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Toxicity Assessment of *Euphorbia esula* L. Extracts on HCT116, SW480, HEK293 Cell Lines, *Artemia salina* Larvae, and Its Bactericidal Effects

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ABSTRACT

The aim of this study is to evaluate the potential toxicity of acetonic and methanolic extracts derived from the *Euphorbia esula* L. plant on various cell lines of human colorectal cancer (HCT116 and SW480), human embryonic kidney normal cells (HEK293), *Artemia salina* larvae, and its bactericidal effects. The cytotoxic effect of *E. esula* extracts on cell lines was performed using the MTT assay. *In vitro* toxicity and biocompatibility of extracts were also evaluated on *A. salina* and red blood cells by hemolysis test, respectively. The ability of the extracts to inhibit bacterial growth was examined by using the disc diffusion method, as well as the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microtiter broth dilution method. Results showed acetonic extract contains the highest concentration of flavonoid (16.17 µg Qu/mg) and phenol (34.84 µg GA/mg) compared to methanolic extract. The anti-proliferative effects of acetonic extract had the highest effect on HCT116 and HEK293 with IC_{50} of 64.80 µg/mL and 47.82 µg/mL at 72h, respectively. The hemolysis degree of the methanolic extracts was <2% at 400 µg/mL. LC_{50} for the acetonic and methanolic extracts exhibited moderate and low toxicities on the brine shrimp larvae, with LC_{50} of 381.969 µg/mL and 1905.77 µg/mL, respectively. The bactericidal effect of 50 mg/mL acetonic extracts showed a clear zone inhibitory growth on *Staphylococcus aureus* and *Klebsiella pneumoniae* with 34 mm and 35mm at the MIC and MBC values of 1000 and 2000 mg/mL, respectively. These findings could help to elucidate the anti-tumor, anti-bacterial, and toxic properties of *E. esula* extracts.

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Introduction

The incidence of colorectal cancer is about 9.4% of cases worldwide and has been ranked as the second and third cancer in males and females, respectively (Kuipers *et al*., 2015). Euphorbia genus belongs to the Euphorbiaceae family and encompasses approximately 2,000 species. These plants are widely spread in Iran, India, and Pakistan (Jassbi, 2006). Iran with 93 species of Euphorbia (21 endemic and 33 sub-endemic species) is one of the most important biodiversity centers and the richest country in West Asia (Pahlevani, 2022). *Euphorbia esula* L. is an herbaceous plant whose aerial parts have been employed in traditional medicine. Active components of Euphorbia species possess diverse biological activities, such as anti-tumor, cytotoxic, anti-inflammatory, anti-oxidant, anti-bacterial, and anti-nociceptive properties (Lanhers *et al*., 1991; Ashraf *et al*., 2015). Multiple studies have revealed that *E. esula* extracts were capable of suppressing cell proliferation and inducing apoptosis in several human cancer cell lines (Gao *et al*., 2013; Fu *et al*., 2016). Additionally, *E. esula* extracts

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exhibited a low anti-viral activity (Halaweish *et al*., 2003). Diterpenoids and flavonoids are two classes of compounds isolated from the *E. esula* extracts that have demonstrated anticancer functions (Guo *et al*., 2018). The present study focuses on the biological activities of *E.esula* and its importance as a source of natural products. To the researchers' knowledge, there is no study to evaluate the anti-cancer properties of *E. esula* acetonic extract on colorectal cancer cell lines (HCT116 and SW480). Therefore, in this study, the cytotoxic effects of crude acetonic and methanolic extracts of *E. esula* were evaluated on HCT116 and SW480 cells. The antibacterial evaluation was carried out to assess the applicability of the therapeutic natural products in the inhibition of bacteria resistance. Furthermore, *in vitro* toxicity and biocompatibility of extracts were evaluated on *Artemia salina* and red blood cells (RBCs) by haemolysis, respectively.

