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THE ANTIOXIDANT AND ANTI-TUMOR ACTIVITIES OF THE LEBANESE *ERYNGIUM CRETICUM* L.

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ABSTRACT

To determine the phytochemical screening of *Eryngium creticum* L. in order to investigate its *in vitro* anti-oxidant, anti-proliferative and cytotoxic activities. The effect of period of growth of the plant on the chemical composition was also studied. Aqueous and ethanolic extracts from different parts (leaves, stems, roots, and the whole plant) of the fresh plant *E. creticum* from the first and second harvest were performed to fractionate the chemical constituents into individual fractions or extracts. The extracts were tested for different secondary metabolites content by classical phytochemical screening tests, antioxidant (DPPH radical scavenging, superoxide radical scavenging), cytotoxic and cell viability (Neutral red assay on HeLa cells), and apoptotic activity (DNA fragmentation assay on HeLa cells). Our results showed that the different parts of first and second harvest of *E. creticum* contain alkaloid, tannin, coumarin, saponin, flavonoid, polyphenol and reducing sugars in different concentrations. Moreover, the 4 parts of this plant have exerted antioxidant activity that may be due to their phenolic content and they have also inhibited the viability of HeLa cell line in a time-dependent (0–72 h) and dose-dependent (0–250 µM) manner. Finally, we demonstrated that the ethanolic extracts from leaves of the second harvest were the most potent (at 48 h) with an IC50 value ≤ 47.24 µg/ml.

The in vitro anti-oxidant and antiproliferative effects of crude aqueous and ethanolic extracts from first and second harvest of *E. creticum* leaves, stems, root and whole plant indicate that it has sufficient potential to warrant further examination and development as a new anti-cancer agent.

Keywords: *Eryngium creticum*, Phytochemical Screening, Antioxidant Activity, Antiproliferative Activity, Cytotoxicity, Cell Viability

INTRODUCTION

According to the Global Burden of Disease, cancer is a leading cause of death worldwide, accounting for 8 million deaths in 2010 **[1]**. It is a complex disease caused by numerous factors ranging from environmental factors to hereditary genetics. Current treatment of cancer can be categorized into three groups: surgery, chemotherapy, and radiation therapy **[2-4]**. Surgical removal of tumor and cancerous growth can effectively treat 50% of cancer but cannot eliminate all cancer cells, which results in high recurrence rate **[3]**. Chemotherapy and radiation therapy have the potential of removing all cancer cells but both lack the specificity to cancer cells since normal cells are also often targeted by most of these treatments, thus harming normal body cells in the process and causing countless side-effects. The focal point of cancer research is still the search for effective and specific anti-cancer treatments **[5]**.

There is widespread use of Complementary and Alternative Medicine (CAM) in developed countries **[5, 6]**. A recent study estimated the overall prevalence for the use of herbal products to be 13% to 63% among cancer patients **[6]**. In a study on the use of CAM by breast cancer survivors in Canada, the investigators found that 67% of the respondents in a randomized survey reported using CAM **[7]**. The study concluded that CAM use is common among Canadian breast cancer survivors and that many are discussing CAM therapy options with their physicians **[7]**. Thus, many investigations are now being carried out to discover naturally occurring compounds, which can suppress or prevent the process of carcinogenesis **[8, 9]**. Lebanon is among the countries that are highly rich in medicinal plants in the Mediterranean region. In this study, we are interested by a Lebanese food plant, *Eryngium creticum*.

Eryngium creticum, a perennial plant which belongs to the Umbellifereae family. It is found principally in Lebanon, Palestine, Jordan, and Syria. It is cultivated for use as vegetable mainly in salad. E. creticum is

traditionally used as diuretic, laxative. Submerged roots and seeds in water had been drunk to treat the kidney stone and the infections, skin diseases and tumors. It is an antidote, used in the treatment of the snakebite **[10]**. *E. creticum* also showed anti-inflammatory and anti-microbial activities **[11]**. It was also used in the treatment of liver diseases, poisoning, anemia and infertility **[12]**. This plant has shown an antioxidant property by inhibiting the lipid peroxidase in the liver of the rat **[13]**.

Very little data on cytotoxicity and anti-tumor activities of this plant exist in literature. For that, our study aimed to determine the phytochemical screening for aqueous and ethanolic extracts of two harvest periods of *E. creticum* leaves, stems, roots and the whole plant, and to evaluate their antioxidant power using hydrogen peroxide radical and DPPH radicals and for the first time the cytotoxicity and the anti-proliferative activity against cervical cancer cell line (Hela) by using neutral red cytotoxicity assay. The mechanism of anticancer activity of this plant extracts has however not been reported. Therefore, the anticancer mechanism of the *E. creticum* extract via apoptosis induction was investigated in the current study.

MATERIALS AND METHODS

Plant Material

E. creticum plants were collected during 2013 in 2 different periods, the first one in March (the plant is edible and maturing) and the second one in May (the plant is mature and non-consumable) from South Lebanon.

Preparation of Extracts

The stems, leaves and fresh roots, as well as the whole plant of *E. creticum* were well washed and cut into small pieces that are then placed in the selected solvent (100 g of each of the plant in 500 ml of the selected solvent: distilled water or ethanol). After a period of maceration and stirring for 8 hours at room temperature and then at 37 ° C, the macerate obtained was filtered to remove insoluble residues. The filtrate was then condensed with a rotary evaporator to half evaporation and the filtrates were then frozen before being lyophilized powder to be processed.

Phytochemical screening tests

To study the phytochemical composition of the aqueous and ethanolic extracts of the stems, leaves, roots and the whole plant, qualitative detection of secondary metabolites was performed according to Muanda **[14]**.

Detection of Alkaloids

The detection and determination of alkaloids are practiced by using the reagent of Dragendorff. The appearance of orange-red precipitate indicates the presence of alkaloids.

Detection of Tannins

After filtration of 10 ml of each of the plant parts extract, the filtrate obtained was mixed with 2 to 3 ml of a solution of ferric chloride ($FeCl_3$ 1%). The occurrence of blue color indicates the presence of tannins.

Detection of Resins

10 ml of each filtered extract is mixed with 20 ml of hydrochloric acid (HCl 4 %). The presence of the resins is indicated by the observation of the turbidity of a mixture.

Detection of Coumarins

Test tubes containing 5 ml of each extract are covered by filter paper by a saturated solution of sodium hydroxide (NaOH). These tubes are then placed in a water bath and are boiled for 15 minutes. The filter papers are subjected to UV radiation, and the appearance of a bright yellow color indicates the presence of coumarins.

