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ORIGINAL ARTICLE

Phenolic Profile with Biological Activities Assessment of Ethanolic and Aqueous Extracts from *Ephedra alata*

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Abstract

Ephedra alata aerial parts are often used in Tunisian traditional medicine, especially patients with cancer. The current paper highlights, for the first time, the pro-apoptotic properties of its aqueous and ethanolic extracts on leukemic, colon and pancreatic cancers. Furthermore, their anti-inflammatory and antioxidant activities were evaluated. Both extracts phenolic profile's was assessed by HPLC analysis. A total of eight and seven compounds respectively in ethanolic and aqueous extracts, were identified as tyrosol (31.86–50.17%) followed by catechin (9.26–10.52%) and syringic (6.94–17.06%), coumaric (10.20-13.56%) and para-hydroxybenzoic (5.67–13.48%) acids as main constituents. Caffeic acid was found only in the ethanolic extract (9.56%). The β-carotene bleaching test and ferric reducing power assay were used to evaluate the antioxidant activity. Both extracts displayed antioxidant potency. The strong antioxidant abilities (i.e. $IC_{50} = 0.023 \pm 0.003$ mg/mL and $EC_{50} = 0.157$ mg/mL for β-carotene bleaching test and ferric reducing power assay, respectively) were recorded in aqueous extract. In vitro anti-inflammatory activity was assessed by flow cytometry by following TNF-α production after lipopolysaccharide challenge of THP-1 cells. The results show that both extracts have the ability to inhibit significantly the TNF-α secretion. An effect more pronounced for the ethanolic extract. The pro-apoptotic potency of the extracts was estimated using viable and apoptotic markers assessed by flow cytometric. Here, both extracts, especially the ethanolic one, showed a noticeable stronger pro-apoptotic effect on all three tested human cancer cell lines (monocytic, colon and pancreatic carcinoma). Such promising anti-inflammatory and anticancer properties of both extracts are certainly related to their richness in tyrosol, catechin and various phenolic acids present in *Ephedra alata* aerial parts.

Keywords *Ephedra alata* · Antioxidant · Anti-inflammatory · Pro-apoptotic · Flow Cytometry · Hematopoietic Monocytic THP-1 · Colon Carcinoma SW-620 · Pancreatic Carcinoma AsPC-1

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1 Introduction

Cancer is a burden on humanity and is among the second leading cause of mortality worldwide; it accounts for around 10 million per year [1]. The rapid multiplication of abnormal growing cells is one of the most characteristic traits of cancer. These cells can occupy nearby organs and then move to other parts of the body [2]. Chemotherapy, radiotherapy, or resection and/or their combinations are frequently employed as therapeutic strategies for the fight against several types of cancers [3]. However, side effects, toxicity on cells of the healthy tissue and drug resistance in cancer are considered as principal limitations to chemotherapy treatment [4]. Around three quarters of the world's population use extensively medicinal plants for the treatment of a variety of human diseases among others, cancers [5]. The richness of medicinal plants with various phytochemical classes leads them to have promising therapeutic effects behind their potential anticancer activity [3]. For instance, aging and chronic diseases, such as obesity, cardiovascular, inflammatory and diabetes could be prevented using foods that contain phenolic compounds [6]. Furthermore, almost all of the anticancer drugs with clinical uses are plant-derived compounds like Taxol, vinblastine, vincristine, topotecan, campthotecin and irinotecan [7]. These derivatives act in cancer treatment through various molecular mechanisms including apoptosis, autophagy, activating tumor suppress genes, inhibiting tumor-relevant enzymes and stimulating the DNA damage repair mechanism [8, 9]. Recent studies have highlighted the important of the synergistic effects of phytochemicals in cancer prevention and therapy. In addition, medicinal plants that are rich in bioactive substances could use as adjuvant therapy to enhance chemotherapies and reduce their side effects [10, 11].

Therefore, the search for plants rich in bioactive substances or contain new pharmacological compounds with anticancer potential has dramatically increased in importance.

Ephedra alata is an edible, non-flowering seed plant from the Ephedraceae family. Commonly recognized as light small green shrub, very branched, with articulated stems. In Tunisia, it occurs the steppes as well as the deserts of the southern regions (Rjim Maâtoug and El Borma) [12, 13]. It has been proven that high intake of *Ephedra herba* aqueous extract (1000 mg/mL) induced basophils in renal tubules rats. Toxic effects of this plant were well identified and attributed to the presence of alkaloids [14]. Capsulated form of *Ephedra* species with other plants was clinically applied against Covid-19 [15]. Therefore, it is expected that this plant will attract the pharmaceutical companies due to its benefit therapeutics without ignoring the toxicity [16].

Ephedra alata is known for its anti-inflammatory, anticancer, antimicrobial, allelopathic antioxidant, anti-obesity, anti-hyperglycemia, anti-antipyretic and analgesic effects [17–20]. Moreover, spectrum of secondary metabolites found in *E. alata*, including phenolics, flavonoids, alkaloids, proanthocyanidins, saponins, and reducing sugars, are responsible for various pharmacological activities [18, 19]. Indeed, aerial parts of *E. alata* are frequently used in Tunisian traditional medicine, as an aqueous hot maceration, by patients with cancer. Various extracts of *E. alata* were previously described for their in vitro and in vivo anticancer properties on breast cancerous cells (MCF-7 & 4T1) [21–23].

