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RESEARCH ARTICLE

IN VITRO EVALUATION OF ANTIOXIDANT AND CYTOTOXIC ACTIVITIES ON HUMAN CERVICAL CANCER CELLS IN THREE DIFFERENT PLANT EXTRACTS FROM TURKEY

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ABSTRACT

The objective of this investigation was to assess the total phenolic and flavonoid content within acetone and water extracts derived from *Erica manipuliflora*, *Ferula communis*, and *Stevia rebaudiana* plants. Additionally, the *in vitro* antioxidant and cytotoxic effects of these extracts were evaluated using the human cervical cancer (HeLa) cell line as a model. Among the three plant species examined, the highest concentrations of total phenols and flavonoids were found in the acetone extract of *E. manipuliflora*, measuring 365.29 mg GAE/g DW and 105.42 mg QE/g DW, respectively. Acetone and water extracts of *E. manipuliflora* showed higher DPPH scavenging activities (IC_{50}: 37.57 and 33.27 µg/mL, respectively) compared to other plants. furthermore, E. manipuliflora acetone extract (IC_{50}: 97.35 µg/mL) exhibited the highest inhibition in the HeLa cell line treated with the extracts, thus being the most effective extract on this cell line. As a result of the analyzes, it has been shown that *F. communis* and *S. rebaudiana* and especially *E. manipuliflora* are important natural antioxidant sources with their strong radical scavenging effects, as well as the anticancer potential of these plants.

Keywords: *Erica manipuliflora, Ferula communis, Stevia rebaudiana, Antioxidant, Cytotoxic Activities, Phenolic Content.*

1. INTRODUCTION

Medicinal plants, employed both in traditional and contemporary medical practices, harbor an array of phytochemicals within their tissues and organs, showcasing diverse potential benefits such as antimicrobial, antioxidant, anticancer, and antifungal properties [1]. Due to the natural and synthetic therapeutic properties used today, interest in medicinal and aromatic plants is increasing both in different industrial areas such as medicine, cosmetics, agriculture and in academic research. In recent times, there has been a growing emphasis on conducting *in vitro* and *in vivo* research involving plant extracts, driven by the recognition of their multifaceted health benefits. Phytochemicals, found

abundantly in these extracts, exhibit a spectrum of therapeutic properties, including but not limited to antioxidants, anticancer agents, and anti-inflammatory agents [2].

Reactive oxygen species (ROS) that occur in plant tissues under biotic and abiotic environmental stress conditions and cause DNA and cell damage are scavenged by many enzymatic [PPO (Polyphenol oxidase), CAT (catalase), APX (ascorbate peroxidase), POD (peroxidase)] and non-enzymatic (ascorbic acid, phenolic compounds, carotenoid, glutathione etc.) antioxidants [3]. Antioxidants are acknowledged for their capacity to promote well-being and mitigate the likelihood of numerous conditions, including various forms of cancer, hypertension, diabetes, asthma, and heart disease [4, 5]. Numerous investigations have demonstrated the utility of plant polyphenols, which encompass essential biological properties such as antioxidant activity, in combatting a variety of oxidative stress-related ailments [6, 7]. Although synthetic antioxidants increase the shelf life of foods, some studies have mentioned their disadvantages for human health. For this reason, many researchers have focused on the development of easily accessible naturally sourced antioxidants [8].

Erica manipuliflora Salisb. (Ericaceae) is a plant species in the form of an upright bush that can reach a height of about 4 m and is known as "broom grass" or "puren" in Turkey. *E. manipuliflora* is commonly found in parts of the Eastern Mediterranean (Turkey, Cyprus, Lebanon, Syria, Greece), Albania, Bosnia and Herzegovina, Croatia, Italy, North Macedonia, Serbia, Montenegro, and Slovenia. The aerial parts (leaf and flower) of *E. manipuliflora* have been used in the treatment of many diseases for ages due to their many medicinal benefits (antioxidant, anticholinesterase, diuretic, astringent) [9]. The main compound of monoterpenoids, which are abundant in the aerial parts of the plant, is germacrene D (13.58%-15.55%) [10]. Although it has been used for therapeutic purposes in traditional medicine in Turkey for many years, there is not enough data on both its phytochemicals and biological activities in the literature. In this context, *E. manipuliflora*, which is accepted as a medicinal plant with various pharmacological effects, is a species worth examining in terms of its biological activities.

