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Original Article

EVALUATION OF PHARMACEUTICAL POTENTIALS OF NATURALLY AND *IN VITRO*-GROWN WILD STRAWBERRY (*FRAGARIA VESCA* L.)

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ABSTRACT

Wild strawberry (*Fragaria vesca* L.) is a perennial herb of the Rosaceae family that grows a rosette of leaves from a central rootstock. It has been used in traditional medicine to treat digestive disorders, inflammation, and skin diseases, as well as for its nutritional value and pleasant flavor. This plant has attracted the interest of the pharmaceutical sector due to its medicinal properties. The objective of this study was to screen and compare the pharmaceutical potentials of various extracts of *F. vesca* obtained from field-grown leaves and fruits, and *in vitro*-grown leaves and callus using specific bioassays (antibacterial, antioxidant, antitumor, toxicity and anticancer) with different extraction methods. Field-grown leaves and fruits revealed better antibacterial activity than *in vitro*-grown leaves and callus. *E. cloacae* was the most vulnerable to the antibacterial activity of the field grown plants extracts. Field grown-leaves showed the highest antioxidant activity and total phenol-flavonoid content among the tested extracts. Potato disc tumor induction assay revealed that aqueous and methanolic extracts of field-grown leaves, as well as a hot ethanolic extract of fresh fruit, had the highest anti-tumor activity. In the brine shrimp toxicity test, extracts of field-grown leaves and fruits were more toxic than extracts of *in vitro*-grown leaves and callus. Extracts of field-grown leaves and fruits had the strongest anticancer action on Human Breast Adenocarcinoma (MCF-7) cell lines. Our findings have revealed that naturally grown-*F. vesca* leaves were the most efficient in terms of pharmaceutical potential.

KEYWORDS: Antibacterial, antioxidant, antitumor, cytotoxicity, wild strawberry

1. Introduction

Fragaria vesca L. is a low-growing perennial herb from the Rosaceae family that grows in meadows, forests, and along roadsides [1-4]. *F. vesca*, often known as wild strawberries or forest strawberries, is a plant that grows in temperate and subtropical areas of the northern hemisphere [1-5]. Basal leaves of *F. vesca* are trifoliate and arranged as a rosette [6]. Wild strawberry leaves are employed in traditional phytotherapy as diuretic as well as for gastrointestinal, cardiovascular, urinary and skin problems [1-5]. The general composition of the *Fragaria* species includes a variety of biologically active phenolic components, including tannins, anthocyanins, flavonoids, and phenolic acids [1-3,7,8]. Some reported activities of *F. vesca* include anti-inflammatory, anticoagulant, antimutagenic, antithrombotic, vasodilatory, analgesic, anti-diabetic, anti-apoptotic, antibacterial, anticarcinogenic and antioxidant properties [1-3,9,10].

Antibiotic overuse or misuse in the public or agricultural sectors has led to the diversification and spread of resistant bacterial strains [11]. Oxidative stress, on the other hand, causes several disorders, including Alzheimer's disease, atherosclerosis, and several types of cancer [12,13]. These challenges highlight the growing importance of researching new potential medication candidates to lower pathogen resistance, alleviate oxidative stress, and prevent cancer progression while minimizing side effects. Natural chemicals have a substantial impact on modern drug discovery. Identifying and generating new medicinal chemicals from various biological sources, such as vascular plants, marine organisms, fungi, and prokaryotes, is critical for screening [14].

F. vesca is especially well-known for its ability to promote health and provide therapeutic benefits. Leaves and fruits of *F. vesca* are a potential source of bioactive compounds with high antioxidant potential [1,8,10].

Therefore, the aim of this work was to reveal the pharmaceutical potentials (antibacterial, anti-tumor, toxicity, antioxidant, phenolic constituent and anti-proliferative) of different extracts (direct and gradual) obtained from different sources (field-grown leaves and fruits, and *in vitro*-grown leaves and callus) of *F. vesca*.

2. Materials and Methods

2.1. Plant Material and Extraction

Leaves and fruits of *F. vesca* obtained from field-grown plants were collected from Campus, Bolu/Turkey (Figure 1A and B). Leaves and callus of *in vitro*-cultured plants were collected from *F. vesca* plants that were previously micropropagated in the laboratory [3] (Figure 1C, D and E). All used leaves included petioles. Plants were identified by using "Flora of Turkey and the East Aegean Islands" [6] and voucher specimens (AUT-1919) were deposited in Bolu Abant Izzet Baysal University Herbarium.

Different extracts were prepared from four different sources of plant (field-grown leaves and fruits, and *in vitro*-grown leaves and callus). The collected plants were dried in a room away from the sun. Fresh fruit samples were also used and all plant parts then were ground into a fine powder before extraction. Powdered plant materials have been extracted with different solvents by using different extraction methods. Plant materials, extracts, their designation and extraction yields (%) were summarized in Table 1. Extract yields were determined with the formula "Yield (%) = Weight of extract (g)/powdered plant material (g) X 100".

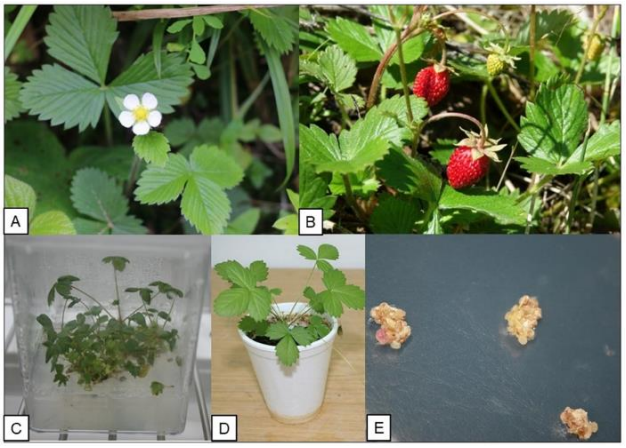

Figure 1. Field-grown leaves (A) and fruits (B); *in vitro*-grown leaves (C and D) and callus (E).

2.1.1. Direct extraction (in water bath)

2.1.1.1. Hot maceration

Field-grown and *in vitro*-grown leaves, fresh and dried fruits and *in vitro*-grown callus of *F. vesca* were extracted with water, ethanol, methanol or acetone at 45 °C in a water bath for 18 h (Table 1).

2.1.1.2. Cold maceration

Fresh and dried fruits were extracted with water or ethanol at 25 °C in a water bath for 18 h (Table 1).