According to the International Agency for Research on Cancer and the World Health Organization, colorectal cancer is ranking the second cause of cancer-related mortality in the world. Pancreatic cancer represents 2.5% of cancer cases worldwide and will be the second leading cause of cancer death in 2025. While, leukemia cancer is the tenth leading cause of cancer human deaths [24, 25].

To the best of our knowledge, the anticancer activity of *E. alata* extracts on leukemia, colorectal and pancreatic cancers have not yet been elucidated. Based on these considerations, the current study focuses on examining the pro-apoptotic potency of *E. alata* aqueous and ethanolic extracts on the human colorectal (SW620), pancreatic (AsPC-1) and leukemic monocyte (THP-1) cancer cell lines. Furthermore, the antioxidant and the anti-inflammatory proprieties of these extracts are elucidated. Also, the phenolic profile of the aerial part of *E. alata* was assessed using HPLC-UV.

2 Materials and Methods

2.1 Plant Material and Extracts Preparation

Ephedra alata aerial parts were collected from Kef Derbi area (latitude: 34°41', longitude: 9°29') in the Governorate of Gafsa, TUNISIA in March 2020. A voucher specimen (Ea-Gaf/20) was deposited at the Laboratory of Analysis, Treatment and Valorization of Environmental Pollutants and Products, Faculty of Pharmacy, University of Monastir, Tunisia.

The air-dried, powdered aerial parts of *E. alata* were extracted with distilled water or absolute ethanol at the ratio of (1:10 w/v). The next day, the aqueous maceration was filtered twice using Whatman No. 1 filter paper and then, lyophilized using a freeze dryer. After 48 h, the resulting alcoholic solution was concentrated under vacuum by using a rotary vacuum evaporator. The dried extracts were then weighed and stored at + 4 °C in an amber vial to be analyzed later.

2.2 Phenolic Profile Analysis

HPLC separation and identification of phenolic extracts was realized out on a Hewlett Packard system (Waldbronn, Germany) comprising a HP-1100 pump, a Rheodyne model 7725 injector (Cotati, CA, USA, loopvolume 20 µL), a UV detector (280 nm) and a C18 Technochrom Eurosphere 100 analytical column (250 mm × 8 mm). The mobile phase [Acetonitrile and sulfuric acid/water (2:98 v/v)] was pumped at a flow rate of 0.5 mL/min with linear gradient. Three milliliters of each extract were filtered through 0.45 µm membrane filter and 20 µL were directly injected into the HPLC. The column temperature was 25 ± 1 °C. The data were stored and processed by an HPLC Chemstation (Dos Series; Hewlett Packard). Phenolic compounds were identified on the basis of their retention times and quantified using external standard calibration curves.

Compounds (catechin, tyrosol, *p*-hydroxybenzoic acid, gallic acid, syringic acid, coumaric acid, vanillin, ferulic acid, quercetin, caffeic acid, and cinnamic acid) used as standards in HPLC analysis were acquired from Sigma-Aldrich.

2.3 Measurement of Antioxidant Activity

2.3.1 β-carotene Bleaching Assay

The β-carotene-linoleic acid bleaching activity of the extracts was performed according to the protocol of [26]. In brief, 500 µL of each extract at different concentrations (1-1000 µg/mL) was mixed with 5 mL of β-carotene-linoleic acid emulsion and incubated for 120 min at 50 °C. The absorbance of the mixtures was measured at 470 nm at 0 min and 120 min. The antioxidant activity (AA) of the tested extracts was determined by the following equation:

$$AA\% = \left[\frac{\text{Absorbance of } \beta\text{−carotene after 120 min of incubation}}{\text{Absorbance of } \beta\text{−carotene at 0 min of incubation}}\right) \times 100$$

2.3.2 Ferric Reducing Power Assay

The reducing power of *E. alata* extracts was tested based on the spectrophotometric detection of the iron reduction method as described by [27] with some modifications.

Aliquots (200 µL) of each extract at different concentrations (1-1000 µg/mL) was mixed with equal amount (200 µL) of sodium phosphate buffer (0.2 M; pH 6.6), and 500 µL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v). After 20 min of incubation at 50 °C, the reaction was stopped by adding 500 µL of trichloroacetic acid (1%, w/v) and the mixture was centrifuged for 10 min at 3000 rpm. Finally, 20 µL of ferric chloride (1%, w/v) and distilled water (100 µL) were added to the 100 µL of the supernatant. Absorbance of blend was measured at 700 nm. The effective concentration (EC_{50}) was deduced from linear regression analysis.

2.4 Cell Cultures

All human cell lines were acquired from ATCC (LGC Standards, Molsheim, France). All investigated human cell lines were acquired from ATCC (LGC Standards, Molsheim, France) pancreas adenocarcinoma (AsPC-1, ATCC® CRL-1682) and colon adenocarcinoma (SW-620, ATCC® CCL-229™) cell lines were kept in DMEM high glucose medium (Dominique Dutscher, 67,172 Brumath, France, Cat No. L0102-500), while human acute monocytic leukemia cell line (THP-1, ATCC® TIB-202) was maintained in RPMI-1640 medium (ATCC® 30-2001™, LGC Standards, Molsheim, France). Both culture medium were supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Life Technologies, Paisley, UK, Cat No. 10270-106) and 1% (v/v) penicillin–streptomycin (10.000 units/mL and 10.000 µg/mL, Life Technologies, Paisley, UK, Cat No. 15140-122).

Cells were conserved at 37 °C in a humidified environment containing 5% (v/v) CO_2 during their exponential development stage and throughout incubation with examined compounds.