Materials and Methods

Herbal materials and extraction

The *E. esula* was obtained from Zanjan, Iran (36°41'56.8"N; 48°30'47.3" E) and validated at the Department of Pharmacognosy, School of Pharmacy, Zanjan. Then, 20 g of the powdered aerial part of *E. esula* was refluxed three times with 200 mL of acetone (3 h) and methanol (8 h). The filtered extracts were concentrated by an evaporator (35-45°C) for 120 min and the obtained extracts were allowed to be dried at room temperature and in the shade for two weeks.

After acetonic and methanolic extraction, the content of flavonoids and phenolic was assayed. For this aim, the aluminum chloride colorimetric approach was applied to assess the total content of flavonoids (TFC) and customized for 96-well microplates (Chang *et al*., 2002). Extracts with a concentration of 5 mg/mL were prepared, and then, standard quercetin solutions were made in the range of 15.625 to 100 µg/mL. Samples (30 µL) were diluted with methanol (90 µL), and 6 µL of aluminum chloride (10%) which was substituted with distilled water in a blank probe, then 6 µL of potassium acetate (1 mol/L), and distilled water (170 µL) were also added. The absorbance of samples at λ max 415 nm was recorded after 30 min. The samples were evaluated in triplicate, and the average flavonoid content was represented as µg of quercetin equivalents per mg of extracts estimated corresponding to the standard calibration curve.

Total Phenolic Content (TPC) was assessed by the approach proposed by Singleton *et al*. (1999) and adapted for 96-well microplates. Extracts were prepared at 5 mg/mL concentration. Gallic acid solutions were made in 9 concentrations (0.48 to 125 µg/mL) as the standard. 30µL of samples (only solvent was applied in a blank probe) was added to 150 µL of Folin–Ciocalteu reagent (0.1 mol/L) and combined with 7.5% sodium carbonate (120 µL) after 10 min. Absorbance at λ max =760 nm was determined after 2 h. TPCs were estimated based on the standard calibration curve. The findings were reported as an average of triplicate tests. TPC was declared as µg of gallic acid equivalents per mg of extract.

MTT assay

Human Embryonic Kidney cell line (HEK293) and Human colorectal cancer cell lines (SW480 and HCT116) were provided from Pasteur Institute, Tehran, Iran, and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (1%) and incubated at 37 °C in 5% CO_2.

The MTT assay evaluated the cytotoxicity of acetonic or methanolic extracts of *E. esula* aerial parts on the cell lines (Hseu *et al*., 2017). After seeding the cells onto a 96-well plate (7×10^3 cells/well), they were attached and grown for 24 h. The filtration of the extracts was performed by 0.22-µm membrane filters. Then, the extracts (10 mg) were dissolved in 100 µL of dimethylsulfoxide (DMSO) and DMEM culture medium (900 µL) to obtain the stock solution. The final concentrations (25, 50, 100, 200, and 400 µg/mL) were prepared by dilution of the initial stock in DMEM (Rahamouz-Haghighi *et al*., 2022). DMSO was considered as control. Following 24-72 h of incubation, 20 µL of 5 mg/mL MTT was added, and incubation was performed for another 4 h. Then, the medium was discarded and 200 µL of DMSO was added into each well to dissolve the formazan crystals. Finally, the absorbance was read at 570 and 690

nm by an ELISA microplate reader (Tecan Infinite M200, Austria). The cell growth inhibitory rate was considered by the below formula, in which A stands for absorbance.
Inhibitory (%)= 1- ([A sample ÷ A negative control] × 100). The selection index was also calculated according to the following formula (Singh *et al*., 2019): Selectivity index = (IC_{50} of a sample in a noncancerous cell) ÷ (IC_{50} of a sample in a cancerous cell).

Hemolytic toxicity

According to the MTT assay, the methanolic extract was selected for biocompatibility testing. The biocompatibility of the methanolic extract of *E. esula* aerial parts was characterized using a hemolysis test. Freshly prepared human RBCs assembled in tubes containing ethylenediaminetetraacetic acid (EDTA) were rinsed using isotonic PBS solution, pH 7.4, and centrifuged in 1663 g for 5 minutes. The tube with erythrocyte was resuspended to a 5% hematocrit in the same medium, followed by adding methanolic extracts (25-400 µg/mL) to 0.4 mL of the diluted human RBCs suspension. Three replicates were considered for the extracts while shaking the suspension before incubation at 37°C for 4 h. The centrifugation of samples at 5400 g over a 5-minute interval deleted non-lysed human RBCs. Then, transferring the supernatant (100 µL) was in the sample tube on a 96-well plate, followed by applying the supernatant and evaluating hemoglobin release at 545 nm. A total of 0.1% sodium dodecyl sulfate and PBS were the positive and negative controls to case 100% and 0% hemolysis. The following equation calculated % hemolysis, in which A shows the absorbance: Hemolysis (%) = ([A sample- A negative control] ÷ [A positive÷ A negative control])× 100.