Ferula communis L. (Apiaceae) is a tall (1.5-3 m), herbaceous and perennial plant species known as "Caksır otu" or "Atkasnagi" in Turkey. *F. communis* is distributed in the forests and bushes of the Mediterranean, East Africa and Central Asia. In the phytochemical analysis of *F. communis* fruit flower root parts, bioactive compounds such as tannic acid, ferulic acid, catechin, syringic acid, gallic acid, coumarin were determined in varying proportions of each part [11]. Although the mainly roots (β-farnesene, β-cubene, caryophyllene) of this plant, which is rich in especially sesquiterpenes, are used for therapeutic purposes, it has been stated in previous studies that leaf (β-eudesmol, α-eudesmol, hedycariol), flower (α-pinene, γ-terpinene, hedycariol), and fruit (α-pinene, β-pinene, myrcene) extracts also contain different bioactive compounds [12]. *F. communis*, a plant historically employed in traditional pharmacopoeia for treating diverse conditions like fever, skin infections and dysentery, has been the subject of research revealing its multifaceted properties, including anti-inflammatory, antineoplastic, anticoagulant, antiproliferative, cytotoxic, antimicrobial and herbicidal activities [13-15]. In another study, it was reported that this plant has toxic effects for humans and animals [16].

Stevia rebaudiana Bert. (Asteraceae) is a perennial herbaceous plant species that reaches 30-60 cm in length, known as "sugar grass" in Turkey and its natural habitat is subtropical regions (such as

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Paraguay and Brazil). The stevioside found in the leaves of *S. rebaudiana* adds sweetness to this plant and is known to be 100-300 times sweeter than table sugar. Therefore, it is a natural sweet source worldwide, especially as an alternative to sucrose and synthetic sweeteners [17]. As a result of GC-MS analysis of leaves, major phytochemicals (1-heptatriacotanol-antihypercholesterolemia; dihydroxanthin-antitumor; β-amyrin and lupenone-anti-inflammatory; phytol-antidiabetic) responsible for different and multiple biological activities were obtained [18]. This plant not only serves as a rich source of numerous antioxidant compounds but is also harnessed for its therapeutic potential in managing neurodegenerative diseases such as Parkinson's and Alzheimer's disease [19]. These therapeutic attributes have propelled it into the spotlight of numerous scientific investigations, including in vitro studies involving cell cultures, callus cultures, as well as tissue and organ cultures [20,21].

The common features of these three species used in the current study are that they belong to the class of medicinal and aromatic plants that contain extremely important compounds in the pharmacopeia and are suitable for the geography of Turkey. In this context, some biochemical contents of *E. manipuliflora, F. communis, S. rebaudiana* plants were investigated, and antioxidant potentials and cytotoxic activities of these plant extracts were determined and compared with each other.

2. MATERIALS and METHOD

2.1. Plant Material and Preparation of the Extracts

The plant specimens used in this study were sourced from the Zeytinburnu Medicinal Plant Botanic Gardens located in Istanbul, Turkey. Plant samples [E. manipuliflora-aerial (leaf and flower), F. communis-root, S. rebaudiana-leaf] were dried at room conditions and ground into powder in a grinder. To obtain the extracts, 20 g of finely ground plant samples were subjected to extraction in 250 mL of both acetone and water separately using a Soxhlet apparatus for a duration of 12 h. Following extraction, the resulting extracts were subsequently filtered through Whatman No.1 filter paper. The acetone extract was evaporated under vacuum at 40°C with a rotary evaporator, the water extract was lyophilized. All samples were stored in a closed container at -20°C until analysis. The data presented in Table 1 showcases the yield rates of extracts derived from plant samples, specifically those obtained through the use of acetone and water.