2.5 Determination of Anti-inflammatory Activity

The anti-inflammatory power of *E. alata* extracts was accomplished by the measurement of TNF-α secretion by lipopolysaccharide (LPS)-stimulated THP-1 cells.

Briefly, THP-1 cells were seeded in 96-well plates (9×10^4 cells/mL). Each extract (500 µg/mL) was dissolved in culture medium and was freshly prepared from a stock solution prior to each experiment. To appropriate wells containing equal amount (100 µL) of cells in a culture medium were pretreated with extracts in culture medium, and then were incubated for 1 h at 37 °C and 5% CO_2. Then, cells were stimulated with lipopolysaccharides (LPS- Sigma-Aldrich, catalog number L7770), at 10 µg/mL. The cells were then incubated for 2 h under the same conditions. Ultimately, incubation period is required after the addition of the anti-TNF-α antibody conjugated to R-phycoerythrin (yellow fluorescence) according to the manufacturer's instructions (TNF-α Secretion Assay– Detection Kit (Miltenyi 200 Biotec. Paris, France). The level of TNF-α in non-stimulated and LPS-stimulated cells in the presence or absence of vehicle (DMSO) and tested extracts were detected by using microcapillary flow cytometry system (Guava1 EasyCyte). Yellow laser, is used for the evaluation of secreted TNF-α.

Here, Celastrol (50 µM) was used as an internal control known for his inhibition of TNF-α secretion. Dot plots were obtained by applying InCyte software 2.6 (Guava/Millipore/Merck) methods.

2.6 Apoptosis Analysis

AsPC-1, SW-620, and THP-1 cells, at a density of 1×10^5 cell/mL, were seeded in 96-well plates and then incubated overnight at 37 °C, 5% CO_2. The cells were then treated with *E. alata* aqueous and ethanolic extracts at increasing concentrations, (i.e., 62.5, 125, 250, 500 and 1000 µg/mL). After 24 h of exposure, for adherent AsPC-1 or SW-620 cells, a trypsinization protocol was applied each time prior to flow cytometry analysis. Suspended cells were then incubated with 2 µL of Annexin V-APC (ImmunoTools GmbH, Friesoy the Germany, Cat No. 31,490,013) and 2 µL Calcein-AM (Sigma-Aldrich., Cat No206700- Calbiochem) for 20 min prior cytometric analysis.

A minimum of 5000 cells were acquired per sample and analyzed with the InCyte software. Gates were drawn around the appropriate cell population using forward scatter (FSC) versus side scatter (SSC) acquisition dot plot to exclude any debris. In addition, by using log scale, set up dot plots were done to detect Calcein-AM as a marker for viable cells (green fluorescence) and APC-conjugated Annexin V that specifically labels apoptotic cells (Red fluorescence).

2.7 Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism software (Version 7.04, GraphPad Software, CA, USA). The results were expressed as means and standard deviation (SD) of three independent experiments. The analysis of variance (one-way ANOVA) was carried out following the Tukey's test at the $p < .05$ significance level.

Table 1 Phenolic profile of the ethanol and the aqueous extracts from *E. alata*

N°	Compounds	Ethanol extract		Aqueous extract	
		RT[a]	%	RT	%
1	Catechin	5.79	19.26	5.56	10.52
2	Tyrosol	6.36	31.86	6.44	50.17
3	*p*-Hydroxybenzoic acid	6.84	5.67	6.84	13.48
4	Gallic acid	7.86	nd[b]	8.17	nd
5	Syringic acid	9.78	17.06	9.72	6.94
6	Coumaric acid	10.20	10.59	10.15	13.56
7	Vanillin	10.40	nd	10.40	nd
8	Ferulic acid	10.68	2.21	10.62	2.09
9	Quercetin	14.79	nd	14.41	nd
10	Caffeic acid	15.91	9.56	16.00	nd
11	Cinnamic acid	16.52	3.74	16.44	3.20

[a] Retention time (min) [b] not detected

3 Results and Discussion

3.1 Identification of the Phenolic Compounds by HPLC

The polyphenolic profiles' qualitative analyses of the *E. alata* ethanolic and aqueous extracts were given by HPLC. A total of 8 and 7 compounds in ethanolic and aqueous extracts respectively were identified (Table 1, Figure S1 and Figure S2).

The HPLC analysis of the ethanol extract showed that tyrosol (**2**, 31.86%) is the major compound followed by catechin (**1**, 19.26%), syringic acid (**5**, 17.06%), coumaric acid (**6**, 10.59%) and caffeic acid (**10**, 9.56%). *p*-hydroxybenzoic acid (**3**), ferulic acid (**8**) and cinnamic acid (**11**) were detected in lower amounts (2.21–5.67%). For the aqueous extract, tyrosol (**1**, 50.17%) represented the major compound followed by coumaric acid (**6**, 13.56%), *p*-hydroxybenzoic acid (**3**, 13.48%) and catechin (**1**, 10.52%). Whereas caffeic acid (**10**) was not highlighted.

Comparing the composition of the ethanol and aqueous extracts, it was found that tyrosol (**2**) was the major compound in both extracts, but its proportion in aqueous extract was higher (50.17%) compared to that in ethanol extract (31.86%). It was also found that in ethanol extract the content of catechin (**1**) and syringic acid (**5**) was high compared to aqueous extract. Also, no caffeic acid (**10**) was detected in aqueous extract, whereas it is present in the ethanol extract in a non negligible amount (9.56%).