Detection of Saponins

2 ml of each part extracts were stirred vigorously for 5 minutes on Vortex. The presence of saponin is indicated by the appearance of more or less significant foam.

Detection of Phenols

In 5 ml beakers, each filtered part extract is mixed with 1 ml of $FeCl_3$ (1%) and 1 ml of $K_3(Fe(CN)_6)$ (1%). The appearance of a blue color indicates the greenish presence of phenols.

Detection of Terpenoids

1 ml of acetic anhydride and 2 ml of concentrated sulfuric acid are added to 1 ml of each filtered part extract. The presence of a reddish brown color to the surface of mixture indicates the presence of terpenoids.

Detection of the Volatile Oil

10 ml of each extract were filtered through a filter paper to saturation of the paper by the extract. Then, it is placed under UV light. The appearance of a pink color gloss on the paper indicates the presence of the volatile oil.

Detection of Flavonoids

In test tubes each containing 5 ml of part extract, 5 ml of potassium hydroxide (KOH 50%) were added. The observation of a yellow color indicates the presence of flavonoids.

Detection of Carbohydrates

Benedict Test: To 0.5 ml of the filtrate, 0.5 ml of Benedict has been added and then boiled for 2 minutes. The red brick precipitate indicates the presence of sugar.

Evaluation of the Antioxidant Activity

For each of the prepared samples, an evaluation of the antioxidant activity was performed according to the method of Rammal *et al*., **[15]** using the free radical 2,2 - diphenyl- 1 - picrylhydrazyl (DPPH) and the H_2O_2 test.

DPPH Radical Scavenging

Increasing concentrations of each sample (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) have been prepared. To 1 ml of each dilution of the part extract prepared, 1 ml of the DPPH reagent was added. The solution was incubated for 30 minutes at room temperature in the dark, and then the absorbance was measured at 517 nm. The antioxidant activity is calculated using the following equation:

% Antioxidant activity = [(Abs control - Abs sample) / Abs control] x 100

The control was prepared by mixing 1 ml of DPPH with 1 ml of used solvent (distilled water or ethanol) and the blank was composed of 1 ml of the selected solvent.

Scavenging with Hydrogen Peroxide (H_2O_2)

A solution of H_2O_2 (40 mM) was prepared in PBS (pH 7.4). Various concentrations of plant part extracts were added to a solution of H_2O_2 (0.6 ml, 40 mM), and the absorbance was measured at 230 nm after 10 minutes against a blank containing PBS without H_2O_2. The antioxidant activity is calculated using the following equation:

% Antioxidant activity = [(Abs control - Abs sample) / Abs control] x 100

Cells and Cell Culture

Cervical cancer (HeLa) cell line were grown in 25 cm² tissue culture flasks (Nunclon TM, Nunc) at 37°C, 5% CO_2 in DMEM: Dulbecco's Modified Eagle Medium (Sigma Chemical Company), containing 10% fetal bovine serum, penicillin 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml). Once cell confluence had been reached, Hela cells were transferred, under sterile conditions, into 48-multiwell plates (10.000 cells/well) for the toxicity tests.

Neutral Red Analysis

Cell viability was performed using Neutral Red assay based on the initial protocol as described earlier **[16, 17]**. Neutral Red, a chromogenic dye, is an indicator of lysosomal activity. Live cells demonstrate a chromogenic change with Neutral Red that is detected spectrophotometrically. Briefly, cells were detached from the tissue culture flask with 2 ml of trypsin solution. The cell pellet was obtained by centrifugation at 1.000 rpm for 5 minutes. The density of the viable cells was counted by the trypan blue exclusion in a haemocytometer. Cells were then plated in 96-well microtiter plate, at a concentration of 1×10^4 cells/well and incubated in a humidified *37°C, 5%* CO_2 incubator that allows the cells to adhere. After 24 h, the cells were treated with five different concentrations of aqueous and ethanolic extracts: 50, 100, 150, 200, and 250 μg/ml each being tested in three replicates. The plates were incubated for 24, 48 and 72 h at

37°C in a 5% CO_2 incubator. The untreated cells were regarded as a negative control, whilst cells incubated only with ethanol (0.5%, v/v) were used as a vehicle control. No effect due to the ethanol was observed. Arsenic was used as the positive control. At 24, 48 and 72 h, the old medium was replaced with 100 μl of fresh medium containing 40 μg/ml neutral red and incubated for 3 h. This is to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. Then, the media was discarded and cells were washed twice with 100 μl of 1X PBS. The intracellular accumulation of neutral red dye was extracted in 200 µl of a 50% ethanol-1% acetic acid lysing solution.

The optical density (OD) of the eluted dye was read at 490 nm using a microplate reader. The experiments were conducted in triplicates. The percentage of inhibition of each of the test samples was calculated according to the following formula using the OD values obtained:

Percentage of inhibition (%) = (OD control − OD sample)/OD control × 100

The average of three replicates was then obtained. Cytotoxicity of each test agent is expressed as IC50 value. The IC50 for each extract was extrapolated from the graphs of the percentage inhibition versus concentration of test agents **[16–20]**.

DNA Fragmentation Detection Assay

The DNA fragmentation was used to identify apoptosis by observing the biochemical changes. Briefly, cancer cells were treated with the 200 µg/ml of ethanolic root extracts of first and second harvest of *E. creticum* for 48 h. Cells were then collected and washed with media. Then cell suspension was transferred to microcentrifuge tube (1.5 ml) and centrifuged at 1500 rpm for 5 min to collect the cell pellet. The DNA in the cell pellet was extracted using "QIAGEN kit (Zymo Research, Belgique), and 5 µg of each DNA sample was analyzed by electrophoresis on 2% agarose gels containing 0.1 mg/ml ethidium bromide. The migration was done at 75V. After electrophoresis, DNA fragment was analyzed by using UV luminated camera.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) of three independent experiments and statistical significance was determined using the Independent Student's t-test and Prism 6.0 software (GraghPad Software, Inc.). A significant difference was considered if $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Screening

The results of the phytochemical screening represented in **Tables 1 and 2** show that the different parts of *E. creticum* are rich in

various secondary metabolites at different concentrations depending on the solvent used. Indeed, we note the presence of saponins and coumarins in the aqueous extract from the different studied parts of this plant.

The ethanolic extract is rich in secondary metabolites including: phenols, flavonoids, alkaloids, carbohydrates and resins. Thus, there is a difference in the distribution of metabolites extracted using the same solvent between the leaves, stems and roots of this plant. Leaves are richer in metabolites compared to stems and roots, which assigns them greater biological availability. Consequently, *E. creticum* by its richness in different secondary metabolites may have several medical importances.