Toxic effect on *A. salina*

The toxic effect of the methanolic or acetonic extracts of *E. esula* aerial parts was assessed on the brine shrimp larvae (*A. salina* Leach) (Atchade *et al*., 2015). *A. salina* eggs were supplied from Urmia University. The cysts were cultured in a flask containing 35g/L NaCl/distilled water, and incubated for 36-48 h at 28 °C. Then, 10 mg of methanolic or acetonic extracts were dissolved in 100 µL DMSO and 900 µL Roswell Park Memorial Institute (RPMI-1640) medium to obtain the stock solution (10 mg/mL). Various concentrations (0.78125 to 10 mg/mL) were prepared by dissolving the initial stock solution in RPMI. The wells were filled with the extracts (20 µL) containing 200 µL RPMI-1640 and 10 nauplii to obtain 7.8125 to 1000 µg/mL concentrations. Then, the 96-well plates were subjected to incubation at 25 °C for 24 h. The number of live nauplii was evaluated using a binocular microscope after 24 h. Moreover, the negative control had only 10 nauplii and artificial seawater as well as DMSO. The average percent mortality of nauplii was specified by calculating the number of living nauplii in the control and test wells. Abbott's formula was used to determine the lethality:
Lethality (%) = ([Sample - Control] ÷ control) × 100

Antibacterial assay

The standard pathogenic bacterial cultures of gram-negative bacteria, including *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Salmonella paratyphi* (ATCC 5702), and *Proteus vulgaris* (PTCC 1182), and gram-positive ones (*Staphylococcus aureus* (ATCC 29737) were provided by the Iranian Biological Resource Center. The bacteria were cultured in Mueller-Hinton broth (MHB) at 37°C for 18h. Bacterial suspensions were then prepared based on 0.5 McFarland ($1.5\times10^{\times8}$ CFU/mL) followed by inoculation on the Mueller-Hinton Agar (MHA).
The antimicrobial activity of *E. esula* extracts was estimated using the agar disc diffusion method with recommendations of the National Committee of Clinical Laboratory Standards (NCCLS, 1997). The sterile blotting paper discs (Whatman no.2; 6 mm) were impregnated with 5µL of DMSO-dissolved methanolic or acetonic extract (10- 50 mg/mL) and then allowed to be dried completely. The discs were then employed for antibacterial tests through the disc diffusion approach. The turbidity of inoculums was adapted with the 0.5 McFarland standard. Subsequently, a sterile cotton swab was used to spread the inoculums onto the MHA to reach uniform microbial growth. Gentamicin (10 µg/mL) and DMSO-soaked discs were regarded as positive and negative controls, respectively. The plates underwent 24 h of incubation at 37°C,

and consequently, the antibacterial properties were evaluated according to the inhibition zone diameter (mm) and reported as the mean of values.

Minimum inhibitory concentration assays

MICs were evaluated using the Mueller Hinton broth microdilution in 96 well-plates based on the Clinical and Laboratory Standards Institute recommendations (CLSI, 2010). The same 0.5 McFarland suspensions were diluted with MHB to inoculate wells containing extracts (2-fold serial dilutions). Extract concentrations ranged from 125 to 2000 µg/mL. Finally, the volume of solution in each well was 200 µL. The final inoculum size was calculated by colony counting from the growth control wells (10^5cfu/well). The seeded plates were incubated at 37°C for 24 h. MICs were the lowest concentration of the extract that indicated no visible growth in MHB. In addition, bacterial suspension and MHB were considered positive and negative controls, respectively. To determine the MBC value of extracts, 100 µL from each well were spread on MHA plates and incubated at 37 °C for 24 h.