Table 1. Plant materials, extracts, their designation and extraction yields (%).

	Designation of Extractions and Yields									
Extraction methods	Field-grown leaves	Yield (%)	*In vitro*-grown leaves	Yield (%)	*In vitro*-grown callus	Yield (%)	Field-grown dried fruits	Yield (%)	Field-grown fresh fruits	Yield (%)
Direct extraction (in water bath)										
Hot Maceration (45 °C)										
Water	DHFLW	14.3	-	-	-	-	DHDFW	25.5	DHFFW	5.6
Ethanol	DHFLE	2.9	-	-	-	-	DHDFE	15	DHFFE	7.1
Methanol	DHFLM	20	DHILM	36	DHICM	9	-	-	-	-
Acetone	DHFLA	18.2	-	-	-	-	-	-	-	-
Cold Maceration (25 °C)										
Water	-	-	-	-	-	-	DCDFW	30	DCFFW	3.5
Ethanol	-	-	-	-	-	-	DCDFE	7.5	DCFFE	3.3
Gradual Extraction (in soxhlet extractor)										
Hexane	GFLH	8	-	-	GICH	1.4	GDFH	7	-	-
DCM	GFLD	2	-	-	GICD	4	GDFD	1	-	-
Methanol	GFLM	20	-	-	GICM	42.7	GDFM	35	-	-
Water	GFLW	2.5	-	-	GICW	7	GDFW	12	-	-

2.1.2. Gradual extraction (in Soxhlet extractor)

Field-grown leaves and fruits, and *in vitro*-grown callus were gradually extracted by using hexane at 65-70 °C, dichloromethane (DCM) at 55-60 °C, methanol at 60 °C and then water at 80 °C, respectively, in Soxhlet extractor for 24 hours (Table 1).

For aqueous extracts, frozen filtrate was lyophilized by using freeze-dryer at -65 °C. Other extracts (hexane, dichloromethane, ethanol, methanol and acetone) were evaporated under vacuum using rotary evaporator at 40 °C to get dry extracts.

2.2. Biological Activities

2.2.1. Antibacterial assay

The antibacterial activity of 17 different plant extracts against 10 human pathogenic bacterial strains was evaluated by using disc diffusion method as modified by Turker et al. [15]. Sterile distilled water was used to dissolve each extract, resulting in a final concentration of 100 mg/ml. After that, all dissolved extracts were sterilized by passing them through a 0.22 µm filter (Pal-Gelman Laboratory®) and then impregnated (13 µl) into sterile filter paper discs (Whatman®; 6 mm in diameter). Three types of Gram (+) bacteria (*Streptococcus pyogenes* (ATTC® 19615), *Staphylococcus aureus* (ATTC® 25923), and *Staphylococcus epidermidis* (ATCC® 12228) and seven types of Gram (-) bacteria negative [*Escherichia coli* (ATCC® 25922), *Enterobacter cloacae* (ATCC® 23355), *Salmonella typhimurium* (ATCC® 14028), *Serratia marcescens* (ATCC® 8100), *Klebsiella pneumoniae* (ATCC® 13883), *Proteus vulgaris* (ATCC® 13315) and *Pseudomonas aeruginosa* (ATCC® 27853)] were used and before inoculating Mueller Hinton agar plates with cotton swabs, the turbidity of each broth culture of bacteria was adjusted to 0.5 using the McFarland Densitometer (Biosan®). Five different antimicrobial susceptibility test discs were used as positive controls which consist of (Bioanalyse®): Erythromycin (15 µg) (E-15), Ampicillin (10 µg) (AM-10), Carbenicillin (100 µg) (CB-100), Tetracycline (30 µg) (TE-30) and Chloramphenicol (30 µg) (C-30). Three independent experiments were performed for each extract.

2.2.2. Antioxidant capacity

2.2.2.1. DPPH radical scavenging activity

Antioxidant capacity of methanolic extracts of *F. vesca* was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazil) photometric assay [15]. The assay is based on spectrophotometrically monitoring the degradation of DPPH by samples at 517 nm. In this method, DPPH was dissolved in methanol to obtain approximately 1.4 absorbance (0.1 mM DPPH solution) at 517 nm and ascorbic acid was used as an antioxidant standard. Briefly, different concentration of extracts and ascorbic acid were prepared in methanol and 1.5 ml of such solution was mixed vigorously with 0.5 ml DPPH solution. The samples were incubated at room temperature in dark for 30 min and then the decrease in the absorbance of these solutions was measured at 517 nm with UV-VIS Spectrophotometer (Hitachi U-1900®) against blank samples to determine antioxidant potential of the extracts.

2.2.2.2. Total phenol and flavonoid content

Total phenolic content in *F. vesca* methanol extracts was determined using Folin-Ciocalteu assay [15]. Gallic acid as a reference phenol was used to prepare calibration curve. Briefly, in short, 100 µL of Folin-Ciocalteu reagent (Sigma®) was combined with 20 µL of gallic acid, extracts, or distilled water (blank). After two min, 300 µl of 20% (w/v) sodium carbonate was added and incubated at 25 ºC in dark for 30 min. The absorbance was measured at 765 nm with UV-VIS spectrophotometer against blank to determine total phenolic content of the extracts expressed as mg gallic acid equivalents (GAE)/g dried extract.

Total flavonoid content in *F. vesca* methanol extracts was determined by aluminum colorimetric assay [15]. Catechol as a reference flavonoid was used to prepare calibration curve. Briefly, 150 µl of $NaNO_2$ (5%) was combined with 500 µl of extract or catechol solution in methanol as a standard, and the mixture was let to stand for five minutes. After that, 150 µl of 10% $AlCl_3$ was added and in the sixth minute, 1000 µl of NaOH (1 M) was added to each mixture and incubated at 25 ºC in dark for 10 min. Absorbance of the mixture was measured at 765 nm against the blank using UV-VIS spectrophotometer against blank to determine total flavonoid content of the extracts as expressed mg catechol equivalents (CE)/g dried extract.