In addition, comparing the first and second harvest we see a difference in the distribution of metabolites for the first harvest which contains more metabolites than the second one, except roots that are rich in secondary metabolites in the second harvest more than the first. This can be attributed to the effect of the maturation of the plant, wherein in the first harvest (in March, the plant is maturing and consumables) it scored more water and less temperature, whilst in the second harvest (in May, the plant is mature and non-consumable) it had received less water and more temperature, and this can in turn influence the distribution of metabolites between the two harvests especially in the second harvest where these metabolites could have been replaced by other compounds which may play important roles in various biological activities. This was not the case with the roots, because in fact the roots are in the soil, so the variation of the temperature and water is negligible. The extract of the whole plant which represents the mixture of 3 parts of the plant (leaves + stems + roots) shows a composition richer in secondary metabolites in comparison with the aqueous extract in the first and the second harvest, but in lower quantities than the other portions. This result seems to be normal because the proportion of each part is divided by 3. This shows that the effect of the combination of plant extracts is additive.

Antioxydant Activity

The activity of chemical compounds draws our attention because of their potential role in the prevention of certain human diseases. A number of studies indicated that flavonoids, polyphenols and triterpenoids have an antioxidant activity and ability to scavenge free radicals (Frankel et al. 1995). These phytoconstituents can have multiple biological effects against tumors, heart disease and various diseases due to their trapping free radicals. Hence our study was to

evaluate the antioxidant power of the extracts through two different *in vitro* methods: DPPH and H_2O_2.

DPPH

Figures 1 (A, C) show the increase in the antioxidant activity of the aqueous extract of leaves, stems and roots of *E. creticum* with increasing concentrations. This increase reached 77% , 89% and 70% at the concentration of 0.5 mg/ml of leaves, stems and roots, respectively, for the first harvest, while for the second one, it reached 73%, 59% and 34% at the same concentration of the leaves, stems and roots respectively. The aqueous extract of the whole plant shows an antioxidant activity of 72% at the same concentration for the first harvest and 49% for the second. This confirms, as phytochemical screening, that in both the effect of the combination of plant extracts is additive, and the antioxidant effect obtained is nearly equal to the average of the effect of three separate parts **(Figure 1 C)**.

The ethanolic extract shows an antioxidant activity of 93%, 82% and 44% for leaves, stems and roots, respectively, at a concentration of 0.5 mg/ml for the first harvest whilst for the second harvest this activity was about 56%, 65% and 61% at the same concentration for the leaves, stems and roots, respectively **(Figure 1 B, D)**. Concerning the whole plant, this extract showed an antioxidant activity of 55% and 59% for the first and the second harvest respectively, which represents an activity equal to the average of the effect of three separate parts **(Figure 1 D)**.

The obtained results show a difference in the antioxidant activity between the aqueous and the ethanolic extract in favor of the ethanolic which is in agreement with our results obtained in the phytochemical screening. Indeed this extract was richer in flavonoids and polyphenols, which have an important role in the antioxidant activity.

Furthermore, comparing the antioxidant activity between the first and second harvest indicates that it is higher in the first one.

To confirm the results obtained by the DPPH method, we used another *in vitro* method; the H_2O_2.

The aqueous extracts at the concentration of 0.5 mg/ml of the leaves, stems, roots and whole plant of *E. creticum* from the first harvest showed an antioxidant activity of 92%, 87%, 68% and 74% respectively. This antioxidant activity was approximately 87%, 76%, 66% and 68% for the leaves, stems, roots and whole plant of the second harvest, at the concentration of 0.5 mg/ml **(Figure 2 A, C)**.

On the other hand, the ethanolic extracts of leaves, stems, roots and the whole plant showed maximum antioxidant activity of 92%, 78%, 71% and 66% respectively for the first harvest, whilst in the second harvest this activity was 75%, 73%, 66% and 70% for these same parts respectively **(Figure 2 B, D)**. Hydrogen peroxide is an important reactive species of oxygen because of its ability to penetrate biological membranes. But, it can be toxic if it's converted into hydroxyl radical in the cell. Scavenging of H_2O_2 by the plant extracts can be attributed to the phenolic compounds giving an electron to H_2O_2, and therefore reduce the H_2O. Our results showed that extracts from the plant are capable of scavenging H_2O_2 depending on the concentrations used. These results showed that *E. creticum* has an important antioxidant activity and therefore it can be considered a good natural source that could be used in the treatment of diseases associated to the oxidative stress.

Cell viability and cytotoxicity effect of crude aqueous and ethanolic extract of *E. creticum* in HeLa cancer cells

An evaluation of the antiproliferative activity of each of the extracts prepared from leaves, stems, roots and of the whole plant of *E. creticum* was done by measuring the viability of the HeLa cell line using the Neutral Red Cytotoxicity/ Viability Assay after the treatment of this cancerous cell line for 24, 48 and 72 hours with increasing concentrations (50, 100, 150, 200, and 250 µg/ml) of these extracts. The effect of inhibition by these extracts from the first and second harvest was dose and time-dependent **(Figures 3, 4)**. The percentage of inhibition was calculated according to the formula mentioned above.

The results of the neutral red assay shows that the different concentrations of aqueous extracts of the stems from the first and second harvest have no antiproliferative effect at different studied times (Data not shown) whilst the different concentrations of aqueous extract of the leaves and the whole plant from the first harvest and the different concentrations of aqueous extract of the roots plant from second harvest showed little antiproliferative effect (because they do not exceed 30% inhibition) at different studied times **(Figure 3 A, B and E)**. The aqueous extracts of the second harvest showed an antiproliferative activity greater than that of the first for the leaves and for the whole plant **(Figure 3 C, F)**. On the other hand, the aqueous extracts of the roots, from the first harvest showed an antiproliferative activity greater than that of the second harvest **(Figure 3 D)**. At the 150, 200, and 250 µg/ml

dose, leaf extracts from second harvest were effective, and treatment with these doses for 48 and 72 hours resulted in about 90 % inhibition on Hela cells **(Figure 3 C)**. As shown in **Figure 3 D**, treatment with root extract from first harvest at the 250 µg/ml dose for 72 h also resulted in significant inhibition (90%) of viability of HeLa cells. The results indicated that *E. creticum* aqueous extracts from the first and second harvest exerted anti-proliferative activity by decreasing the viability of HeLa cancer cells.