3.2 Antioxidant Ability

In the present study, the antioxidant activity of the tested extracts was accomplished by β-carotene and FRAP methods, on the basis of their IC_{50} and EC_{50} values determined from a calibration curve. The obtained results are shown in Table 2. Interestingly, aqueous and ethanolic extracts exhibited strong antioxidant ability for β-carotene assay with IC_{50} values of 23 ± 3 µg/mL and 25 ± 1 µg/mL, respectively. In addition, both extracts were capable of reducing ferric properties with similar capacities ($EC_{50} = 157 \pm 7$ and 161 ± 2 µg/mL) recorded in aqueous and ethanolic extracts, respectively. Taken as a whole, these data suggested that, aqueous and ethanolic extracts contain molecules with antioxidant properties.

Tyrosol is a phenolic compound abundantly found in olive oil, white wine and plant extracts. In the present work, tyrosol was identified as the main compound of the two extracts and can be one of the essential active principles at the origin of the antioxidant activities. This compound has been reported exhibiting antioxidant capacity [28]. Previous studies have also demonstrated that tyrosol has shown

Table 2 Antioxidant potencies of E. alata extracts

Extracts	IC_{50} (µg/mL)	EC_{50} (µg/mL)
	β-carotene	FRAP
Ethanolic	25 ± 1^a	161 ± 2^a
Aqueous	23 ± 3^a	157 ± 7^a
Ascorbic acid	17 ± 2^b	21 ± 1^b

Data represent the mean ± SD ($n = 3$)

Values followed by the same letter in the same column are not significantly different ($p < .05$) as assessed by Tukey test

IC_{50}: The concentration of samples at which 50% of antioxidant activity were shown

EC_{50}: The effective concentration to reduce 50% of the absorbance

an interesting antioxidant potency and a capacity in inhibiting the pro-oxidation processes on human LDL particles in several in vitro experiments [29, 30]. Moreover, the two extracts from *E. alata* were found to contain other phenolic compounds which can certainly contribute to the noted antioxidant activity. The presence of catechin, well known by its antioxidant power [31], in a relatively high percentage (10.52%) in this extract (aqueous) reinforces the observed activity. On the other hand, synergic acid present in both ethanolic and aqueous extracts with respective percentages of 17.06 and 6.94% is also well known for its fairly high antioxidant power [32, 33].

Coumaric acid, one of the major compounds in both extracts, is a natural ligand abundant in many vegetables, fruits and cereals with many health benefits. Previous studies have demonstrated the significant antioxidant capacity of this phenolic acid [34, 35]. Caffeic acid, detected only in the ethanolic extract, is a phenolic secondary metabolite biosynthesized by most medicinal plant species. It is well known by its antioxidant properties which is related to the position and the number of its phenolic functions in its structure [36]. *p*-Hydroxybenzoic acid, belonging to the family of phenolic compounds, is known in turn for its antioxidant properties against different types of free radicals and for its potency to prevent or reduce the overproduction of reactive species [37]. All the literature data cited above support the antioxidant activities of the two ethanolic and aqueous extracts noted. The slight difference in activity observed between the two extracts could be explained by the proportion and the structure of each compound contributing to these activities, obviously adding to the specific synergistic effect in each case.

3.3 Anti-inflammatory Activity

It has been proven that inflammatory status is characterized by the release of several pro- inflammatory mediators, like prostaglandins, proteinases, histamine and also cytokines. Among these, the pro-inflammatory cytokine (TNF-α) that possess a wide range of biological activities linked to the immune-pathology of acute or chronic inflammatory illness. It is well known that pretreatment of human monocytes (THP-1 cell line) with LPS results in a significant release of TNF-α. Therefore this model of cells is highly used to discover new anti-inflammatory agents. Thus, in this study, this model of cells was used to evaluate the anti-inflammatory properties of the extracts. As potent anti-inflammatory agent, Celastrol was used as control.

Dot plots obtained by flow cytometric analysis shown in Fig. 1, displayed the level of TNF-α secretion. In the opposite of non-stimulated cells, an increase in yellow fluorescence intensity (x-axis) was observed in LPS-stimulated cells compared to the control, which reflects the release of TNF-α by the cells.

While, treatment of LPS-stimulated cells with 500 µg/mL of aqueous and ethanolic extracts strongly inhibited the production of the cytokine compared to LPS/vehicle-treated cells. The level of TNF-α decreased from approximately 80% in the vehicle to 41.79%, 29.27%, and 19.14% in the aqueous, ethanol, and Celastrol treatments, respectively. Furthermore, the bar graph presented in Fig. 2, revealed a significant inhibition level of TNF-α in LPS-stimulated cells when exposed to aqueous and ethanolic extracts compared to the vehicle.

Moreover, ethanolic extract showed greater inhibition of the TNF-α secretion level (21.91%) than aqueous extract with a lower value (42.29%). Therefore, these data suggest that these extracts could contain important anti-inflammatory molecules, since it exhibit well their ability to inhibit TNF-α similarly to Celastrol that known to be pure inhibitory substance.

Polyphenols are promising secondary metabolites that may help to control oxidative stress and consequently inflammatory response. Additionally, they are implied in a diversity of reactions as a result of inflammation [38]. Several works have been undertaken to study the anti-inflammatory power of polyphenols, and developed methods in cell and molecular biology helped to understand the possible interactions of these compounds with basic inflammation response mechanisms [39, 40].