Table 1. Yield (%) of obtained the plant extracts.

Samples	Abbreviation of the extracts	Yield (%)
Acetone extract obtained from *E. manipuliflora*	EE_A	6.14 ± 0.47
Water extract obtained from *E. manipuliflora*	EE_W	18.21 ± 1.31
Acetone extract obtained from *F. communis*	FE_A	8.6 ± 0.54
Water extract obtained from *F. communis*	FE_W	22.34 ± 1.14

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Acetone extract obtained from *S. rebaudiana*	SE_A	3.47 ± 0.67
Water extract obtained from *S. rebaudiana*	SE_W	26.52 ± 1.23

2.2. Quantitative Determination of Secondary Metabolites in Plant Extracts

2.2.1. Determination of total phenolic content (TPC)

The quantification of total phenolic contents (TPC) in the extracts followed the method outlined by Ulusu and Şahin [22], employing the Folin-Ciocalteu colorimetric assay. A standard calibration curve using gallic acid was used as a reference. 100 µl of extracts (10 mg/mL), 9 mL dH_2O, 200 µl of Folin-Ciocalteu reagent and 600 µl of Na_2CO_3 (2%, w/v) were added, after mixing, the total volume was adjusted to 10 mL with dH_2O. After the samples were incubated for 2 h under dark room conditions, the absorbances of the samples were measured at 750 nm in the Shimadzu UV-1800 spectrophotometer. The total phenolic content was calculated as the mean ± standard error from the calibration curve obtained as gallic acid equivalent (GAE). All experiments were performed in triplicate.

2.2.2. Determination of total flavonoid content (TFC)

The quantification of total flavonoid contents (TFC) in the extracts followed a slightly modified protocol based on the method originally described by Bouasla [23], utilizing quercetin as the standard. In this procedure, 250 µl of extracts at a concentration of 10 mg/mL were combined with 1.25 mL of dH_2O, 75 µl of $NaNO_3$ (5%, w/v), 150 µl of $AlNO_3$ (10%, w/v), and 0.5 mL of NaOH (1M). The total volume was adjusted to 2.5 mL using dH_2O to complete the assay. Following thorough mixing, the solution was allowed to incubate at ambient room temperature for a duration of 40 min. Subsequently, the absorbance values were recorded at a wavelength of 415 nm using a spectrophotometer. The quantification in quercetin equivalent (QE) was carried out by applying the standard calibration curve established with quercetin.

2.3. DPPH (1-1-diphenyl 2-picryl hydrazyl) Radical Scavenging Assay

In measuring the DPPH scavenging activity of plant extracts, each extract and DPPH were dissolved in methanol. 0.2 mL of each extract at different concentrations (10, 25, 50, 100, 200, 400 µg/mL) and 1.8 mL of DPPH (0.06 mM) solution was added to it. The solutions were kept in dark room conditions for 30 min (incubation time). Absorbance values were determined at 517 nm following incubation. In this experimental setup, ascorbic acid served as the designated positive control. In addition, all experiments were performed in triplicate. The following equation was used to compute the extracts' percent inhibition of DPPH radical scavenging:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \left[\frac{A_0 - A_s}{A_0}\right] x100$$

A_0 = The absorbance of the control
A_s = The absorbance of the extract

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2.4. Cell Culture

In this study, the human cervical cancer (HeLa) cell line was cultured in a medium comprising DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.01% gentamicin. The culture plates were maintained in a sterile microculture environment within an incubator set at 37°C, with a controlled humidified atmosphere containing 5% CO_2. Subsequent to the incubation period, confluent cells were dislodged from the culture surface employing the standard trypsinization procedure. The cytotoxicity assays were conducted in triplicate for each sample during *in vitro* testing.