On the other hand, our results showed that the majority of the ethanolic extract of *E. creticum* from the first and second harvest showed a significant antiproliferative effect at different doses and different times **(Figure 5)**. The different concentrations of the stem ethanolic extract from first harvest showed little antiproliferative effect (because they do not exceed 35% inhibition) at different studied times **(Figure 4 B)** whilst the ethanolic extracts of the stem from the second harvest showed an antiproliferative activity greater than that of the first harvest. As shown in **Figure 6 F**, treatment with root extract from the second harvest at the 250 µg/ml dose for 72 h also resulted in significant inhibition (70%) of viability of HeLa cells. At the 250 µg/ml dose, leaf, root and whole plant extracts from first and second harvest were very effective, and treatment with this dose for 72 hours resulted in about 87 to 97% inhibition in Hela cells viability **(Figure 4 A, C, D, E, G and H)**.

In order to determine the concentration required to achieve a 50% inhibition of cells induced by each aqueous and ethanolic extracts, the dose response curve was plotted. The results of cytotoxic assay were mentioned as IC_{50} (µg/ml) in **Table 3 and Table 4**. It is clear by our results that ethanolic leaves (at 24 h) and aqueous whole plant extracts (at 48 h) from second harvest have the highest antiproliferative activity on the HeLa cell lines with an IC50 of 47.24 ± 1.23 to 52.33 ± 0.91 µg/ml respectively.

Apoptotic Effects of the Extracts from *E. creticum* in HeLa Cells

In order to determine whether the investigated plant extracts have pro-apoptotic activities, we examined occurrence of internucleosomal DNA fragmentation (180 bp laddering), the hallmark of apoptosis by agarose gel electrophoresis of genomic DNA isolated from Hela cells treated with plant extracts of *E. creticum*. The morphological changes of the nuclei DNA after being treated with ethanolic root extracts from the first and second harvest of *E. creticum* (200 µg/ml) for 48 hours are shown in **Figure 6**. The results showed a destruction of genomic DNA and

appearance of band at approximately 180 bp due to apoptosis of the HeLa cells treated with *E. creticum*. These analyses confirmed that the cytotoxicity of *E. creticum* extracts is based on their prominent proapoptotic effects.

Table 1: Comparison of the phytochemical composition of crude aqueous extracts from leaves, stems, roots and the Whole Plant (WP) of *E. creticum*

	First harvest				Second harvest			
	Leaves	**Stems**	**Roots**	**WP**	**Leaves**	**Stems**	**Roots**	**WP**
Alkaloids	-	-	-	-	+	-	+	-
Tannins	+	-	-	+	-	-	-	-
Resins	-	-	-	-	-	-	-	-
Coumarins	+	++	+	+	+	++	++	++
Saponins	+++	+++	+	+++	++	+	++	+++
Phenols	-	-	+	+++	-	++	-	++
Terpenoids	-	-	-	-	-	++	-	++
Volatil oils	-	-	-	-	-	-	-	-
Flavonoids	-	-	-	-	+++	+	-	+
Carbohydrates	-	-	-	-	-	-	++	+
Glucosides	-	-	-	-	-	-	-	-

Table 2: Comparison for the phytochemical composition of crude ethanolic extracts from leaves, stems, roots and the Whole Plant (WP) of *E. creticum*

	First harvest				Second harvest			
	Leaves	**Stems**	**Roots**	**WP**	**Leaves**	**Stems**	**Roots**	**WP**
Alkaloids	+	-	++	+++	-	+	++	+
Tannins	-	+	+	++	-	-	-	-
Resins	+++	+	-	++	-	+	+	-
Coumarins	-	++	+	+	+	+	++	+
Saponins	++	++	+	+++	+	+	-	+
Phenols	-	+	-	+	+	+	-	+
Terpenoids	-	-	+	+	-	-	-	-
Volatil oils	-	-	-	-	-	-	-	-
Flavonoids	++	++	+	+++	-	+	++	+++
Carbohydrates	++	+	++	+	-	++	+	+
Glucosides	-	-	-	-	-	-	-	-

Table 3: Concentrations of eight aqueous *Eryngium creticum* extracts, which induced 50% decrease in Hela cancer cell survival, determined by Neutral red cytotoxicity assay.

		24 hours	48 hours	72 hours
Leaves 1	**IC50 [µg/ml]**	-	148.53 ± 2.3	167.63 ± 6.43
Whole plant 1	**IC50 [µg/ml]**	-	194.21 ± 5.2	250.01 ± 8.07
Leaves 2	**IC50 [µg/ml]**	122.22 ± 2.45	64.82 ± 1.23	62.03 ± 2.12
Stems 2	**IC50 [µg/ml]**	199.48 ± 6.34	194.26 ± 4.7	184.79 ± 4.94
Roots 2	**IC50 [µg/ml]**	-	-	122.65 ± 3.23
Whole plant 2	**IC50 [µg/ml]**	187.27 ± 6.42	52.33 ± 0.91	112.67 ± 2.67

(1) Aqueous extracts from the first and (2) second harvests. Tabulated values are mean ± SD of three replicates

Table 4: Concentrations of eight ethanolic *Eryngium creticum* extracts, which induced 50% decrease in Hela cancer cell survival, determined by Neutral red cytotoxicity assay.

		24 hours	48 hours	72 hours
Leaves 1	**IC50 [µg/ml]**	212.16 ± 10.34	195.58 ± 9.65	129.67 ± 3.23
Stems 1	**IC50 [µg/ml]**	150 ± 7.52	140 ± 6.52	85.34 ± 1.20
Roots 1	**IC50 [µg/ml]**	100.42 ± 4.43	164.15 ± 6.91	69.39 ± 1.12
Whole plant 1	**IC50 [µg/ml]**	205.25 ± 9.65	206.5 ± 6.23	131.26 ± 3.76
Leaves 2	**IC50 [µg/ml]**	47.24 ± 1.23	90.99 ± 3.32	85.37 ± 2.14
Stems 2	**IC50 [µg/ml]**	172.80 ± 8.10	141.73 ± 3.87	198.41 ± 7.82
Roots 2	**IC50 [µg/ml]**	54.83 ± 2.13	56.99 ± 2.12	62.48 ± 2.34
Whole plant 2	**IC50 [µg/ml]**	190.88 ± 9.23	186.15 ± 5.43	208.44 ± 8.87

(1) Ethanolic extracts from the first and (2) second harvests. Tabulated values are mean ± SD of three replicates