Statistical analysis

The tests were performed in triplicate. Group comparisons and statistical analysis were accomplished by Duncan's multiple range test ($p < 0.05$) using SPSS v. 21. The IC_{50} and LC_{50} values were also analyzed by ED_{50} plus v 1.0 software.

Results

Total flavonoids and phenolic content

The quantities of total flavonoids and phenols identified in the tested extracts are represented in Table 1. The acetonic extract had the highest concentration of flavonoids (16.17 µg Qu/mg), followed by the methanolic extract of *E. esula* (15.96 µg Qu/mg).

Table 1. Total flavonoids and phenolic contents in *Euphorbia esula* extracts.

Euphorbia esula	Total Flavonoids	Total Phenolics
Extracts	(µg quercetin equiv /mg extract)	(µg gallic acid equiv /mg extract)
Methanolic	15.96 ± 0.30	22.20 ± 0.24
Acetonic	16.17 ± 0.82	34.84 ± 0.31

Values are the average of three analyses ± standard deviation.

The content of phenols in various extracts of *E. esula* was reported in terms of gallic acid equivalent, µg of GA/mg of extract. The acetonic extract showed a higher content of phenols (34.84 µg GA/mg extract) in comparison to the methanolic extract.

MTT assay

To assess the growth-inhibitory activity of *E. esula* aerial parts extracts, colorectal cells were treated with various concentrations of total acetonic or methanolic extracts. The antiproliferative effects of the extracts on cell lines are presented in Fig. 1. The results suggested a decline in the cell viability after treating with acetonic or methanolic extracts of *E. esula* in time- and dose-dependently. HCT116 cells receiving *E. esula* acetonic extract showed a significant decrease in cell viability compared to SW480 (Fig. 1A-C).

However, the acetonic extract exhibited remarkable proliferation inhibition in HCT116 and HEK293 cells with IC_{50} values of 64.80 and 47.82 µg/mL at 72 h, respectively (Table 2). When the concentration of *E. esula* acetonic extract was higher than 200 µg/mL, more significant inhibition could be detected in SW480 cell viability. Additionally, the inhibition was more obvious when treatment was prolonged to 72 h (Fig. 1C).

Similar to acetonic extract, the methanolic extract exhibited the highest anti-proliferative activity against HCT116 cells. Among tested concentrations of the methanolic extract on the SW480 cell line, only the 400 µg/mL concentration actively inhibited the growth of the cells at 24 to 72 h (Fig. 1D-F). However, IC_{50} values of acetonic and methanolic extracts on SW480 were the highest (682.03 and 875.67 µg/mL, respectively) (Table 2). The selectivity index showed the selectivity of *E. esula* extracts between cancer and non-cancer cells. The higher the magnitude of the selectivity index, the greater its selectivity. In this regard, the methanolic extract of *E. esula* showed the highest selectivity against SW480 at 24 h (Table 2). However, the methanolic extract showed good selectivity against HCT116 at 24-72 h (>2) (Badisa *et al*., 2009).

