried (lyophilized). All extracts were stored immediately in dark bottles at -20°C until further investigations. Stock solutions of the plant extracts (200mg/mL) were prepared by weighing the powder and dissolving it in 10% DMSO/PBS as a vehicle and kept at −20°C, until used (Solowey *et al.,* 2014 and Bouyahya *et al.,* 2018).

Cell line

Human Cervical Cancer cells (HeLa) were kindly provided by Dr. Matthew Friedersdorf, Molecular Genetics and Microbiology Dept., Duke University Medical Center, NC, USA. The stored cells were first suspended in DMEM, to wash away DMSO, and collected by centrifugation at 1200rpm for 5min and 25°C. The washed cells were re-suspended and cultured in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented by 10% heat-inactive Fetal Bovine Serum (FBS) and 1% Pen-Strep Antibiotic (100U/mL Penicillin and 100μg/mL streptomycin); at 37°C in 5% CO_2 incubator according to the method of Artun *et al.* (2016) and Makridakis *et al.* (2009). Cells were subcultured after they formed a monolayer on the culturing flask and detached by treating with trypsin for 15min. cell suspension was mixed by pipetting to remove any clumps of cells just before a sample was taken. Then complete medium was added to inhibit the reaction (Kontostathi *et al.,* 2017). All media and supplements were obtained from Gibco (Life Science Technology, Waltham, MA, USA).

Table I. Mean of gross weight (g), net weight (g) and lost weight percentage of plants under study.

Plant specie	Scientific name	Gross weight (g)	Net weight (g)	Net weight (%)	Lost weight (%)
Italian Parsley	*Petroselinum neapolitanu*	119.86±09.08	90.19±08.40	75.24	24.75
Fennel	*Foeniculum vulgare*	305.01±29.36	236.44±32.94	77.52	22.48
Celery	*Apium graveolens*	647.21±69.67	592.02±63.91	91.47	08.53
Cilantro	*Coriandrum sativum*	144.03 ±11.72	112.47±05.77	78.09	21.91
Dill	*Anethum graveolens*	120.101±6.65	105.22±07.55	87.61	12.39

Values are Mean ± SE of three independent experiments; Net weight (%) = (net weight/gross weight)*100; Lost weight (%) = 100 – [net weight %]

Table II. Means of fresh weight (g), dry weight (g) and extract yield percentage of selected plants under study.

Plant specie	Part used	Fresh weight (g)	Dry weight (g)	Extract yield (%)
Italian Parsley	Leaves	38.79±3.42	6.73±0.65	5.22
	Stems	51.41±5.15	5.62±0.59	4.66
Fennel	Leaves	9.83±1.23	1.35±0.15	-
	Bulb	131.97±21.31	8.21±1.21	2.43
	Stalk	104.14±14.46	7.22±1.29	1.04
Celery	Leaves	16.82±3.36	1.62±0.35	0.244
	Stems	575.19±60.63	28.30±2.73	1.81
Cilantro	Leaves	44.62±2.16	5.22±0.21	7.26
	Stems	67.85±4.15	3.89±0.21	6.72
Dill	Leaves	50.16±2.62	5.57±0.35	9.35
	Stems	55.07±5.92	3.73±0.39	3.91

Values are Means ± Standard Error (SE) of three independent experiments; Extract yield (%) = weight of extract (g)/ 50g of plant sample × 100 (Karakas *et al.,* 2012)

Cell viability

Viability of cells was determined by trypan blue dye assay using haemocytometer. Cells were washed with HBSS (Hank's Buffered Salt Solution) and centrifuged for 10-15min at 10.000rpm. The procedure is repeated twice. Then, cells were suspended in known quantity of HBSS. Cells were exposed to extracts (100µL/well) and incubated at 37°C for 24, 48 and 72h. After incubation period, dye exclusion test took place, that is, equal quantity of the drug treated cells were mixed with tryphan blue (0.4 %) and left for 1min., then mixture were loaded in a haemocytometer and viable cells were recorded within 2min. Viable cells do not take up color, whereas dead cells take up color. However, if kept longer, live cells also generate and take up color (Unnikrishnan and Ramadasan (1998) and Sumitra and Nagani, (2013)). Number of cells per 1mL was 11.33×10^4. "Actinomycin D" was used as positive control (25µL/mL) while DMSO was used as a negative control.