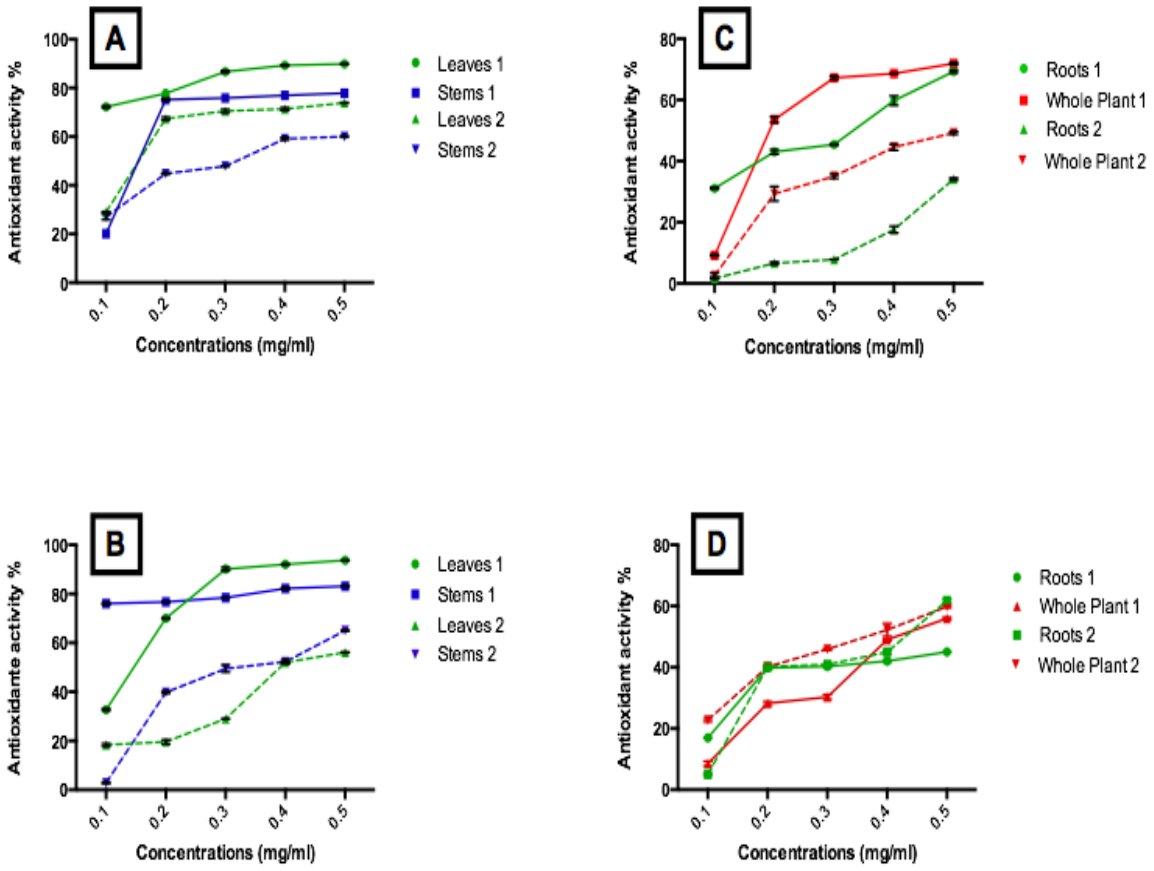

Figure 1: The in-vitro antioxidant activity of the aqueous (A, C) and ethanolic (B, D) crude extracts of different parts of *E. creticum* measured by the DPPH radical scavenging activity

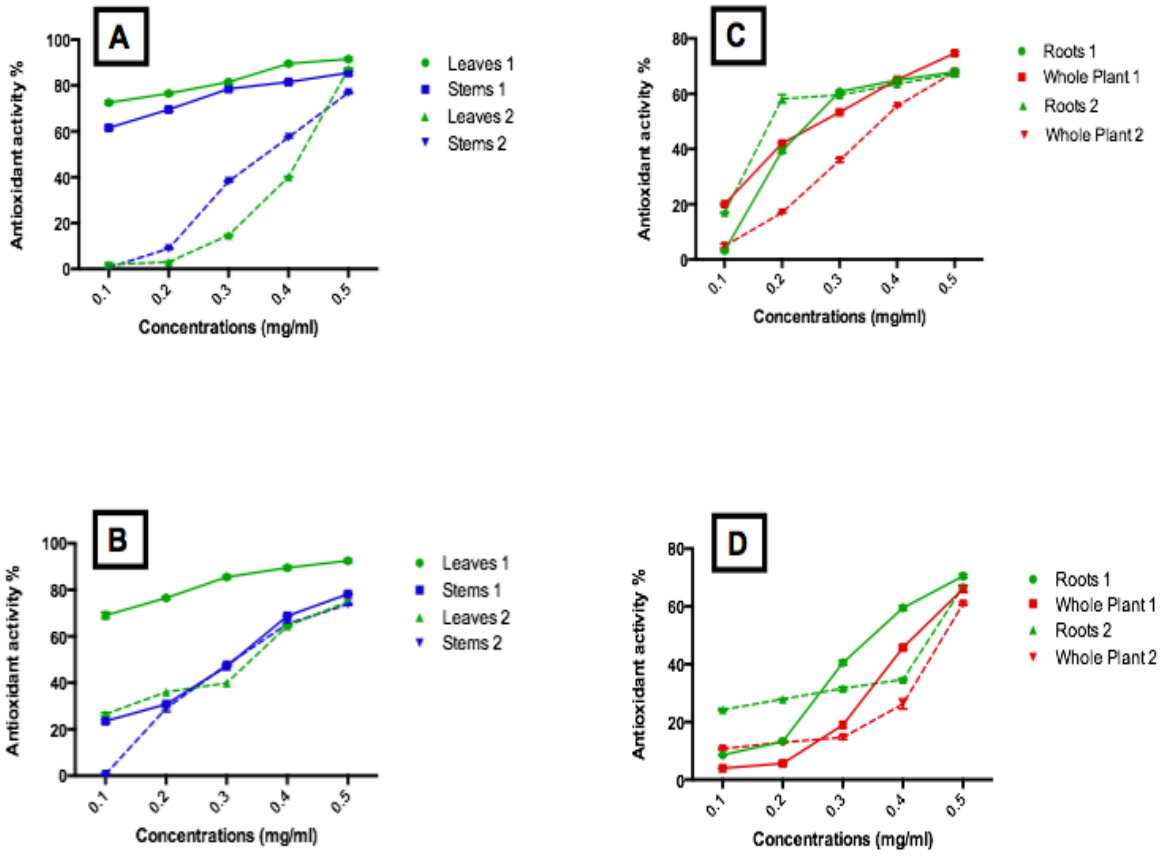

Figure 2: The in-vitro antioxidant activity of the aqueous (A, B) and ethanolic (C, D) crude extracts of different parts of *E. creticum* measured by the H_2O_2 radical scavenging activity