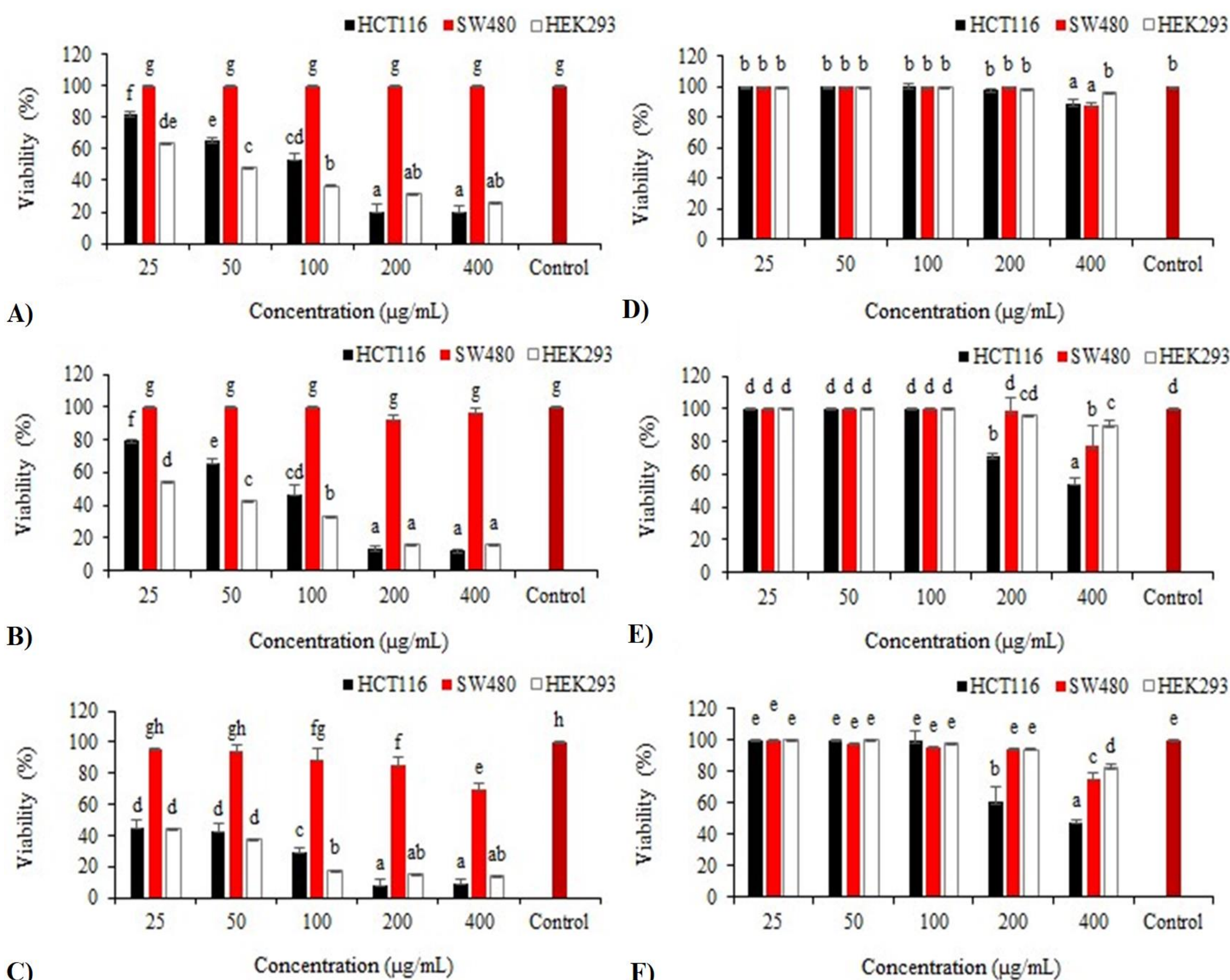

Fig. 1. Cytotoxicity activity of *E. esula* extracts against HCT116, SW480, and HEK293 cell lines by MTT assay: A, B, and C cell lines treated with acetonic extract of *E. esula* for 24, 48 and 72 h, respectively; D, E, and F cell lines treated with methanolic extract of *E. esula* for 24, 48 and 72 h, respectively. Reported data are the average of three replications ± standard deviation. Letters show significant differences based on Duncan's multiple range test ($P<0.05$). Graphs depicting similar letters are not statistically significant.

Table 2. IC_{50} value and selectivity index values of *E. esula* extracts against different cell lines.

Time (h)	Methanolic extract			Acetonic extract		
	HCT116	**SW480**	**HEK293**	**HCT116**	**SW480**	**HEK293**
A) IC_{50} value						
24	1784.65±8.2	1689.75±5.5	10492.30±6.2	165.54±4.2	Nd*	136.29±3.5
48	423.74±4.5	961.72±5.6	1450.64±4.2	142.22±4.6	4259.18±5.8	87.95±6.4
72	359.50±8.5	875.67±7.3	1063.59±9.3	64.80±7.5	682.03±6.6	47.82±12.5
B) Selectivity index						
24	5.87	6.20	-	0.82	-	-
48	3.42	1.50	-	0.61	0.02	-
72	2.95	1.21	-	0.73	0.07	-

*Nd: not determined at 25 to 400 μg/mL concentrations using ED50plus v1.0 software.