2.2.3. Potato disc tumor induction assay

Antitumor efficacy was evaluated using the potato disc tumor assay caused by *A. tumefaciens* [16,17]. Antitumor activities of 11 different extracts of *F. vesca* were evaluated. In this method, *Agrobacterium tumefaciens* (ATCC® 23341) suspensions in phosphate-buffered saline (PBS) were standardized to $1x10^9$ Colony Forming Units (CFU). All extracts were dissolved in sterile distilled water (100 mg/mL) and so water was used as a negative control. Camptothecin (Sigma®) (tumor suppressant) was used as a positive control. Extracts and control solutions were filter sterilized with 0.22 µm filter. The test solutions contained 600 µl of the adjusted bacterial suspension, 150 µl of water, and 600 µl of control extracts. Potato discs (10 mm in diameter) were placed into 24 well plates filled with water-agar and then 50 µl of prepared extract or control was transferred on top of each potato disc. After incubation of all plates at 28 °C for 2 weeks in the dark, the number of tumors were recorded.

A bacterial viability test was also performed for the *A. tumefaciens*-induced potato disc tumor assay to show that the extract under test should not have antibacterial activity toward *A. tumefaciens* [16,17]. Serial dilution of *A. tumefaciens* ($1×10^9$ CFU in PBS) was performed until $1×10^3$ CFU and inoculated into yeast extract medium (YEM) using the spread plate technique (control). Also, 0.1 mL of the inoculum (bacteria + extract) was inoculated on YEM media to test the bacterial growth. After 24 h incubation of inoculated YEM media at 28 °C, colony counts were performed.

2.2.4. Brine shrimp toxicity assay

Brine shrimp lethality test was used to predict the cytotoxic activity (LC_{50} value) of six different *F. vesca* extracts [17,18]. Tricaine methane sulfonate (MS222) (Sigma®), common fish anesthetic, was used at different concentrations (10, 100, 1000 and 10000 mg/L) in seawater as a positive control and seawater was used as a negative control. Briefly, 2.5 ml of prepared extracts or controls were placed on twenty-four well culture plates. Meanwhile, brine shrimp eggs were cultured for 48-72 hours in seawater to hatch and develop into nauplii. Following incubation, 10 hatched nauplii were added to each test solution well and allowed to grow at room temperature for 24 hours. Each concentration was examined three times. By counting the dead nauplii after incubation, the lethality of the larvae for each concentration was determined. Thus, the lethal concentration for 50% mortality and 95% confidence intervals were determined using the Read Muench method [17,18].

2.2.5. Antiproliferative assay

All of the gradually prepared *F. vesca* extracts were used to evaluate the cytotoxic effects on MCF-7 Human Breast Adenocarcinoma (ATCC®). The cells were kept in Dulbecco's modified eagle's medium (DMEM, Invitrogen®), which contained 100 ng/ml of streptomycin and penicillin (Sigma®) and 10% fetal bovine serum (FBS) and incubated at 37 °C in humidified environment containing 5% CO_2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed for the determination of cytotoxic activity of plant extracts [19,20]. Briefly, the cells were harvested using a tripsin/EDTA solution and viable cells were then counted and plated in 100 µl of medium/well in 96-well plates (Corning®). Different concentrations of each extract (100 µl) were added to each well ($1x10^4$ cells/well) in triplicate and incubated at 37°C for 24 h. After incubation, 10 µl MTT solution (5 mg/mL) was treated into each well and incubated for additional 4 h. Following 4 hours, 100 µl of DMSO/well was added to each sample in order to dissolve the formazan, which is the final product of the MTT reaction. The samples were then left to incubate for one night at 37 °C in a humidified incubator with 5% CO_2. After incubation, viable cell absorbance (A) was measured spectrophotometrically at 570 nm using a Multiscan FC microplate photometer reader. Antiproliferative activity (%) was calculated according to their control groups [Cell viability (%) = 100 × A570 nm (sample)/A570 nm (control).

2.3. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc. Chicago, IL, USA). All data in the tables were presented as a mean number ± standard error (SE). Correlations were assessed using Pearson correlation analysis.

3. Results and discussions

3.1 Antibacterial Assay

Seventeen different extracts of *F. vesca* were used to screen for antibacterial activity (Table 2). Bacterial growth was generally sensitive to the tested reference antibiotics. Since distilled water was utilized to modify the extracts' final concentrations, it served as a negative control and showed no inhibition.

Direct extractions of field-grown leaves displayed the most effective activity among the tested extracts, and acetone extract (DHFLA) showed the most potent activity (Table 2). Generally, most of the tested extracts exhibited a broad-spectrum activity against both Gram (+) and Gram (-) negative bacteria (Table 2). This activity against both types of bacteria may be an indication of the presence of broad-spectrum antibiotic compounds or simply general metabolic toxins. Among gradually prepared extracts of field-grown leaves, the best antibacterial activity was obtained with methanolic (GFLM) and aqueous extracts (GFLW) against *E. cloacae* (18.1 mm and 18.9 mm, respectively). *E. cloacae* was the most vulnerable to the antibacterial activity of the extracts, and the extracts with the highest antibacterial activity were GDFW (20.8 mm), DHFLA (19.5 mm), GFLW (18.9 mm), and GFLM (18.1 mm) against this bacterium. Prominent antibacterial effects could potentially be attributed to a strong phenolic constituent of *F. vesca* (Table 3 and Figure 2).

Among all tested extracts, hexane and dichloromethane extracts of all gradual extraction were not effective against any bacteria. Generally, all tested 3 Gram (+) bacteria, and 4 Gram (-) bacteria seemed to be susceptible to the inhibitory effects of the *F. vesca* extracts. On the other hand, none of the tested extracts showed antibacterial activity against *S. marcescens*, *S. typhimurium* and *E. coli* (Table 2). Direct hot extraction of *in vitro*-grown leaves (DHILM) demonstrated better activity than this of *in vitro*-grown callus extracts. Among gradually prepared extracts of *in vitro*-grown callus, only aqueous extract (GICW) exhibited good antibacterial activity against only *E. cloacea* (15.7 mm). The remaining callus extracts did not show antibacterial activity against any tested bacteria (Table 2). Among gradually prepared extracts of dried fruits, aqueous dried fruit extract (GDFW) and then methanolic dried fruit extract (GDFM) showed the best antibacterial activity against *E. cloacae* (Table 2).