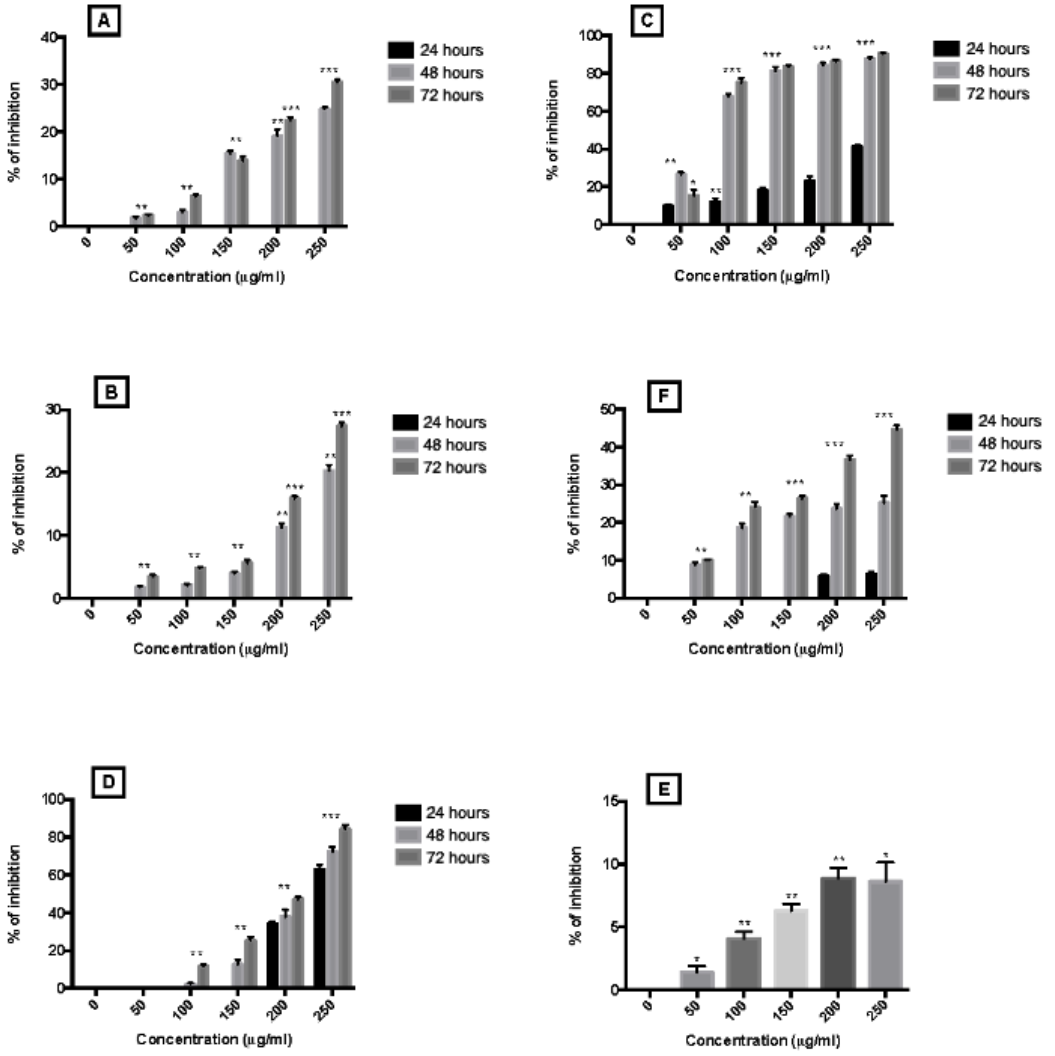

Figure 3: Effect of crude aqueous extract of *E. creticum* plant on viability (% of inhibition) of HeLa cancer cells as determined by Neutral Red assay. (A-C) HeLa cells were treated with (0-250 µg/ml) of aqueous extract from the first (A) and second harvest (C) of *E. creticum* leaf for 24-72 h. (B-F) HeLa cells were treated with (0-250 µg/ml) of aqueous extract from the first (B) and second harvest (F) of the whole plant for 24-72 h. (D-E) HeLa cells were treated with (0-250 µg/ml) of aqueous extract from the first for 24-72 h (D) and second harvest (E) for 72 h of *E. creticum* roots. Each experiment was done in triplicate. Data are expressed as mean ± SD. *p < 0.05 was considered to be statistically significant, **p < 0.01 very significant, and ***p < 0.001 highly significant