The potential anti-inflammatory activity of tyrosol has been proven against different enzymes, including cyclooxygenase-2 (COX-2) [41], TNF-α [42] and methyl glyoxal-induced inflammation in RAW264.7 cells [43]. It has also been shown that tyrosol is able to inhibit TNF-α, IL-1β and IL-6 production by LPS-stimulated human peripheral blood mononuclear cells [44]. Previous works reported to suppress inflammation [45], and attenuates pro-inflammatory cytokines [46]. Also, it displays several health-promoting properties, including anti-inflammatory, and antioxidant effects [47–50]. Therefore, in our study by using a different technique we expected that this molecule will be responsible

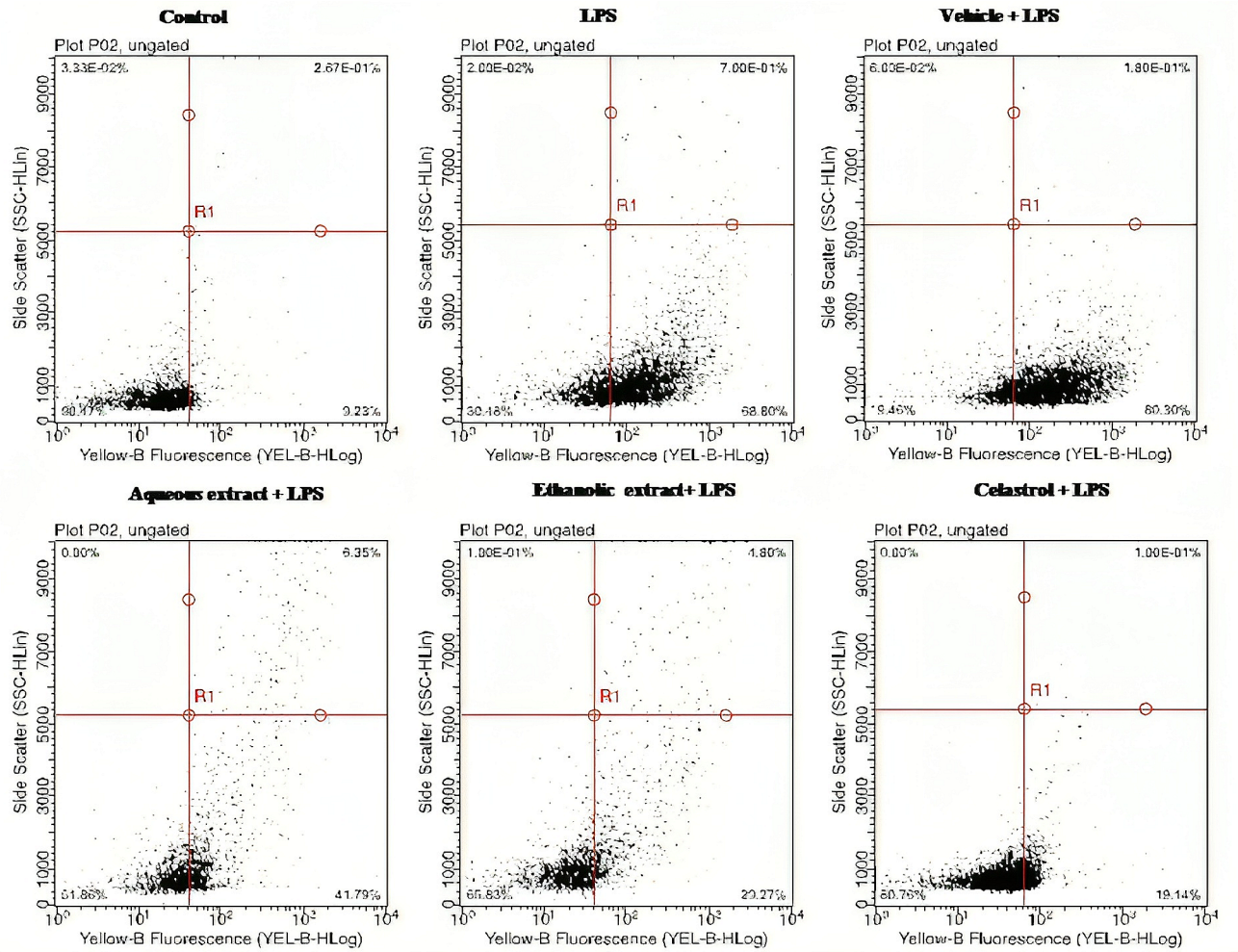

Fig. 1 TNF-α secretion assay. THP-1 monocyte cells were treated with 0.5 mg/mL of each sample for 1 h, then were stimulated with LPS for 2.5 h tracked by incubation with TNF-α antibody, and then detected by flow cytometry. Dot plots are given here representative the level of TNF-α secretion non-stimulated and stimulated cells in the presence or absence of vehicle and extracts

for inhibiting TNF-α in monocyte cells and so will reinforce previous studies showing anti-inflammatory properties of this plant or its extract.

On the other hand, catechin, the second predominant compound in ethanol extract (19.26%) but also found at 10.52% in the aqueous one, has previously been verified for its anti-inflammatory potency [51, 52]. The role of catechin in the prevention of TNF-α mediated inflammation resistance in 3T3-L1 adipocytes was proved. Regulation of AMPK/SIRT1 activity by catechin is considered as a promising therapeutic way towards a broad variety of chronic inflammation and related illnesses [53]. On the other hand, syringic acid one of the main compounds, highlighted in both studied extracts, displays several significant bioactivities in medical field such as antioxidant, anti-inflammation, anti-cancer, cardiovascular, anti-diabetic and liver and brain/CNS [54]. In the current study, catechin is expected to play a role for inhibiting TNF-α in monocyte cells.