Toxic effect of extracts on *A. salina*

The general toxic effect of acetonic and methanolic extracts of *E. esula* aerial parts was tested on *A. salina*. The lethality (%) was applied as an index for evaluation of the toxicity level. The lethality of nauplii at 125 to 1000 μg/mL of acetonic extracts was 45% to 93.33%, suggesting the significant toxicity of acetonic extract. However, the methanolic extract at 250 to 1000 μg/mL exhibited moderate toxicity as 15 to 22% of the nauplii lethality (Fig. 2).

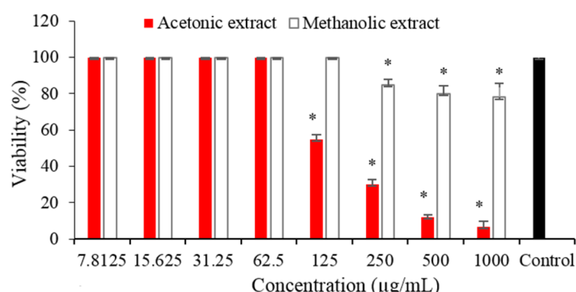

Fig. 2. The lethality of the *E. esula* extracts on *Artemia salina* after 24 h exposure time* implies a significant difference from the negative control (seawater) ($p < 0.05$).

Biocompatibility assay

Hemolysis is introduced as an index to predict toxicity for RBCs. Hemolysis assay investigated the toxicity of methanolic extract of *E. esula* at different concentrations. The methanolic extract exhibited less than 2% cytotoxicity at 400µg/mL for 4 h.

Antibacterial assay

The findings revealed the antibacterial activities of methanolic and acetonic extracts of *E. esula* aerial parts against gram-negative and gram-positive strains. The methanolic extract showed a lower inhibitory effect than the acetonic extract on all tested bacteria except for *E. coli*. The antimicrobial activity of extracts showed the maximum inhibitory activity of acetonic and methanolic extracts against *S. aureus* (34 and 28 mm) and *K. pneumoniae* (35 and 23 mm) at 50 mg/mL. However, 50 mg/mL acetonic extract exhibited minimum inhibition against *E. coli*, with 11 mm, (Table 3). The acetonic extract exhibited a stronger inhibitory effect against *K. pneumoniae* at 50 mg/mL in comparison to gentamicin (10 µg/mL). Thus, it can be concluded that this plant possesses favorable antibacterial properties.

The MICs of methanolic and acetonic extracts were determined at various concentrations of 1000 to 4000 µg/mL. The MIC results compared bacterial growth receiving the extracts to bacterial growth with no treatment (control). Accordingly, extracts at the tested concentration inhibited the growth of tested bacteria after 24 h of exposure. Generally, such findings showed acceptable anti-bacterial properties of these extracts after 24 h (Table 4). The acetonic extract showed more inhibitory effects against target bacteria. The lowest MICs and MBCs were observed for *E. esula* acetonic extracts at 1000 µg/mL and 2000 µg/mL against *S. aureus* and *K. pneumoniae*.

Table 3. Antimicrobial activities of *E. esula* on bacteria strains by disc diffusion method.

Microorganism	Extracts (50 mg/mL)		Standards (10 µg/mL)
	Acetonic extract	Methanolic extract	Gentamicin
A) Gram-positive bacteria			
Staphylococcus aureus (ATCC 29737)	34.0±3.2	28.3±2.3	18.0±2.2
B) Gram-negative bacteria			
Klebsiella pneumoniae (ATCC 10031)	35.0±1.8	23.0±1.5	26.0±2.2
Proteus vulgaris (PTCC 1182)	18.0±1.5	15.0±2.0	17.0±2.5
Salmonella paratyphi (ATCC 5702),	16.5±1.4	12.0±1.2	14.0±0.0
Escherichia coli (ATCC 10536),	11.0±1.0	12.3±3.4	16.0±1.7

The results are expressed as mean ± standard deviation.