2.4.1. Cell viability assay

The evaluation of cytotoxicity on the HeLa cell line was conducted using the Alamar Blue® assay. To facilitate testing, all plant extracts were initially dissolved in a 1 mg/mL DMSO (dimethyl sulfoxide) stock solution. Importantly, the DMSO concentration in the culture medium did not exceed 0.1%. Subsequently, we prepared a series of dilutions to achieve various sample concentrations, including 10, 25, 50, 100, 200, and 400 µg/mL. Cells were introduced to 96-well microculture plates, with each well receiving a seeding of $2x10^4$ cells. They were then incubated for 24 h at 37°C to facilitate proper adhesion to the plate surface. After incubation, fresh medium containing different concentrations of plant extracts (10, 25, 50, 100, 200, 400 µg/mL) was added. The negative control group was culture medium containing 0.1% DMSO. The HeLa cell line was exposed to plant extracts in triplicate for 24 h at 37°C. After incubation, Alamar Blue® reagent (1:10, v/v) was added to each microplate well. Subsequently, the plates underwent a 4 h incubation period at 37°C, during which measurements were taken at wavelengths of 570 nm and 600 nm using a spectrophotometric microplate reader (Multiscan Go, Thermo Fisher Scientific, USA). Cell viability was then determined as a percentage of the initial cell count.

3. RESULTS AND DISCUSSION

3.1. The Yield of Crude Extracts

Phytochemical extraction from plant material is the focus of research. Secondary metabolites with different polarities can be included in the solution depending on the polarity of the solvents used in the extraction [24]. In this study, extractions of *E. manipuliflora*, *F. communis* and *S. rebaudiana* were carried out using polar (water) and apolar (acetone) solvents. From the plant materials used, the highest yield was obtained from SE_W (26.52%), while the lowest yield was obtained from SE_A (Table 1). The water extract was effective in obtaining the highest yield of all plant extracts. This result shows that increasing solvent polarity significantly increases the plant extraction yield. Zaidan [18] emphasized that water was the best solvent compared to methanol, ethanol and acetone solvents in *S. rebaudiana* extraction and obtained the highest yield from the water extract. Again, among the compounds extracted in water, the inclusion of not only secondary metabolites but also high-soluble primary metabolites (such as protein and carbohydrates) in the extract contributes to increasing the yield [25].

3.2. TPC and TFC of the Plant Extracts

TPC and TFC of acetone and water extracts obtained from *E. manipuliflora*, *F. communis* and *S. rebaudiana* were investigated. The recovery amount of TPC and TFC from *E. manipuliflora* and *F.*

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communis water extracts is significantly higher than from acetone extracts. Also, according to the data, the highest phenol content belongs to EE_W (365.29 mg GAE/g DW), followed by FE_W (132.82 mg GAE/g DW). Similarly, the highest total flavonoid content was found to be EE_W (105.42 mg QE/g DW), while the lowest SE_W (6.53 mg QE/g DW) (Table 2).

It is stated that polar solvents are generally more suitable for revealing the polyphenols in the plant cell, while alcohol-derived solvents are more effective in the degradation of the cell wall and testa. Again, polar solvents such as water and methanol are more efficient than apolar solvents in terms of extraction of phenolic compounds [26, 27]. The dielectric constant of the organic solvents utilized in the extraction process can influence TPC and TFC found in plant extracts [28]. Therefore, this study proves once again that polarity differences of plant phytochemicals and solvents affect phenolic and flavonoid recovery. Prior research has identified the presence of numerous phenolic and flavonoid compounds within the specific plant species under investigation. In these studies, unlike us, methanol and ethanol extracts were used in *S. rebaudiana* plant, and while the highest TPC was obtained from polar solvents (methanol-6.96 and water-6.65 mg GAE/g), the highest TFC was obtained from ethanol extract (10.91 mg QE/g) [18]. Again, TPC in ethanol extracts of four *S. rebaudiana* lines ranged from 55.64 to 58.35 mg GAE/g DW [29]. *E. manipuliflora*, this situation varied in extraction by different solvents (water, methanol, chloroform, ethyl acetate, n-butanol) and the highest TFC was determined with ethyl acetate (735.5 mg GAE/g) [30]. In another study, the TPC of *E. manipuliflora* methanol extract was 260 mg QE/g [31]. TPC and TFC of *F. communis* (stem) methanol extract were obtained as 129.86 mg GAE/g DW and 13.37 mg QE/g DW, respectively [11]. Among the extracts of *F. communis* (aerial part) made by different solvents, the highest TPC (0.031 mg GAE/mg extract) was in the ethanol:water (50:50) mixture [32].