It has been previously demonstrated that coumaric acid could alleviate the symptoms of arthritis in rats according to a mechanism including suppression of inflammatory cell infiltration, as well as the levels of TNF-α and IL-6 [55].

Caffeic acid has been reported to exhibit a wide range of biological effects including anti-inflammatory. It was demonstrated that caffeic acid was able to limit nitric oxide (NO) and prostaglandin E2 (PGE2) production in LPS-stimulated RAW264.7 cells. Moreover, mRNA levels of down regulate tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2, and inducible NO synthase (iNOS) [56].

Given the presence of the aforementioned compounds in the two studied extracts except caffeic acid, which is absent in aqueous extract, we assume that each of them contributes to the noted anti-inflammatory activity in an individual manner and/or in synergy.

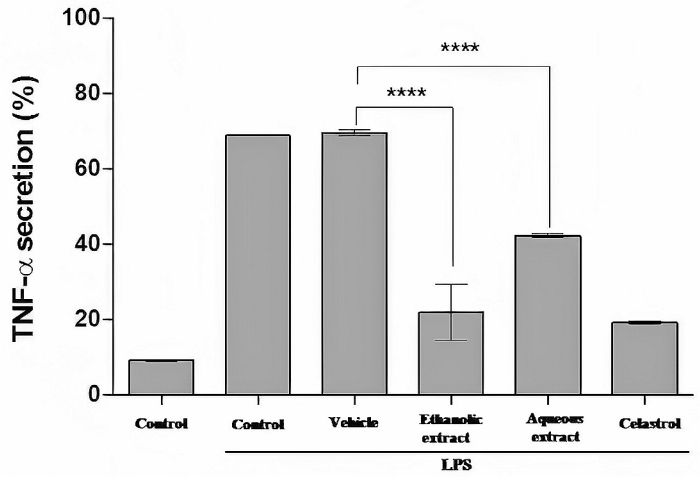

Fig. 2 Effect of extracts of aerial parts of *E. alata* on TNF-α inhibition activity after 2.5 h LPS-stimulation THP-1 cells. Data represent the mean ± SD. Bars with asterisks are statistically different ($P < .001$). The experiments were performed three times, with each individual treatment being run in triplicate

3.4 Apoptotic Activity

One of the strategies to discover new candidate anti-cancer compounds is their ability to eliminate cancer cells *via* programmed cell death pathway. Therefore, the induction of apoptosis by *E. alata* extracts was evaluated using Annexin V-APC/Calcein-AM, characteristic markers for cell apoptosis and cell viability, respectively. This double assay was conducted on three human cell lines: the cancerous monocytic THP-1 cell line, the colon carcinoma SW-620 and the pancreatic carcinoma AsPC-1. As evidenced in Fig. 3, flow cytometric analysis clearly demonstrated an increase in red fluorescence intensity (x-axis), which reflect that, these population of cells were positive to the apoptosis marker when the cells exposed to both extracts compared to the vehicle-treated cells. Treatment of THP-1, SW-620 and AsPC-1 with 1 mg/mL of aqueous extract for 24 h resulted 69.3%, 76.16% and 34.3% of Annexin V-APC positive, respectively. Whereas, for ethanolic extract and under the same conditions, were 93.3%, 78.07% and 41.9%, respectively. It is important to mentioned that, the percentage of AnnexinV-APC is estimated as a total of the percentage of cells in early (upper-Right quadrant) and late (Lower-Right quadrant) apoptosis.

In addition, dose-dependent anti-tumor effects of the aqueous and ethanolic extracts were investigated. Three cancer cell lines were exposed to various concentrations of each extract. The bar graphs in Fig. 4A, C, E G, I, K, represent the percentage of total apoptotic cells of three tested cell lines induced by each concentration. The results revealed that, all tested cell lines were significantly undergo apoptosis when exposed to 250 µg/mL of both extracts, except aqueous-AsPC-1 treated cells (Fig. 4E). The later showed minor increase in apoptotic cells at high concentrations but was not significant compared to the vehicle-treated cells. Furthermore, the finding showed that, monocytic THP-1-treated cells were extremely sensitive especially to the ethanolic extract, since the effect started to be significant after exposure to 62.5 µg/mL (Fig. 4G). Furthermore, the half-maximal effects (IC_{50}) values were estimated based on the percentage of viable cell (% of Calcein-positive cells). The curves derived from IC_{50}-normalized data (Fig. 4B, D, F, H, J, L) allow us to determine the half-maximal effects. Aqueous extract exhibited IC_{50} values of 439.2, 237.4 and 413.7 µg/mL, against THP-1, SW-620 and AsPC-1 cell lines, respectively. While, for ethanolic extracts were 52.94, 227.5, and 236.4 µg/mL, respectively. Noteworthy, the ethanolic extract reveals always to have the most potent pro-apoptotic activity on the tested human cancerous cells when compared to the aqueous extract.