Data analysis

Statistical results were presented as Mean ± Standard Error (SE) of data obtained from triplicate experiments. Significance test were performed using two-way Completely Randomized Design ANOVA to detect the difference between the treated cells and the control ones. Comparisons among the means of different treatment were carried out, using Duncan's multiple range test procedure at p = 0.05 and 0.01 level of significance, as explained by Snedecor and Cochran (1980) using CoStat (v6.303). Microsoft Excel 2007 was used for the statistical and graphical presentation of data.

RESULT AND DISCUSSION

Five plants from family Apiaceae; were studied in this research according to their medicinal use history. Plants and Plant parts used in this investigation, with their collection time and patch numbers (Table I). Results of gross weight (g), net weight (%) and lost weight (%)

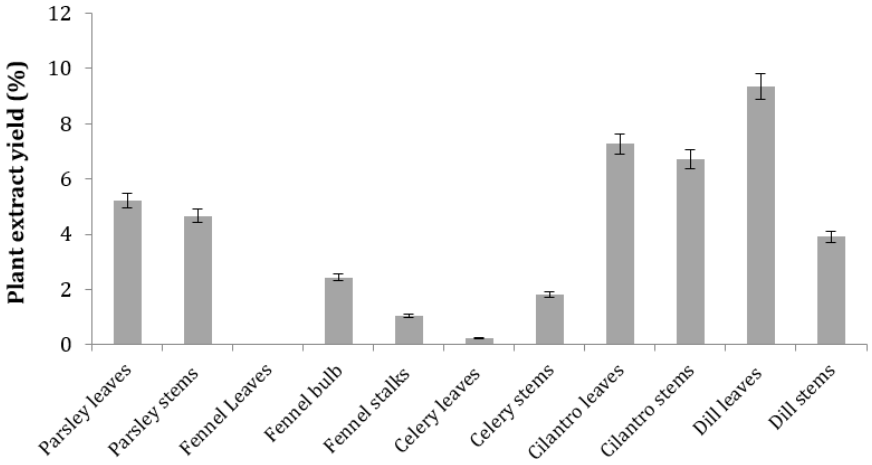

Figure 1. Ethanolic extract yield (%) of plant parts under investigation.