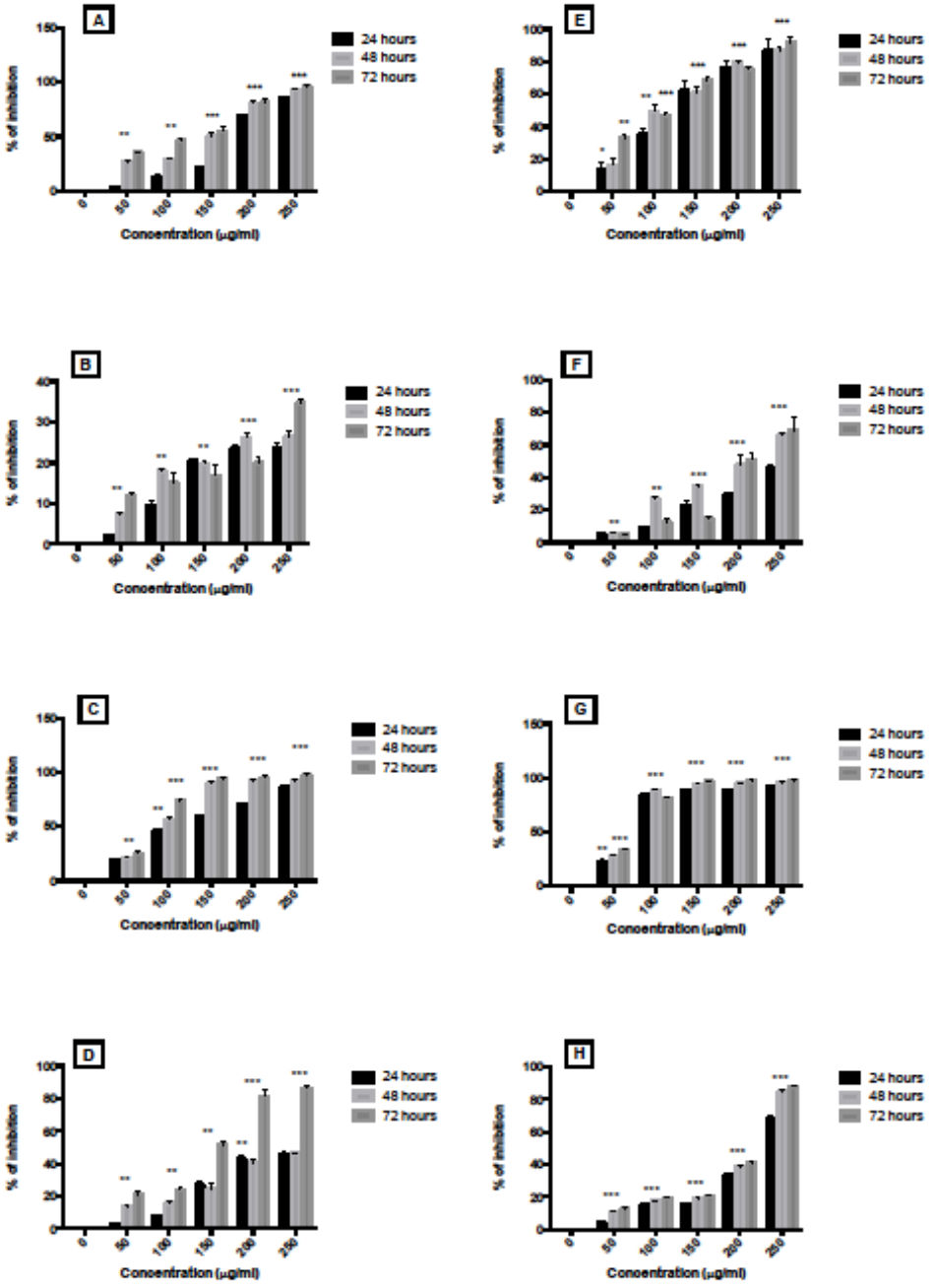

Figure 4: Effect of ethanolic extract of *E. creticum* plant on viability of HeLa cancer cells as determined by Neutral Red assay. (A-D) HeLa cells were treated with (0-250 µg/ml) of ethanolic extract from the first harvest of *E. creticum* leaf (A), stem (B), roots (C) and whole plant (D). (E-H) HeLa cells were treated with (0-250 µg/ml) of ethanolic extract from the second harvest of leaf (E), stem (F), roots (G) and whole plant (H). Proliferation was measured by Neutral Red assay. Each experiment was done in triplicate. Data are expressed as mean ± SD. *p < 0.05 was considered to be statistically significant, **p < 0.01 very significant, and ***p < 0.001 highly significant

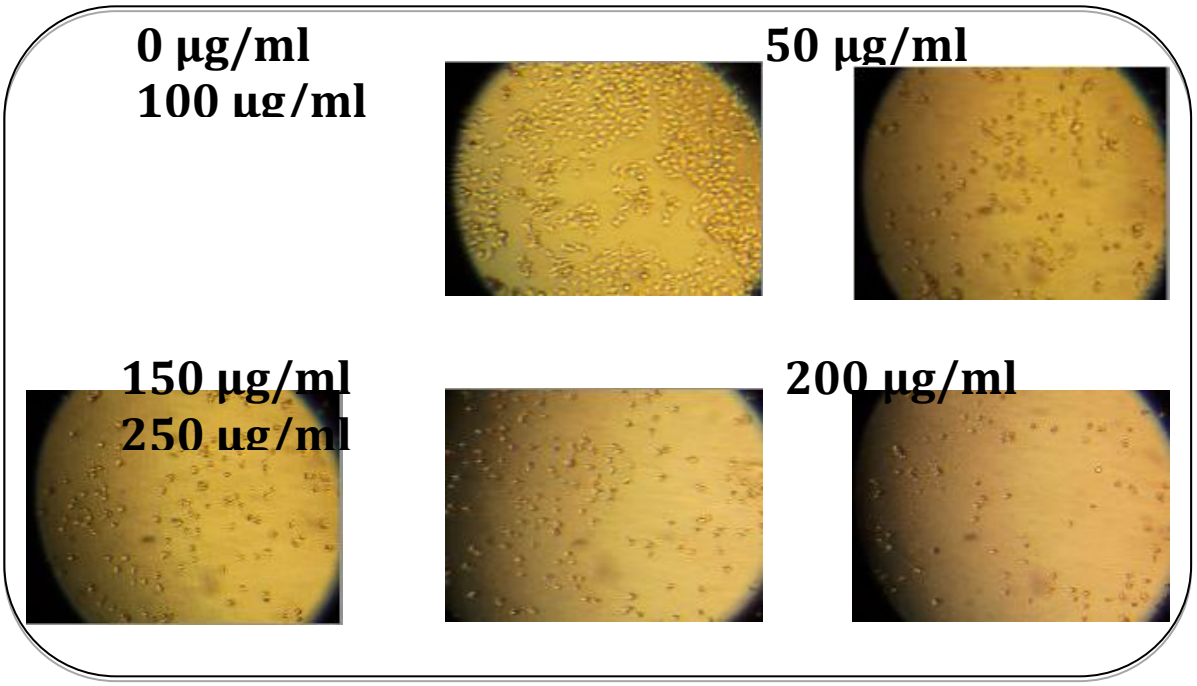

Figure 5: Microscopic view of HeLa cells treated with ethanolic root extracts from the first harvest of *E. creticum* for 72 h. HeLa cells after 72 hours incubation with (0-250 µg/ml) of ethanolic root extracts from the first harvest of *E. creticum*. The results presented are from one experiment representative of three carried out, and were photographed with a microscope (× 40)

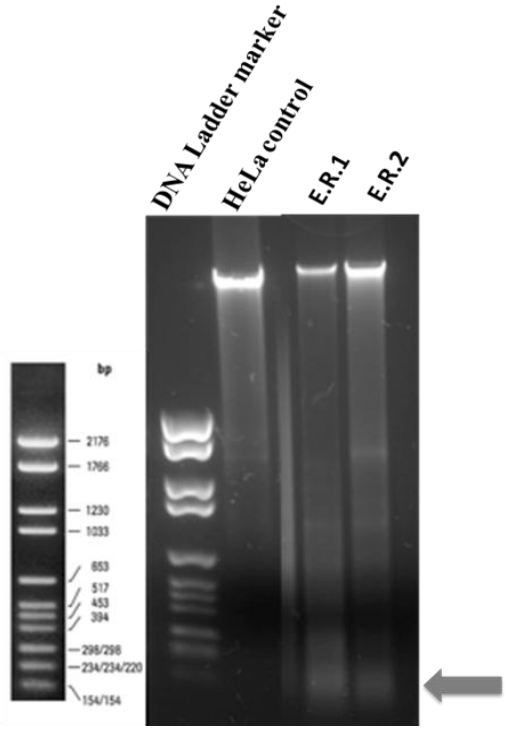

Figure 6: The latter stage of apoptosis (DNA laddering) in HeLa cells induced by ethanolic root extracts (200 µg/ml) from the first (E.R.1) and second harvest (E.R.2) of *E. creticum* for 48 hours. The new band generated at approximately 180 bp due to apoptosis is indicated by arrow