To the best of the authors' knowledge and despite the important anticancer power of *Ephedra* species, its potential apoptotic activity in these human cancer cells has not been reported yet. The differential responses to the treatment could relate to the biological properties of the tumor cell line i.e. genetic alterations in particular cancer including genes that regulate DNA damage, survival and apoptosis and could relate to the presence of specific-resistance

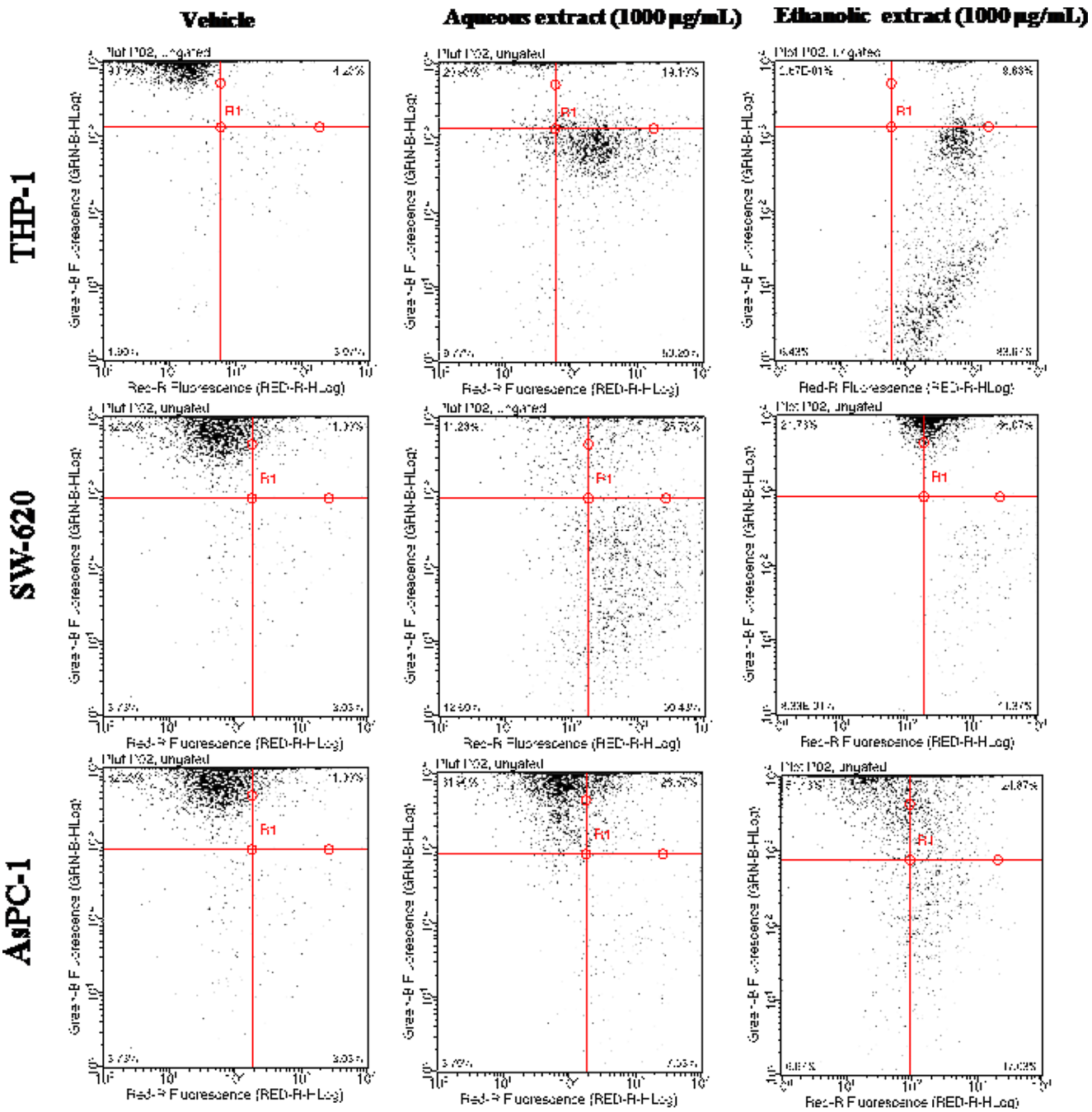

Fig. 3 Apoptosis induction by aqueous and ethanolic extracts on THP-1, SW-620 and AsPC-1 cell lines after 24 h. Dot Plots represent cells in apoptosis (x-axis-red fluorescence/ upper and lower right quadrant) or viable cells (Y-axis-green fluorescence /upper-left quadrant) and necrotic cells (lower left quadrant)

mechanisms that characterize particular tumor. Therefore, heterogeneity among cancers could result variable in treatment effectiveness [57], which may explain the differential sensitivity of tested cell lines to the extracts.

Various polyphenols are reported to be endowed with several anticancer powers, including cell cycle arrest, apoptosis induction, antiproliferative, antioxidant, antiangiogenic, and antimetastatic action against many human cancer cell lines without affecting normal cells [58, 59]. The pro-oxidant properties of natural polyphenols is at the origin of their anti-cancer and chemopreventive potentials [60]. In the current study, Tyrosol the major phenolic compound identified in the aqueous and the ethanolic extracts, exerts a potential antigenotoxic, cytotoxic and pro-apoptotic activities when high concentration are administrated [61]. This is consistent with our finding, as significant apoptosis