Table 2. Total phenolic content (TPC) and total flavonoid content (TFC) of the plant extracts.

Plant extracts	TPC (mg GAE/g DW)[a]	TFC (mg QE/g DW)[b]
EE_A	103.32 ± 5.71	27.43 ± 0.53
EE_W	365.29 ± 1.52	105.42 ± 1.41
FE_A	114.75 ± 2.23	15.21 ± 0.44
FE_W	132.82 ± 2.81	18.43 ± 0.56
SE_A	63.78 ± 0.86	7.87 ± 0.25
SE_W	55.46 ± 0.72	6.53 ± 0.16

Each value is represented as the mean ± standard deviation, based on a sample size of n = 3.
[a]mg gallic acid equivalent per gram of dry weight.
[b]mg quercetin equivalent per gram of dry weight.

3.3. DPPH Scavenging Activities of the Plant Extracts

One of the oldest and most commonly employed *in vitro* techniques for assessing the antioxidant properties of research materials relies on DPPH radical analysis. A dose-dependent increase in DPPH scavenging activities was observed across various concentrations (10, 25, 50, 100, 200, and 400

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µg/mL) of the plant extracts. Among the extracts, the treatment of EE_W and EE_A at concentrations of 200 and 400 µg/mL resulted in the highest antioxidant activity with over 85% inhibition, and the results of these two concentration treatments were statistically different (p<0.05) (Figure 1.). In addition, the lowest IC_{50} value (33.27 µg/mL) was belonged to EE_W, while the highest IC_{50} value (101.11 µg/mL) was found in SE_A (Table 3.). While both water and acetone extracts of *E. manipuliflora* had the highest DPPH scavenging activity, the lowest activity was determined in acetone extracts of *S. rebaudiana*. Antioxidant compounds in water and acetone extracts of the studied plant species showed good rate antioxidant activity. In addition, there are many antioxidant studies conducted in these plant species. In the literature, DPPH activity of *E. manipuliflora* was determined as IC_{50}: 255.9 mg/mL in methanol extract [31], IC_{50}: 0.021 µg/mL in water extract [30]. The DPPH activity of *S. rebaudiana* (leaf) was found to be IC_{50}: 83.45 µg/mL in aqueous extract [33], IC_{50}: 93.46 µg/mL in ethanol extract [34], IC_{50}: 752.6 and 904.4 mg/mL in water and methanolic extract, respectively [35]. *F. communis* (stem) methanol extract has an IC_{50} value of 168 µg/mL in the DPPH activity [11], in another study, the best antioxidant activity was determined with a parallel effect to the ethanol:water extract, from which the highest antioxidant compounds were obtained [32]. Our results show that water and acetone extracts of *E. manipuliflora, F. communis* and *S. rebaudiana* are potent antioxidants. Oxidizing enzymes (such as peroxidase) contribute to the reduction of oxidative damage in cells by being inhibited by some plant-derived molecules (phenols). In this study, it was determined that *F. communis* and *S. rebaudiana*, and especially *E. manipuliflora*, contain high levels of phenolic compounds, and thus, it can be thought that they show high antioxidant capacity due to these phytochemicals.