It was noticeable that the susceptibility of *E. cloacae* was the most prominent with all tested aqueous extracts (Table 2). *E. cloaceae* is a bacteria highly resistant to the widespread usage of broad-spectrum antibiotics. Many medications that were used to kill this organism may no longer be effective against it since the organism may gain genetically generated resistance mechanisms over time [21]. Discovery of new natural antibacterial extracts against resistant bacteria is very worthful. Also, synthetic antibiotics have residual effects on the environment and aquatic life. Natural substances have been shown to be more environmentally friendly and biodegradable [22]. Aqueous extracts of *F. vesca* have very strong effects against *E. cloaceae* and this finding is very valuable. *E. cloacae* is known to result in infections in lower respiratory tract, skin and soft tissue, and urinary tract [21]. The leaves of *F. vesca* have been applied externally to cure inflammation of the skin and mucosal surfaces, as well as to act as an emollient, dermatological protector, and antimicrobial in folk medicine. Additionally, they have been utilized to treat a number of illnesses, including

Table 2. Antibacterial activities of various *F. vesca* extracts against 10 human pathogens. Means with the same letter within columns are not significantly different at $P>0.05$.

Treatment	Mean diameter of inhibitory zones (mm ± SE)									
	S. auerus	*S. epidermidis*	*S. pyogenes*	*S. marcescens*	*S. typhimurium*	*P. aeruginosa*	*P. vulgaris*	*K. pneumonia*	*E. cloacae*	*E. coli*
DHFLW	10.0± 0.0*h*	11.0 ± 0.6*fg*	10.5 ± 0.3*f*	- -	- -	- -	- -	8.0 ± 0.0*d*	16.0 ± 0.0*f*	- -
DHFLE	10.8± 0.5*g*	12.0 ± 1.2*ef*	13.5 ± 0.9*e*	- -	- -	- -	- -	- -	9.0 ± 0.0*h*	- -
DHFLM	10.8± 0.3*g*	12.5 ± 0.3*e*	9.5 ± 0.3*f*	- -	- -	8.3 ± 0.3*e*	12.3 ± 0.6*e*	- -	14.5 ± 0.3*g*	- -
DHFLA	12.8± 0.5*f*	14.5 ± 0.3*d*	10.5 ± 0.3*f*	- -	- -	9.8 ± 0.5*cd*	12.3 ± 0.3*e*	- -	19.5 ± 0.6*de*	- -
DHILM	- -	10.7 ± 0.2*fg*	8.3 ± 0.2*fg*	- -	- -	- -	9.0 ± 0.0*f*	- -	16.3 ± 0.4*f*	- -
GFLH	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GFLD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GFLM	- -	9.2 ± 0.2*hi*	10.2 ± 0.8*f*	- -	- -	- -	- -	- -	18.1 ± 0.2*e*	- -
GFLW	9.1± 0.2*i*	10.4 ± 0.4*gh*	8.5 ± 0.1*fg*	- -	- -	9.9 ± 0.3*c*	9.8 ± 0.2*f*	9.2 ± 0.2*c*	18.9 ± 0.6*e*	- -
GDFH	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GDFD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GDFM	7.6± 0.1*j*	8.9 ± 0.2*i*	7.4 ± 0.1*g*	- -	- -	- -	- -	- -	16.2 ± 0.7*f*	- -
GDFW	9.8± 0.2*hi*	13.2 ± 0.8*e*	10.2 ± 0.2*f*	- -	- -	9.2 ± 0.2*d*	10.1 ± 0.1*f*	- -	20.8 ± 0.3*d*	- -
GICH	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GICD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GICM	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
GICW	- -	- -	- -	- -	- -	- -	- -	- -	15.7 ± 0.2*fg*	- -
Ampicillin	39.2 ± 0.7*b*	30.6 ± 0.2 *c*	48.0 ± 2.5 *b*	15.0 ± 0.5 *d*	27.4 ± 0.2*c*	- -	28.0 ± 1.2*c*	8.4 ± 1.0*cd*	27.0 ± 0.0*c*	21.4 ± 0.6*c*
Carbenicillin	43.2 ± 0.7*a*	38.2 ± 1.3 *a*	50.4 ± 0.7*a*	29.2 ± 0.7*a*	30.0 ± 0.0*a*	20.8 ± 0.5*a*	38.0 ± 1.6 *a*	8.6 ± 1.1*cd*	33.4 ± 1.4*a*	24.8 ± 0.4*b*
Chloramphenicol	25.8 ± 0.5*e*	33.6 ± 0.6 *b*	32.4 ± 1.1*d*	27.2 ± 1.0*b*	28.4 ± 0.7*b*	8.6 ± 0.2*e*	28.0 ± 1.3 *c*	29.8 ± 0.7*a*	30.0 ± 1.1*b*	28.4 ± 0.6*a*
Erythromycin	29.6 ± 0.2*d*	39.2 ± 0.5 *a*	36.4 ± 2.9*c*	11.6 ± 0.6*e*	11.6 ± 0.7*e*	- -	13.6 ± 0.2 *d*	13.0 ± 0.0*b*	9.4 ± 0.2*h*	13.2 ± 0.7*d*
Tetracycline	30.8 ± 0.7*c*	8.0 ± 0.0 *i*	36.8 ± 0.7*c*	23.6 ± 0.7*c*	24.6 ± 0.6*d*	14.8 ± 0.7*b*	35.8 ± 1.8 *b*	29.2 ± 0.9*a*	30.0 ± 0.5*b*	28.0 ± 0.8*a*
Water	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

Table 3. Free radical scavenging capacity and total phenolic-flavonoid content of methanolic *F. vesca* extracts and ascorbic acid (antioxidant standard).

Treatments	DPPH Inhibition(µg/ml) IC50	Total Phenolics (mg GAE/g dried extract)	Total Flavonoids (mg CE/g dried extract)
DHFLM	< 12.5	767.80 ± 0.00	184.43 ± 0.00
DHILM	47.13 ± 3.85	554.86 ± 0.00	121.46 ± 0.00
DHICM	> 200	125.83 ± 0.00	55.59 ± 0.00
GFLM	< 12.5	633.00 ± 0.00	71.66 ± 0.00
GDFM	37.08 ± 3.61	251.50 ± 0.00	46.42 ± 0.00
GICM	175.08 ± 5.17	146.00 ± 0.00	29.62 ± 0.00
Ascorbic acid	< 12.5		