Table 4. MIC values of acetonic and methanolic extracts of *E. esula* against bacteria strains.

Microorganism	Acetonic (µg/mL)		Methanolic (µg/mL)	
	MIC	MBC	MIC	MBC
Staphylococcus aureus (ATCC 29737)	1000	2000	1500	3000
Klebsiella pneumonia (ATCC 10031)	1000	2000	1500	2500
Proteus vulgaris (PTCC 1182)	1500	3000	2000	4000
Salmonella paratyphi (ATCC 5702),	2000	3500	2500	R
Escherichia coli (ATCC 10536),	3000	4000	4000	R

The results are expressed as mean ± standard deviation. R: Resistance.

Discussion

Euphorbia species are currently explored as an adjunct treatment for some types of cancer; however, the lack of sufficient information about the nature of the crude extract of some species is still noticeable. The role of *E. esula* in suppressing tumor cell proliferation, apoptosis, and metastasis has been demonstrated *in vitro* (Fu *et al*., 2016). The ethanolic and hexane extracts of *E. esula* contain diterpenoids which could induce apoptosis and suppress SGC7901/ADR cell proliferation (Fu *et al*., 2018). In this regard, three types of diterpene, including euphoscopin, euphornin, and jatrophane, were isolated from Euphorbia species and showed strong cytotoxicity on HCT116, human hepatocellular carcinoma (HepG2), human cervical carcinoma (Hela), lung adenocarcinoma (A549), human breast adenocarcinoma (MCF7), murine leukemia (P388), and ovarian cancer cell lines (Caov4, OVCAR3) (Lu *et al*., 2008; Tao *et al*., 2008; Wang *et al*., 2012; Yang *et al*., 2014).

The diterpenoids of ingenol-6,7-epoxy-3-tetradecanoate, ingenol-3-myristinate, and ingenol 3-palmitate isolated from *Euphorbia fischeriana* exhibited a cytotoxic effect on colorectal cancer cells (HCT116) with IC_{50} values of 14.62, 16.05, and 14.38 µg/mL at 72 h, respectively (Wang *et al*., 2013). Lathrop-3-phenylacetate-5,15-diacetate, a lathyrane diterpenoid, isolated from Euphorbia (Caper spurge) seeds, was capable of inhibiting the proliferation of A549, KERATIN-forming tumor cell line HeLa, and HCT116 cells, of which A549 cells exhibited the highest susceptibility (Zhang *et al*., 2017). Triterpenoid compositions, such as tirucalla-8, 24-diene-3, 11-diol-7-one and upha-8, 24-diene-3, 11-diol-7-one were isolated from *Euphorbia kansui* and offered moderate cytotoxicity on HCT116 with IC_{50} values of 20.89± 1.28 and 33.97± 2.15µM, respectively (Zhang *et al*., 2017). Euphol was reported as the main constituent of *Euphorbia tirucalli,* which indicated a cytotoxic effect on DLD1 and Caco2 colon cancer cell lines with IC_{50} values of 2.56 to 35.19 µM, respectively (Silva *et al*., 2018). Taraxerone and another mixture of cycloartenol, α-amyrin, lupeol, and β-amyrin triterpenes isolated from *Euphorbia hirta* showed no cytotoxicity on the HCT116 and A549 cell lines (Ragasa and Cornelio, 2013). The growth-suppression effects of the ethyl acetate extract and chloroform extract of *Euphorbia helioscopia* L on SW480 cells were also reported (Wang *et al*., 2012). Hajipour *et al*., (2022) reported the strong inhibitory effects of chloroform and ethyl acetate extracts of *Teucrium persicum* on SW-480 cancer cells and HEK-293 normal cells with IC_{50} values of (4.453, and 6.574 µg/mL) and (1.65, and 5.181 µg/mL), respectively. The present study demonstrated noticeable cytotoxic effects of acetonic and methanolic extracts on human colorectal cancer cell lines including HCT116 and SW480. According to the USA national cancer institute, the IC_{50} of a total extract, as a suitable material for further purification, should be less than 30 µg/mL. (Rajkumar *et al*., 2009). The acetonic extract showed higher IC_{50} values on HCT116 compared to the suitable dose, but their effects are still important.