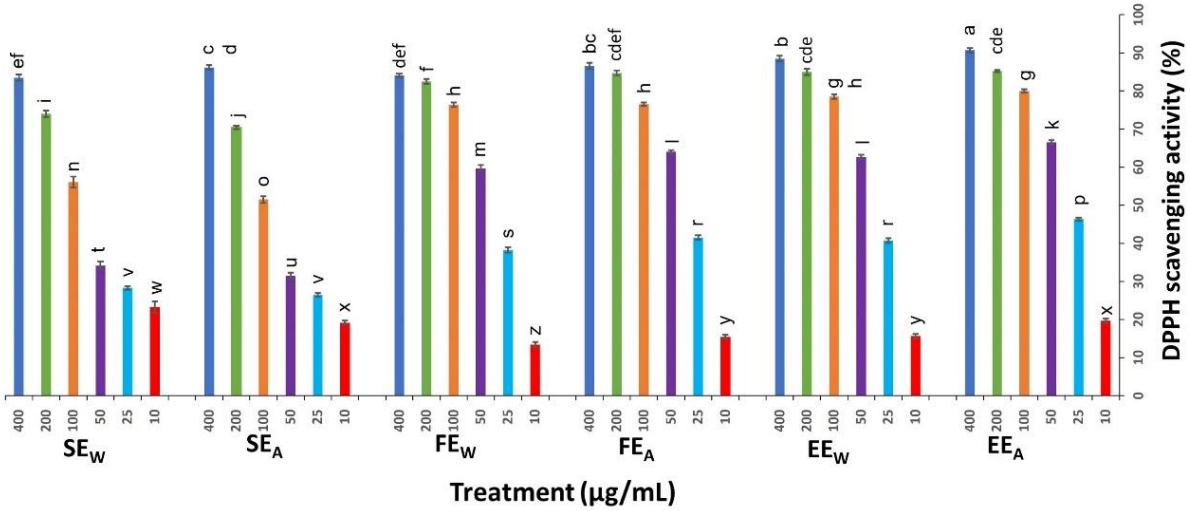

Figure. 1. DPPH scavenging activities of different plants extracts.

3.4. Cytotoxicity activities

The Alamar Blue® assay was employed to assess the viability of HeLa cells following exposure to

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extracts from *E. manipuliflora*, *F. communis*, and *S. rebaudiana*. Across all tested plant extracts, a concentration-dependent cytotoxic effect on HeLa cells was observed. Particularly noteworthy was the significant reduction in cell viability, which reached 22.26% when exposed to EE_A at a concentration of 400 µg/mL. Furthermore, HeLa cells subjected to the highest concentrations of EE_A, EE_W, FE_A, and FE_W exhibited viabilities below 35%, as illustrated in Figure 2. These percentages were also supported by the IC_{50} values, and the lowest IC_{50} value (97.35 µg/mL) belonged to the EE_A. The extract with the lowest effect in this cancer cell line was SE_W (IC_{50}: 341.96 µg/mL) (Table 3). Many different species of *Erica* genus exhibited different biological activities such as cytotoxic [36], antibacterial, antioxidant [37, 38], diuretic [39], anti-inflammatory [40]. These studies indicate *Erica*'s potential for alternative and complementary therapy. In this study, acetone and water extracts of *E. manipuliflora*, which has limited literature, showed cytotoxic activity on HeLa cell line. In the literature, the cytotoxic activities of different species of *Erica* genus on different cell lines (*E. carnea* - MCF-7 [41]: *E. multiflora* - B-16 melonoma [42]) have been investigated and the data obtained support our results. In addition, the fact that *E. manipuliflora* contains more phonolic compounds compared to other species in our study may suggest that these plant extracts support more cytotoxic potential on HeLa cell line.