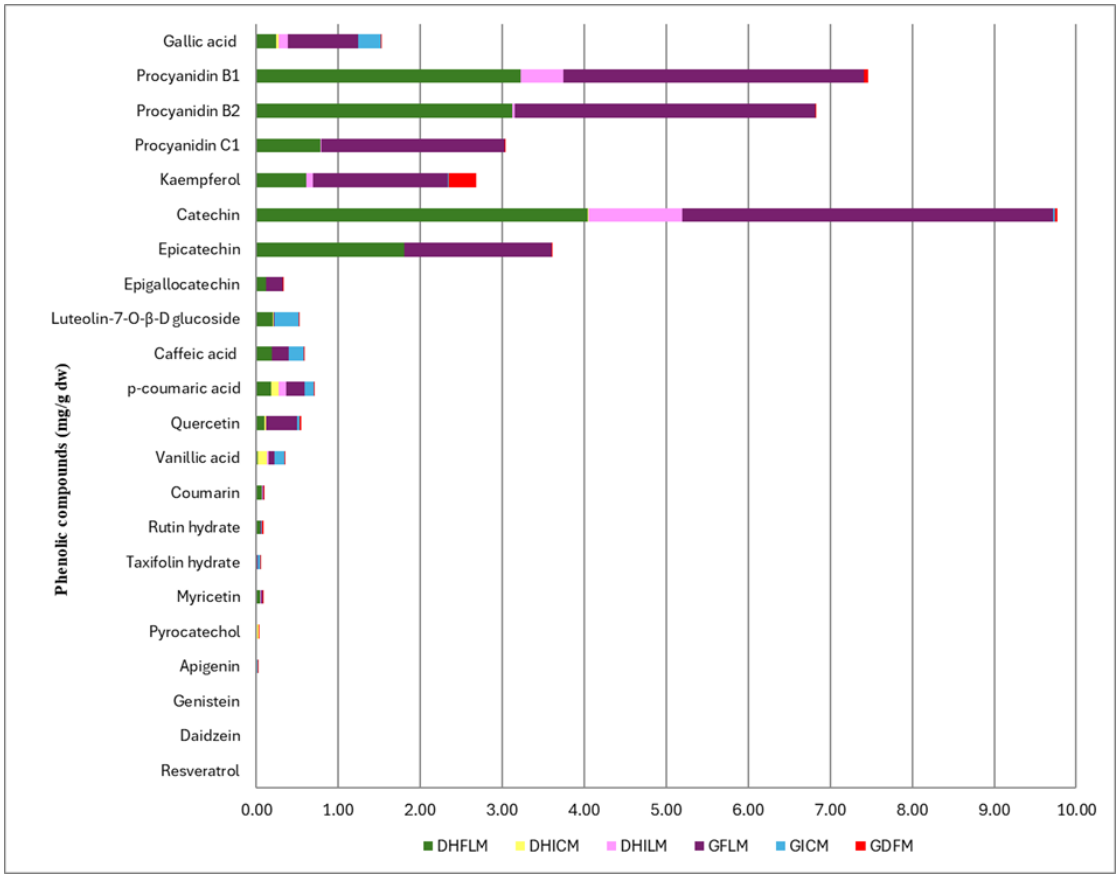

Figure 2. LC-ESI-MS/MS results obtained from 6 different methanolic extracts [3].

urinary, cardiovascular, and gastrointestinal problems [23]. Significant antibacterial activity of *F. vesca* extracts against *E. cloacae* may explain why *F. vesca* is used in folk medicine in skin problems and infections in urinary and respiratory tracts, as well as effective antibacterial activity of *F. vesca* extracts against *S. aureus*, *S. epidermidis* and *S. pyogenes* may explain why *F. vesca* is used in folk medicine to treat diarrhea, rheumatism and skin diseases [9].

Similarly to our findings, Turker et al. [24] evaluated the antibacterial activity of *F. vesca* extracts against five different fish pathogen bacteria and reported that aqueous extract showed a strong activity against *Y. ruckeri* among the tested pathogens. Hydromethanolic extracts of wild *F. vesca* fruits showed high antibacterial properties and the ability to prevent the formation of bacterial biofilms [25]. Cardoso et al. [26] obtained 2 different *F. vesca* extracts (ethanol and ellagitannin-enriched fraction) to evaluate the antibacterial activity against twelve *Helicobacter pylori* clinical isolates by the disc diffusion method. Both extracts presented maximum antibacterial activity, especially the ellagitannin-enriched fraction inhibited growth of all isolates for all used concentrations (15-2.5 mg/L). Gomes et al. [27] studied the antibacterial activity of *F. vesca* leaves and root extracts against *S. aureus* ATCC 25923 and six clinical isolates. The authors observed weak antibacterial activity of hydromethanolic extracts against all *S. aureus* strains (ranging from 5 to 9 mm). Another study showed that *F. vesca* ethanol extract in combination with colistin exhibited strong activity against *P. aeruginosa*. They also reported that *F. vesca* ethanolic extract has ability to reduce biofilm formation [28].

3.4. Antioxidant capacity

A common substrate used to evaluate the scavenging ability of antioxidant compounds is DPPH. The scavenging activity of antioxidants is measured by the decrease in the absorbance of the DPPH radical at 517 nm. The ability of *F. vesca* methanolic extracts to scavenge 50% of the DPPH (IC_{50} value) free radicals was used to assess their antioxidant activity in comparison to ascorbic acid. Direct and gradual methanolic extract of field-grown leaves (DHFLM and GFLM) exhibited the highest antioxidant capacity (IC_{50} value of <12.5 µg/mL) as well as field grown leaves showed higher antioxidant potential than *in vitro*-grown materials (DHILM, GICM and DHICM) (Table 3).

Methanolic extracts of *F. vesca* were also evaluated to determine total phenol and flavonoid content. In parallel to antioxidant capacity, direct and gradual methanolic extract of field-grown leaves (DHFLM and GFLM) followed by direct methanolic extract of *in vitro*-grown leaves (DHILM) had the highest total phenol and flavonoid content (Table 3). Plant materials include antioxidant chemicals that are crucial for inhibiting and scavenging free radicals. The radical scavenging activity of all tested extracts was strongly correlated with total phenolic content ($r = -0.84$, $P < 0.05$). The level of antioxidative effects provided by phenols is largely determined by the structure of a given chemical (or extract), specifically the number and substitution of hydroxyl groups (-OH) on the structure. Phenolic compounds quickly release hydrogen atoms on -OH groups, implying that they oxidize themselves, acting as antioxidants [29].