Extracts of *Euphorbia palustris* L. (marsh or swamp spurge), *Euphorbia amygdaloides* L. (wood spurge), *E. helioscopia* L. (sun spurge), *Euphorbia cyparissias* L. (cypress spurge), and *Euphorbia myrsinites* L. (myrtle or donkeytail spurge) were evaluated on *B. subtilis*, *C. albicans*, *S. aureus, E. coli,* and *E. amygdaloides. E. helioscopia* and *B. subtilis* were the least sensitive bacteria (Papp, 2004). The antibacterial activity of petroleum ether, chloroform, ethyl acetate, and 96% methanol-water extracts of different parts of 15 Yemeni Euphorbiaceae species was also examined on *Staphylococcus epidermidis* (ATCC 12228), *S. aureus* (ATCC 29737), *B. subtilis* (ATCC 6633), and *E. coli* (ATCC 10536) using a qualitative agar diffusion test. The findings exhibited that *Flueggea virosa* and *Euphorbia cuneata* extracts were active on all tested bacteria. Extracts of *Euphorbia ammak* (96% methanol-water) showed the highest activities against *E. coli* and *B. subtilis*, while *Euphorbia uzmuk* (ethyl acetate, and water) was the most toxic against *S. aureus*, *S. epidermidis,* and *E. coli* (Alasbahi, 2012). The ethyl acetate and methanolic extracts of *E. peplus* L. showed high antimicrobial activity against *S. aureus* (15 and 16 mm), *S. typhimurium* (15 and 16 mm), *B. cereus* (12 and

14 mm), and *E. coli* (8 and 9 mm) (Ali *et al*., 2013). The antimicrobial effects of the alcoholic extracts of *Euphorbia helioscobia* L., *Euphorbia granulata* Forssk, and *Euphorbia hirta* Linn were determined using a good diffusion method. *E. hitra* extract exhibited high inhibitory activity against *K. pneumonia* and *S. aureus* (Awaad *et al*., 2017). According to the antibacterial assay findings, the acetonic extract of *E. esula* aerial part could be a more favorable candidate for *in vitro* treatment of gram-negative and gram-positive bacteria compared to alcoholic extracts, as the acetonic extract of *E. esula* demonstrated larger inhibition zones and lower MIC values than methanolic one. In this regard, the acetonic extract of the *E. esula* aerial part may be considered an appropriate source of secondary metabolites that can be used as antibiotic drug precursors.

LC_{50} values of each extract against brine shrimp larvae above 1000 µg/mL were considered non-toxic (Ruebhart *et al*., 2009). The acetonic and methanolic extracts of *E. esula* exhibited moderate and low toxicity against brine shrimp larvae with LC_{50} values of 381.969 and 1905.77 µg/mL, respectively. The methanolic extract was nontoxic as its LC_{50} value was more than 1000 µg/mL. Therefore, by reviewing various *in vitro* studies, the toxicological effect of herbal extracts was investigated using this test on *A. salina*. The hemolysis degree of the methanolic extract was lower than 2% at 400 µg/mL, indicating the non-toxicity on RBCs.

As reviewed by Stanković and Zlatić (2014), obtained amounts for total flavonoids in the methanolic and acetonic extract of *Euphorbia cyparissias* ranged from 59.41 to 134.34 µg Ru/mg and for phenolics varied from 17.97 to 40.72 µg GA/mg. By comparing the effectiveness of the tested extracts, it was observed that the acetonic extract was a very effective agent in the plant extraction process used in the study.

Conclusions

Based on the results of the present research, crude methanolic and acetonic extracts of *E. esula* have cytotoxic effects. These results could help in finding anti-tumor, anti-bacterial, and toxic properties of extracts. Prior to clinical trials on euphorbia extracts for cancer prevention or treatment, special attention should be paid to the design of pharmacokinetics investigations. The findings also revealed that the *E. esula* extracts can well contribute to developing efficient methods to control infectious diseases. All the plant extracts were active against the tested bacteria.

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Conflict of Interest

The authors declare no conflict of interest.

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