F. communis and *S. rebaudiana* are important medicinal and aromatic plants grown in the geography of Turkey. The inclusion of both species in the category of medicinal plants has been supported by many biological activity studies. In the researches, besides the antioxidant activities of these plant species, the antimicrobial [43], cytotoxic [15], anti-neuroinflammatory [44] activities of *F. communis*, and the antidiabetic [45], antimicrobial [46], anticancer [47], anti-hyperuricemic [48] activities of *S. rebaudiana* are remarkable. Studies involving various *Ferula* species have consistently reported a range of bioactivities, including cytotoxic, antioxidant, anticholinesterase, and anti-tyrosinase effects, which are often attributed to the presence of phenolic compounds in these plants [49, 50]. On the other hand, the genus *Stevia* has garnered attention due to the substantial inhibitory effects displayed by its metabolites, such as stevioside, steviolbioside, and isosteviol derivatives, on several cancer cell lines, including MDA-MB-231, Hep3B, BxPC-3 [51], HER2+ SKBR-3 [52] and MCF-7 [53]. These findings underscore the potential significance of *Stevia* in alternative and complementary therapies for cancer. Notably, our study aligns with these observations, as we have observed remarkable cytotoxic potential in the HeLa cell line for both *F. communis* and *S. rebaudiana* species. Future investigations into the gene expression and related aspects in other cancer cell lines exposed to *E. manipuliflora*, a plant with limited anticancer information, hold promise for expanding our understanding of its effectiveness in modulating cancer metabolism.

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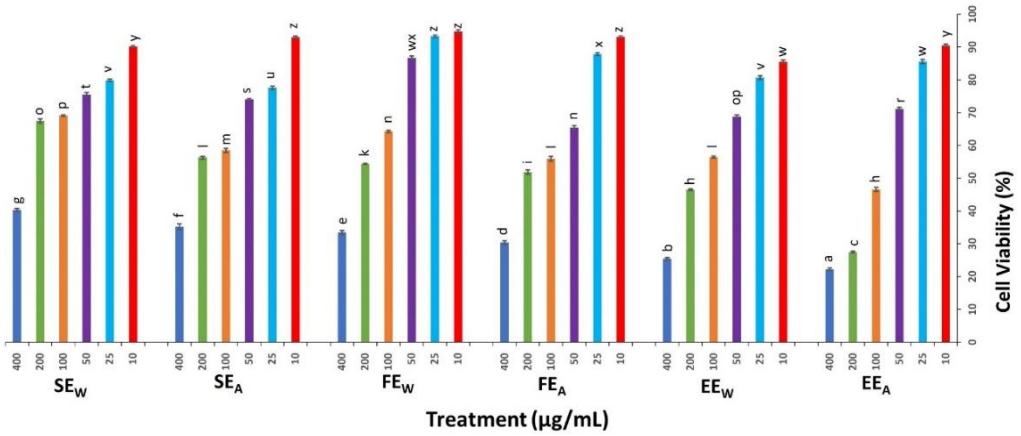

Figure 2. % Cell viability rates on HeLa cells treated with different plant extracts.

Table 3. IC_{50} values (µg/mL) resulting from DPPH scavenging activities and cytotoxic activities of different plant extracts.

Treatment	DPPH scavenging activities	Cytotoxic activities
	IC_{50} (µg/mL)	**IC_{50} (µg/mL)**
EE_A	37.57± 0.52	97.35 ± 0.58
EE_W	33.27 ± 0.18	165.44 ± 1.74
FE_A	40.29 ± 0.70	207.13 ± 9.02
FE_W	37.42 ± 0.29	213.35 ± 0.95
SE_A	101.11 ± 1.87	262.31 ± 6.47
SE_W	88.82 ± 1.44	341.96 ± 3.05

4. CONCLUSION

Total phenol and flavonoid contents, antioxidant activities and cytotoxic properties on human cervical cancer (HeLa) cell line of *E. manipuliflora*, *F. communis* and *S. rebaudiana* acetone and water extracts were determined. Among these three species, *E. manipuliflora* species stood out in terms of both phenolic compound content and antioxidant and cytotoxic activity. This study highlights that the plants under investigation serve as readily available natural sources of antioxidants, demonstrating substantial cytotoxic activity. These findings emphasize the potential utility of these plants in pharmacology, owing to their rich reservoir of valuable phytochemicals, for the management and treatment of various diseases.

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