Although there was no significant difference in the total phenolic contents of DHFLM and GFLM extracts, DHFLM extract had 2.5 times higher flavonoid content than GFLM extract. This is because the hexane and dichloromethane used first in gradual extraction may have pulled out the flavonoids. All tested leaf extracts (field and *in vitro*- grown) showed better total phenol and flavonoid content than fruit extract. Similar to our result, Wang and Lin [30] studied leaves and berries of strawberry and they found that leaves had a greater amount of phenolic compounds than berries. Wang and Lin [30] investigated the antioxidant activity of strawberry, blackberry, and raspberry leaves and fruits. They found that the leaves contained a higher concentration of antioxidants. Likewise, the extracts obtained from field-grown leaves had greater antioxidant activity than fruit extracts in our study (Table 3). A lot of compounds possessing antioxidant activity were identified in the leaves of *F. vesca* [31]. These were (+)-catechin, (-)-epicatechin, epigallocatechin and procyanidins (B1 and B2) [31]. Scalzo et al. [32] investigated the antioxidant activities of *F. vesca* fruit and some cultivated strawberries by using Trolox equivalent antioxidant capacity (TEAC) assay. They found that TEAC value of *F. vesca* fruits was 2.5 times higher than for cultivated strawberries. Dias et al. [33] demonstrated the high antioxidant capacity of wild *F. vesca* roots. Yildiz et al. [34] studied the antioxidant activity of fifteen *F. vesca* genotypes harvested from Black Sea in Türkiye and reported that IC_{50} values in DPPH assay varied from 7.94 µmol to 11.38 µmol per g fruit sample. Besides, they reported that total phenolic content ranged from 170 to 228 mg GAE/100 g fresh fruit. In the present study, it was found that fruit extract (GDFM) showed a high antioxidant activity with IC_{50} value of 37.08 µg/mL and a high phenolic content of 251.1 mg/L GAE/g extract. Similar to our result, they reported that the antioxidant activity of *F. vesca* fruit extract is primarily correlated with total phenolic content. Dyduch-Sieminska et al. [35] also studied the antioxidant effect in fresh fruits of three *F. vesca* cultivars and evaluated their flavonoid content. Their results indicated that at a dosage of 20 µg/mL, antioxidant activity ranged from 12.40% to 14.27% and the water extract from Regina cv fruit had the highest scavenging activity (14.27% DPPH inhibition). It was also reported that total flavonoid content ranged from 0.471 to 0.593 mg QuercetinE/g fresh fruit. Another study on antioxidant activity of hydromethanolic and water extracts of *F. vesca* (leaves and stems) reported that water extract showed the best activity with IC_{50} value of 86.17 µg/mL [25]. Zugic et al. [36] presented better antioxidant results with IC_{50} value of 13.46 µg/mL for methanol extract from *F. vesca* leaves.

LC-ESI-MS/MS results [3] obtained from 6 different methanolic extracts revealed that the concentrations of phenolic compounds in field-grown leaf extracts (GFLM and DHFLM) were at least five times higher than in the other extracts (Figure 2). The phenolic compounds found in high concentrations in GFLM and DHFLM extracts were catechin, epicatechin, epigallocatechin, gallic acid monohydrate, kaempferol, procyanidin C1, procyanidin B1, procyanidin B2 and quercetin. The concentration of these compounds in field-grown leaf extract obtained with gradual extraction method (GFLM) was higher than field-grown leaf extract obtained with direct extraction method (DHFLM). Probably, in gradual extraction, moderate polar solvents such as dichloromethane might have removed some other compounds that would be soluble in methanol. So, the concentration of the phenolic compounds was found to be higher in DHFLM extract (Figure 2). D'Urso et al. [37] investigated the metabolite profiles of different *F. vesca* leaves by LC-ESI/LTQOrbitrap/MS analysis and showed 27 metabolites mainly belonging to organic acids, flavonoids, catechin and its oligomers, and ellagitannins. Similar to other studies [37,38], catechin was found to be one of the major antioxidant compounds in wild strawberry leaves in our study. Catechin derivatives, kaempferol and quercetin derivatives are also well-known antioxidant flavonoids highly reported in the literature [37]. In parallel to literature, the highest antioxidant activity was obtained with DHFLM and GFLM involving the highest phenolic content (Table 3) and individual flavonoids (catechin, procyanidin B1, procyanidin B2, procyanidin C1, kaempferol and epicatechin) (Figure 2).

3.2 Potato disc tumor induction assay

Eleven different extracts at concentration of 100 mg/ml (Table 4) were tested in order to screen and show their potential as anti-tumor agents. The best antitumor activity was observed with aqueous (DHFLW) and methanolic (DHFLM) extracts of field-grown leaves (98.1% and 97.1% tumor inhibition, respectively) and hot ethanolic extract (DHFFE) of fresh fruit (93.2% tumor inhibition) (Table 4). When compared with control (water), the percentage inhibition of all extracts except cold aqueous extract of fresh fruit (DCFFW) and hot aqueous extract (DHDFW) of dry fruit was more than 40%

in three separate experiments. Since final concentrations of all extracts were adjusted with distilled water, it was used as a negative control and no inhibition was observed with water. No tumor formation was observed with camptothecin (100% tumor inhibition) (Table 4). Generally, all extracts (methanol, ethanol and water) of field-grown leaves exhibited effective antitumor activity. Moreover, ethanolic extracts of both dry and fresh fruits exhibited better antitumor activity than those of the aqueous extracts.

Table 4. Antitumor activity of controls (camptothecin - posivite and water - negative) and several *F. vesca* extracts. Means with the same letter within columns are not significantly different at *P*>0.05.

Treatment	Mean Number of Tumors (±SE)	% Tumor Inhibition
Water	20.66 ± 2.0 ^{f}	0.0
Camptothecin	0.0 ± 0.0 ^{a}	100
DHFLW	0.4 ± 0.3 ^{a}	98.1
DHFLE	3.3 ± 0.6 ^{ab}	84.1
DHFLM	0.6 ± 0.2 ^{a}	97.1
DCDFW	10.9 ± 1.1 ^{d}	47.3
DHDFW	12.6 ± 1.5 ^{d}	39.1
DCDFE	7.3 ± 1.2 ^{c}	64.7
DHDFE	3.7 ± 1.2 ^{ab}	82.1
DCFFW	16.3 ± 2.1 ^{e}	21.3
DHFFW	5.9 ± 1.3 ^{bc}	71.5
DCFFE	3.5 ± 0.6 ^{ab}	83.1
DHFFE	1.4 ± 0.5 ^{a}	93.2

A prerequisite for potato disc tumor induction assay is that the extract or substance being tested should not have antibacterial activity toward *A. tumefaciens* [39]. Crown gall formation on potato discs is inhibited by either reducing the viability of *A. tumefaciens* or by anti-tumorogenesis. To differentiate between these options, viability tests were performed on each extract. Plant extracts were incubated with 1 x 10^3 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension for 30 minutes to assess bacterial viability. There was no difference in bacterial growth across the plates between control (only *A. tumefaciens*) and tested extracts (*A. tumefaciens* + plant extracts) in terms of colony counts (ranged from 9.2 x 10^3 to 13 x 10^3 CFU). All tested extracts did not affect the viability of the bacterium. Thus, observed inhibition of tumor formation for these extracts was on the formation of tumors.