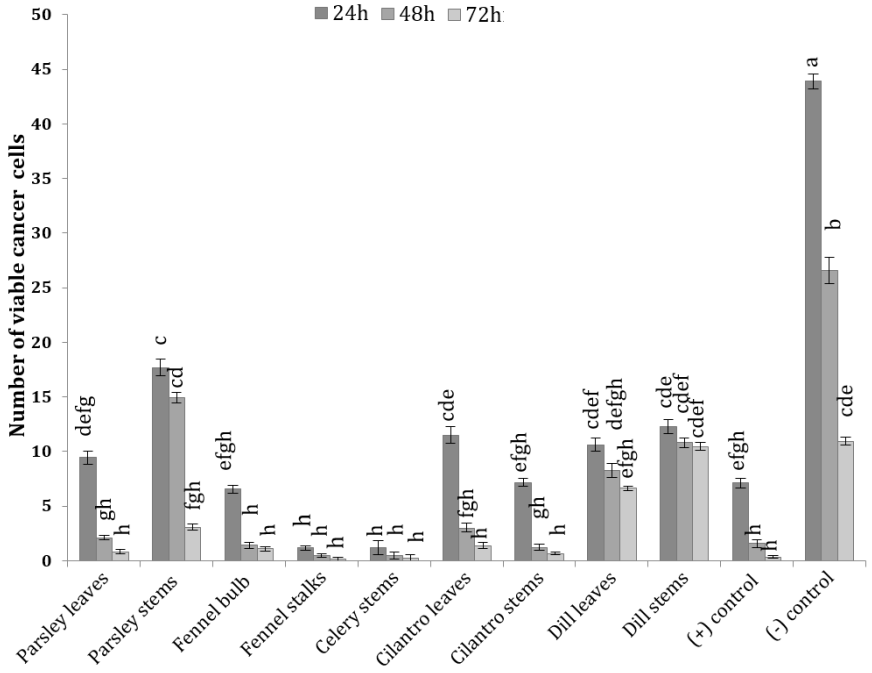

Figure 2. Mean number of viable cancer cells (Hela) after treatment with different plant ethanolic extracts (100µg/mL) through 3 different times: 24, 48 and 72h. (+ve) control stands for "Actinomycin D" and (-ve) control stands for DMSO.

(Table I) showed that celery has the highest net weight percentage of 91.47 followed by dill (87.61%) then cilantro, fennel and Italian parsley with net weight percentages of 78.09, 77.52 and 75.24 respectively. Results of Fresh Weight (g) and Dry Weight (g) (Table II). Leaves of all plants under study showed approximately double higher dry weight than stems and other parts except for fennel leaves which gave low fresh weight (9.83±1.23g) and consequently low dry weight (1.35±0.15g), and thus discarded from the research. Italian parsley showed the highest dry weight of stems among all examined plants (10.93%). Fennel was divided to leaves, blub and stalk according to its morphological structure. On the other hand, plant yield extract percentage results of 50g of plant dry

weight powder, showed in general higher yield extract percentage in all plant leaves when compared to stems extract yield, except for celery leaves which showed very low yield. However, the highest extract yields (9.35%) was obtained from Dill leaves followed by Cilantro leaves and stem (7.26 and 6.72%, respectively). Fennel came in the last place with bulb extract weight of 2.43% and stalk extract weight of 1.04% only (Table II and Figure 1).

Concerning the interaction significance of each extract depending on time manner (Figure 2) clearly demonstrates that all extracts showed significant inhibitory effect on cancer cell. Parsley leaves extract, cilantro leaves extract and cilantro stems extract showed no significant difference with the positive control (Actinomycin D). However, fennel bulb extracts, fennel stalks extracts and celery stems gave better results than the positive control with no significant difference through the 24, 48 and 72h treatment. In case of parsley; leaves extract showed better effect on viable cancer cell number than the stems at 24, 48 and 72h respectively. These results came in agreement with Sarwar *et al.* (2016), who reported that, parsley leaves was used in cancer treatment and also used externally to calm down stings and bites. Agyare *et al.,* (2017) stated also that Parsley was found to have many pharmacological bioactive compounds of antioxidant, antibacterial, antifungal, anticancer, hepatoprotective, antidiabetic, analgesic, spasmolytic and gastroprotective properties.

There were no significant difference between Fennel and the positive control, which showed high effect on the cancer cells survival, and also there were no significant difference through the three timings between fennel bulbs and fennel stalks extracts. This came in agreement with Zaahkouk *et al.* (2015) who investigated the sulphodiamine-B assay (SRB assay) cytotoxicity of methanolic fennel seed extracts and observed that, there were morphological cell line modifications which affected MCF-7 (Human breast cancer cell line), HePG-2 (human hepatocellular carcinoma cell line), and HCT 116 (colon carcinoma cell line), this was thought to be due to the increase of p53 gene expression, and also suggestion of DNA fragmentation due to apoptosis.