DISCUSSION

Many scientific studies show that the plant kingdom provides a rich source of potential cancer chemopreventive and therapeutic. The main agents currently used in clinical practice in the treatment of malignant diseases originate from plants: vinca alkaloids, taxanes, camptothecins and epipodophyllotoxins **[21]**. In the last decades, researchers of anticancer natural products chemistry focused their research in a wide variety of natural compounds, especially on phenolic compounds. Numerous phytochemicals derived from edible plants have been reported to affect different intracellular signaling pathways implicated in the initiation, promotion and progression of cancer. The antitumor effects of plant compounds have been associated with the induction of free radicals, anti-inflammatory activity, induction of cell cycle arrest or apoptosis, inhibition of tumor angiogenesis and metastasis **[22, 23]**.

It is reported that root extracts of the different Eryngium species are used in traditional medicine for diurectic, antidiabetic and antispasmodic purposes as well as in the treatment of asthma, liver diseases, low-back pain, and cough and in certain poisoning conditions. An aqueous extract of the roots of *E. creticum* was found to be able to neutralize scorpion venom and antagonize cobra. It was found that *E. creticum* extract will prolong the life of guinea pig when combined with scorpion venom **[24]**. The *E. creticum* was also tested for their inhibitory activity of different methicillin-resistant *Staphylococcus aureus* **[25]**. It was demonstrated that the ethanolic extracts of the E. creticum have effective antimutagenic activity. However, the aqueous extracts lacked any inhibitory efficacy **[26]**. *E. creticum* showed antimycotic and antihyperglycemic activities **[27, 28]**.

The crude aqueous extract of *E. creticum* leaf and stem was found to possess antioxidant activity **[29-30]**. It was detected that the consumption of 100 g of fresh *E. creticum* leaves and stems could provide antioxidants equivalent to (78.50 ± 0.80) mg of vitamin C and (50.42 ± 0.50) mg of vitamin C, respectively. The ethanol extracts either from the aerial parts or roots of Eryngium species showed apparent antioxidant, antinociceptive and anti-inflammatory activity **[31]**. Regarding the link between inflammation and cancer, chemicals with anti-inflammatory properties targeting the small molecules or regulating signaling cascades implicated in inflammation and carcinogenesis are regarded as key cancer chemopreventive drugs.

Research into the anti-cancer potential of herbal extracts has been studied as a

cancer treatment or prevention **[22]**. On the other hand, data about the potential anticancer activity of extracts and phytochemicals of plants from the genus Eryngium are rare. The cytotoxicity and anti-proliferative effect of E. creticum human breast adenocarcinoma MCF-7 cells have been documented. The aqueous and ethyl acetate extracts of both leaves and stems of *E. creticum* didn't show any cytotoxicity on MCF-7 cell line whilst the methanolic extract of both studied parts had inhibited the growth of MCF7 by 72% and 68% respectively at a concentration of 50 µM **[29]**. However, the molecular mechanisms of the anti-proliferative effect of *E. creticum* phytochemical extracts have not been studied. The anti-proliferative effect of crude aqueous and ethanolic extracts of *E. creticum* aerial parts and roots on human cervical carcinoma HeLa cell line have not yet been studied. In contrast to previous studies, this is the first study to examine the extract effect from the second harvest of plant.

Our results demonstrate the selective dose-dependent cytotoxic actions of the crude aqueous and ethanolic extracts from first and second harvest of *E. creticum* leaves, stems, root and whole plant against human cervical carcinoma HeLa cell line. The observed selectivity in the antitumor effects of the extracts against HeLa cell line could be attributed to the actions of different *E. creticum* constituents on target molecules of the signal transduction pathways that regulate cell proliferation and apoptosis. Furthermore, each of the investigated extracts exhibited considerably stronger cytotoxicity to HeLa cells. The prominent antitumor properties of these extracts need to be examined further in in vivo studies. It would be interesting to investigate their action towards different PBMC subpopulations and elucidate the potential mechanisms through which they stimulate proliferation.

Several studies have shown that saponins, flavonoids, phenols, and tannins have an important role in the anticancer and in the antioxidant activity. Phenolics, flavonoids, and tannins from crude aqueous extracts of *P. indica* were shown to have anticancer potential by the inhibition of ATP-binding cassette transports in cancer cells **[32]**. Saponins from *Radix astragali* were found to suppress colon cancer cell carcinogenic activity by reducing vascular endothelial growth factor **[33]**. Flavonoid intake was found to be associated with a significant reduction in the risk of gastric cancer in women **[34]** whilst proanthocyanidins from grape seeds was found to inhibit pancreatic cancer cell growth and to induc apoptosis **[35]**. The cycle arrest and pro-apoptotic

effects of coumarin were shown against bladder cancer cell line **[36]**. The phenolic and flavonoid coumpouds of *Citrus aurantium* bloom were found to have antioxidant properties, anti inflammatory and anti cancer activity against human cancer cell lines (MCF-7; MDA-MB-231), and human colon adenocarcinoma (HT-29) **[37]**. The presence of tannins, flavonoids, phenolics, and saponins, among others in the extracts used in the present study, may be responsible for the antiproliferative activities on the HeLa cell line. In our study, it was observed that ethanolic leaves and aqueous whole plant extracts from second harvest might be a significant source of novel promising anticancer compounds in view of their pronounced cytotoxic activities against HeLa cells. The cytotoxic activities of leaves and whole plant aqueous extracts may be at least in part due to the phenolic compounds and flavonoids compounds, respectively. It is also important to note that second harvest extracts is found more effective than first harvest. DNA fragmentation assay showed that the treatment of HeLa cells with higher concentration of first and second harvest of *E. creticum* induced apoptotic cell death. Further examinations are needed to isolate and characterize the active components of anti-cancer in the extracts and to identify the caspases implicated in the employed apoptotic pathways.

CONCLUSION

In conclusion, the results of this study suggest that aqueous and ethanolic extracts of first and second harvest of E. creticum has a promising anti-oxidant, antiproliferative and cytotoxic effect on HeLa cervical carcinoma cells. In addition, it was found for the first time that the second harvest of *E. creticum* ethanolic extracts induced tumor apoptosis. Further investigations are needed to determine the entire anti-cancer molecular mechanism of E. creticum extracts. Also, it is very important to determine the cancer- suppressive effect of the tested extracts in in vivo experiments.

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