Lei et al. [40] demonstrated an efficient antitumor activity of procyanidin B1 through *in vivo* experiments on a xenograft mouse model, demonstrating that it significantly suppressed tumor growth. Similarly, the highest antitumor activity of DHFLM may be associated with the highest procyanidin B1 content found in this extract in our study (Table 4 and Figure 2). Some studies have reported that certain dietary flavonoids possess antitumor activity. Molnar et al. [41] reported the antitumor activity of polyhydroxylated flavonoids towards NK/LY ascites tumors in mice. Quercetin and apigenin also inhibited tumor development in other animal models. Quercetin the polyhydroxylated flavonoid exerted potent growth inhibitory effects on several malignant tumor cell lines *in vitro*. Flavones and flavonols, such as 3-hydroxy-flavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone, apigenin and luteolin, also inhibited tumor cell growth [42]. Correspondingly, total flavonoid content in methanolic extract of field-grown leaves (DHFLM) was the highest (Table 3), and the flavonoids such as procyanidins (B1, B2 and C1), kaempferol, catechin, epicatechin, luteolin and quercetin were detected dominantly in DHFLM extract by LC/MS-MS analysis (Figure 2). The antitumor activity of the methanolic extract (DHFLM) may be attributed to the highest flavonoid content in this extract.

3.3 Brine shrimp toxicity assay

The toxicity of methanolic extracts of *F. vesca* was tested with brine shrimp bioassay (Table 5). This bioassay determines the toxicity of materials toward brine shrimp larvae and can predict the ability to kill cancer cells in cell cultures, various pests, and exert a wide range of pharmacological effects [43]. Meyer et al. [18] found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. Among the tested extracts, gradually prepared methanol extracts of fruits (GDFM) and field-grown leaves (GFLM) were more toxic than other tested extracts with lower LC_{50} values (465 mg/L and 696 mg/L, respectively) (Table 5). Moreover, direct methanolic extracts of *in vitro*-grown leaves (DHILM) and callus (DHICM) were the least toxic. When we made a comparison within extraction types, gradually prepared methanolic extracts (GFLM and GICM) were more toxic than methanolic extracts prepared by direct extraction (DHFLM and DHICM). In gradual extraction, hexane and DCM extractions performed before methanol extraction may have drawn some less toxic active ingredients, making the methanolic extract more concentrated and toxic. In LC/MS-MS analysis (Figure 2), gradual and then direct extracts of field-grown leaves (GFLM and DHFLM) had the highest individual phenolic content and high phenolic content of these extracts is likely to increase their toxicity and therefore their cytotoxic properties. In parallel to our results, antiproliferative effects of hydroalcoholic extract of *F. vesca* leaves and its ellagitannin-enriched fraction were demonstrated on human hepatocellular carcinoma cells (HepG2) [23].

Table 5. LC_{50} (lethal concentration for 50% mortality after 24 hours) values for *F. vesca* extracts and MS-222 (positive control).

Extractions	LC_{50} (mg/L)	Confidence Intervals
DHFLM	1002	633 - 1583
DHILM	1246	789 - 1970
DHICM	1407	891 - 2225
GFLM	696	440 - 1099
GDFM	465	294 - 735
GICM	1054	667 - 1665
MS-222	33	21 - 52

3.5. Antiproliferative assay

Fourteen different gradual extracts of *F. vesca* were used to evaluate the cytotoxic activities on Human Breast Adenocarcinoma (MCF-7). The results of the cytotoxicity assay (for 24 h and 48 h) of the tested extracts were presented in Table 6 and 7.

In MTT assay for 24 h (Table 6), among tested extracts, gradually prepared aqueous extract (GDFW) of fruits showed better anticancer activity than rest of the extracts at 100 and 200 µg/mL concentrations. All gradually prepared hexane (GICH) and dichloromethane extracts (GICD) of *in vitro*-grown callus gave higher cell viability than in control. In MTT assay for 48 h (Table 7), two additional extracts were used: methanolic extracts of *in vitro*-grown leaves (DHILM) and field-grown leaves (DHFLM). DHILM extract did not show anticancer activity, and it resulted in higher cell viability than in control at low concentrations (12.5–50 µg/mL). DHFLM extract resulted in the same cell viability (75.2%) at 100 and 200 µg/mL. Direct extract of field-grown leaves had better anticancer activity than this of *in vitro*-grown leaves (Table 7).

Table 6. Percentage of cell viability of several *F. vesca* extracts with 6 different concentrations on MCF-7 cell line over a 24-hour period.

	% Cell Viability (Mean ± SE)					
	Concentrations					
Treaatments	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**	**200 µg/mL**
GFLH	105.2 ± 3.0	107.1 ± 1.7	109.4 ± 0.7	111.1 ± 2.8	113.2 ± 0.6	118.2 ± 1.4
GFLD	97.5 ± 1.0	97.0 ± 1.2	96.4 ± 1.1	96.3 ± 1.6	94.5 ± 2.0	94.0 ± 0.5
GFLM	97.3 ± 3.0	96.8 ± 0.9	93.8 ± 0.7	92.7 ± 1.4	90.0 ± 1.3	85.3 ± 0.4
GFLW	96.9 ± 0.9	93.9 ± 1.0	91.1 ± 1.5	89.8 ± 1.5	86.4 ± 1.2	82.2 ± 1.4
GDFH	105.0 ± 0.4	104.4 ± 1.6	107.1 ± 2.0	106.1 ± 1.5	107.4 ± 0.9	115.3 ± 0.5
GDFD	96.7 ± 0.8	96.0 ± 2.2	94.8 ± 1.3	95.6 ± 1.4	93.2 ± 0.5	90.7 ±.1.1
GDFM	96.7 ± 2.7	94.8 ± 1.7	92.0 ± 1.4	90.2 ± 1.2	87.5 ± 0.8	85.1 ± 0.8
GDFW	96.6 ± 1.8	92.1 ± 1.9	90.9 ± 2.2	87.0 ± 2.6	82.0 ± 1.9	80.8 ± 1.8
GICH	96.7 ± 2.0	98.1 ± 3.9	100.7 ± 3.1	101.0 ± 2.6	105.9 ± 2.3	106.3 ± 3.9
GICD	100.4 ± 2.4	101.4 ± 1.6	101.8 ± 1.0	103.5 ± 1.8	104.0 ± 2.7	106.8 ± 0.9
GICM	96.7 ± 0.4	92.9 ± 1.2	90.0 ± 2.4	89.1 ± 1.5	89.0 ± 1.4	88.5 ± 2.1
GICW	96.5 ± 3.4	91.5 ± 1.9	90.4 ± 0.9	89.8 ± 2.2	89.7 ± 0.3	88.2 ± 3.4