However, in case of Cilantro, there were no significant difference between leaves and stems extracts and the maximal inhibitory effect was obtained at 72h. Gomez-Flores *et al.* (2010) used different parts of Cilantro and reported on antioxidant, anti-inflammatory and anticancer activities. Also Laribi *et al*. (2015) confirmed that phenolic compounds are the bioactive compounds of the aerial parts. This came in agreement with Tang *et al*. (2013) and Sathishkumar *et al.* (2016) who stated that, Cilantro leaves and root extracts have had anticancer effect against MCF-7 breast cancer. Also, Chithra and Leelamma (2000) confirmed that, if cilantro is consumed on daily basis it can protect against colon cancer. Ethanol extracts of cilantro lower down the cell cycle viability, invasion and migration in PC-3 and LNCaP Prostate cancer cell lines since there were a difference in gene expression of apoptosis and proliferation (Elmas *et al*., 2019). Alteration in gene expression implies cell arresting and being forced to apoptosis which ceases cancer progress. (Çıtışlı *et al*., 2015; Fahrioğlu *et al*., 2016). Cilantro act as a protective agent in lipid metabolism of colon cancer when studied on rats, as it reduced the cholesterol and cholesterol to phospholipid ratio while increased phospholipid ratio (Chithra *et al*., 2000).

Regarding Dill, cell number survival for leaves and stems extracts showed no significant difference between both of them on time dependent manner. As also previously reported, Dill extracts has an antiproliferative activity when tested on MK-1, HeLa and B16F10 cell lines (Nakano *et al.,* 1998). Recently, these findings were supported by Javadi and Emami (2015) and Tariq *et al.* (2017) who extracted oils from aerial parts and demonstrated its anticancer effect when tested on uterus cancer in folk medicine formulation. Furthermore, Mohammed *et al*. (2018) fractionated, the ethyl acetate of Dill seeds which blocked the HepG2 (Human liver hepatocellular carcinoma) cell proliferation from the nuclear and cytoplasmic assays. The recent study of Al-Sheddi *et al.* (2019) considered dill essential oils as anticancer agent for hepatocellular carcinoma since it showed cytotoxic/ antiproliferative activity and stimulation of cell mortality rate in HepG2 cells in 24h of treatment. This came in agreement with Oliveira *et al.* (2015), who suggested the anticancer effect of some essential oils on cancer against human cervical adenocarcinoma (HeLa), human colon carcinoma (HT29), human hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (MCF-7). Further phytochemical studies on dill revealed the presence of flavonoids which was reported to have anticancer and anti-mutagenic effect according to their anti-oxidant

and inflammatory properties ((Kasolo *et al.,* 2010; Heamalatha *et al.,* 2011; Dahiya and Purkayastha, 2012).

With respect to Celery, it showed positive effect on the viability of cancer cells when compared with the commercial drug (positive control), with no significant difference between them based on time factor. This was supported by Sultana *et al.* (2005); and Powanda *et al.* (2015) who returned the cytotoxic properties, antioxidant and anti-inflammatory effect to celery seeds. Also, Ahmedy *et al*. (2016) stated that PCNA expression was decreased and caspase-3 expression was increased by celery seed oil that lead to antiproliferation and apoptosis respectively on liver cancer cells, who suggested celery as a dietary supplement against hepatocarcinoma.

It is suggested that, the anticancer activity of extracts was due to phytochemical constitutes presence which should be purified and isolated for a better understanding of its action. Furthermore the antiproliferative activity might not be related to a single phytochemical constituent but suggested to be due to the interaction of more than one constituent (Alison *et al*., 2001 and Bhandarkar, 2003).

CONCLUSION

Fennel bulb extracts, fennel stalks extracts, celery stems exhibited the highest anticancer activity followed by parsley leaves extract, cilantro leaves extract and cilantro stems. However, it is recommended that further investigation for ethanolic extracts of parsley leaves, fennel bulb, fennel stalks, celery stems, cilantro leaves and cilantro stems which showed better results than using the commercial drug Actinomycin D (25µl/mL). Further investigation on the anticancer activities of these extract on different cancer cells are requested. This open a new hope for the development of herbal based potential anticancer drug as safe alternative for the current extensive use of chemotherapy. It is also recommended to increase the regular consumption of fennel and celery in the daily meals especially for those who have cancer history in their family records. Overall results showed that plants used in this investigation are promising candidates as anticancer agent.

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