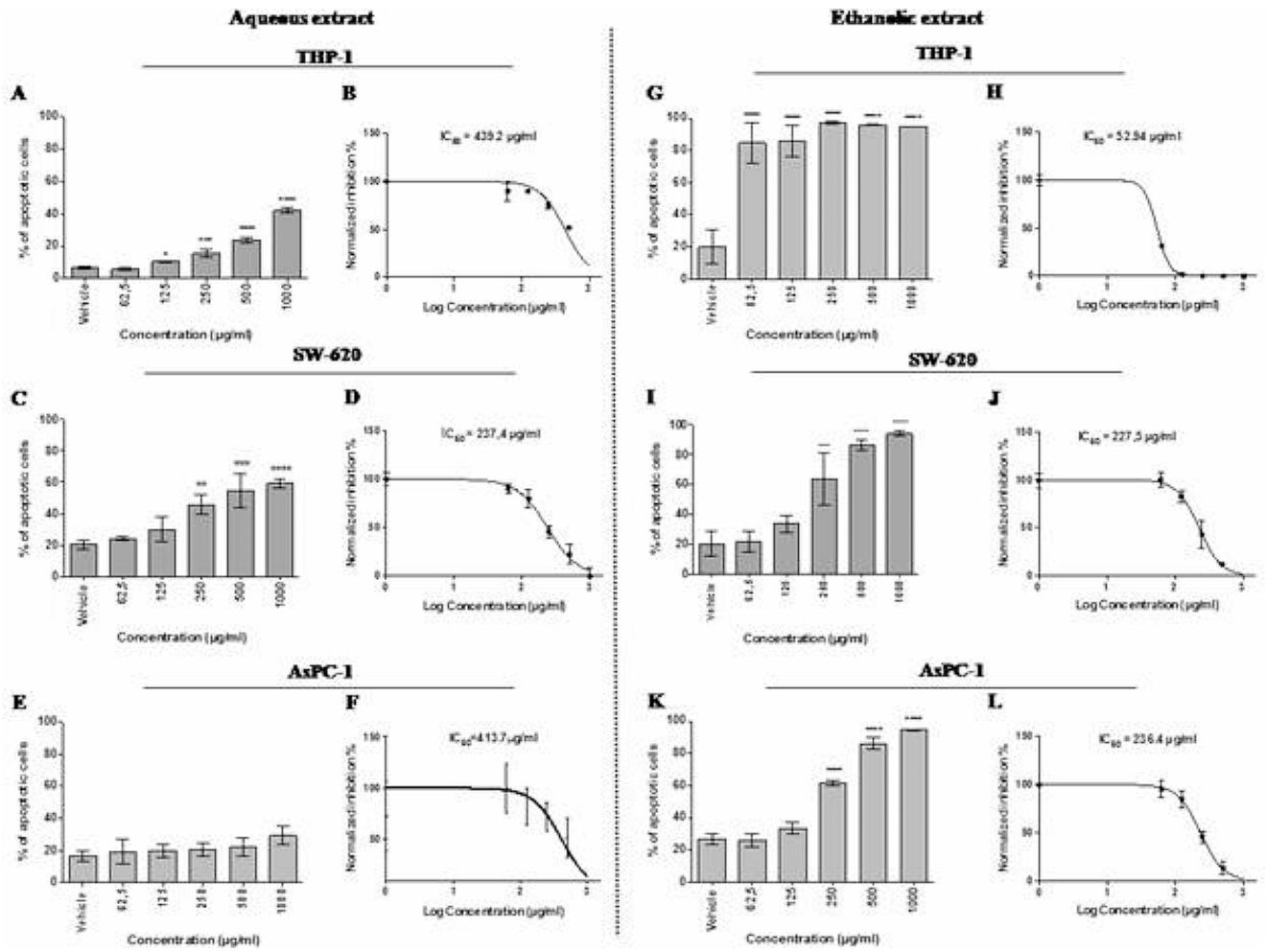

Fig. 4 Concentration-dependent effects of aqueous and ethanolic extracts on THP-1, SW-620 and AsPC-1 cell lines after 24 h. (**A, C, E, G, I, K**) Bar charts represent the percentage of annexin V-positive cells. The number of apoptotic bodies is expressed as percent relative to the total cell number. (**B, D, F, H, J, L**) Log concentration-normalized inhibition curve and IC_{50} values for aqueous and ethanolic extracts

induction is obtained with higher concentrations, especially on colorectal and pancreatic cancers. It has been reported previously that Catechin and syringic acid [62–64], coumaric and ferulic acids [65–67], cinnamic acid [68–70] and caffeic acid [71–73] exhibited antioxidant activity and potential anticancer proprieties on numerous tumor cells.

In addition, a previous work has demonstrated that several polyphenolic compounds act in combination to induce apoptosis and inhibit the growth of different cancer cells [74, 75]. Thus, the results of this current work further confirm the findings of the previous studies and could be interpreted by a synergistic pro-apoptotic effect of the phenolic constituents present in each extract. The pronounced activity of the ethanolic extract compared to the aqueous one may be related to the proportion of each product. One should note that, plant spices, plant part, environmental conditions and experimental procedure could influence bioactive molecules content in the plants.

4 Conclusion

Our work demonstrates here that ethanolic and aqueous extracts of *Ephedra alata* are rich in several phenolic compounds of which tyrosol is the main product, present in a fairly high proportion. The two extracts exhibit potent antioxidant, anti-inflammatory and apoptotic activities against several human cancer cells. These activities may be due to the presence of tyrosol together with the other phenolics such as catechin, synergic, coumaric, cinnamic and caffeic acids well-known that all exhibit such activities individually and/or in synergy. Thus, these properties might permit such extracts to be considered as effective source of bioactive molecules to treat these cancer types. However, further assays, in vitro and in vivo, are needed with compounds separated and in combination so better understand the origin of the described activities. Therefore, we have planned to conduct further phytochemical analysis experiments such

as Thin Layer Chromatography (TLC) to finally isolate the identified phenolic compounds.

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Informed Consent Not applicable.

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