Table 7. Percentage of cell viability of several *F. vesca* extracts with 6 different concentrations on MCF-7 cell line over a 48-hour period.

	% Cell Viability (Mean ± SE)				
	Concentrations				
Treaatments	**12.5 µg/mL**	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**	**200 µg/mL**
GFLH	91.9 ± 0.0	91.7 ± 0.0	88.1 ± 0.0	84.7 ± 0.0	83.3 ± 0.0
GFLD	80.6 ± 0.9	78.5 ± 2.1	73.4 ± 2.2	68.3 ± 2.4	64.3 ± 0.4
GFLM	87.9 ± 1.6	79.6 ± 2.5	76.7 ± 3.7	76.3 ± 2.6	76.3 ± 2.3
GFLW	91.9 ± 2.4	80.1 ± 2.9	73.3 ± 0.9	72.6 ± 0.3	67.9 ± 0.9
GDFH	75.4 ± 1.5	68.9 ± 2.6	67.9 ± 0.3	67.9 ± 0.0	67.7 ± 0.1
GDFD	77.7 ± 1.3	77.5 ± 1.1	74.7 ± 2.6	73.9 ± 0.9	73.0 ± 0.6
GDFM	83.7 ± 1.3	79.9 ± 1.6	77.9 ± 1.4	73.8 ± 1.5	72.6 ± 1.2
GDFW	99.6 ± 3.9	87.5 ± 2.8	77.2 ± 1.9	74.5 ± 3.3	71.9 ± 1.7
GICH	90.0 ± 1.4	85.6 ± 1.7	83.1 ± 0.6	82.6 ± 1.6	81.4 ± 0.7
GICD	76.8 ± 0.1	75.6 ± 1.2	72.6 ± 1.2	70.6 ± 1.6	68.4 ± 1.3
GICM	107.2 ± 0.6	93.4 ± 1.4	93.4 ± 2.3	93.3 ± 1.9	92.7 ± 2.1
GICW	94.4 ± 2.4	93.3 ± 1.8	89.4 ± 1.3	83.9 ± 2.9	81.3 ± 1.2
DHILM	111.5 ± 2.7	104.6 ± 1.8	101.1 ± 3.1	98.4 ± 1.9	98.4 ± 1.9
DHFLM	93.9 ± 1.3	83.1 ± 1.8	80.3 ± 2.6	75.3 ± 1.1	75.2 ± 0.9

Among the tested extracts, gradually prepared dichloromethane extract (GFLD) of field-grown leaves, followed by gradually prepared hexane extract (GDFH) of fruits, showed better anticancer activity than the rest of the extracts. Increasing concentration of the extracts had no effect on the anticancer activity. This indicates that the anticancer activity is not concentration-dependent with respect to the result. However, it slightly increased by increasing time (from 24 h to 48 h). In general, the extracts from field-grown leaves and fruits exhibited slightly better anticancer activity than those from *in vitro*-grown leaves and callus, however all extracts showed minimal anti-proliferative activity.

Some studies showed antiproliferative effects of *F. vesca* leaves on different cell lines. Liberal et al. [23] investigated anticancer potential of hydroalcoholic extract of *F. vesca* leaves and extract (ellagitannin-enriched fraction) fractionated from hydroalcoholic extract on human hepatocellular carcinoma cells (HepG2). They reported that hydroalcoholic and fractionated extracts showed antiproliferative activity against HepG2 cell line with IC_{50} values of 690 ± 1 µg/mL and 113 ± 1 µg/mL. On the other hand, extracts exhibited no substantial antiproliferative action against the MCF-7 cell line in our study. After being treated with MCF-7 cell lines for 48 hours, the extracts exhibited minimal antiproliferative action. On the other hand, Somasagara et al. [44] showed the cytotoxicity of methanolic extract of Indian strawberry [*Potentilla indica* (Jacks.) Th.Wolf.] fruits on leukemia (CEM) and breast cancer (T47D) cell lines in a concentration-dependent manner *ex vivo* and anti-proliferative effect on tumor cells *in vivo*. Forni et al. [45] demonstrated the antiproliferative and differentiation potential of an anthocyanin-rich strawberry (*Fragaria* × *ananassa* Duch.) fruit extract on B16-F10 murine melanoma cells.

4. Conclusions

Pharmaceutical potentials (antibacterial, antitumor, toxicity, antioxidant, and anticancer) of various extracts of *F. vesca* obtained from field-grown leaves and fruits, and *in vitro*-grown leaves and callus were revealed with this study. In addition to their phenolic components, field-grown leaves had notable antibacterial, anticancer, and antioxidant properties. Antioxidant capacity was correlated with the levels of phenolic contents in the extracts. The most remarkable result is the antibacterial activity of field-grown leaves, especially against *E. cloacae*. Additionally, the field-grown leaves exhibited the highest levels of brine shrimp toxicity and mild antiproliferative efficacy on MCF-7 cell line. The naturally grown *F. vesca* leaves were the most efficient in terms of medicinal potential. This study's findings emphasize the potential of *F. vesca* as a valuable natural source of bioactive chemicals with potential applications in the pharmaceutical and nutraceutical industries. Further research is suggested to investigate this species' various medicinal potential and methods of action. Also, future studies should focus on the anticancer potential of different extracts of *F. vesca* leaves with different cell lines, as well as more specific fractionation of the extracts having antibacterial, antitumor and antioxidant activities to identify more active components of different extracts of *F. vesca*.

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