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Original Article

The difference between the extracts of *Erica manipuliflora* in flowering and fruiting periods in terms of the cytotoxic effects

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Abstract: The genus *Erica* comprises five species (*E. arborea, E. bocquetii, E. manipuliflora, E. sicula, E. spiculifolia*), in Turkey. In traditional Turkish medicine, the aerial parts of *E. arborea* and *E. manipuliflora* are used as diuretic and astringent and in the treatment of urinary infections. Additionally, the decoction of *E. manipuliflora* is used as weight-loss medicine, diuretic and in the treatment of diabetes, in Mugla (Turkey) and Western Mediterrenean Region in Turkey. This present study aimed to determine the cytotoxicity potentials of different extracts from this species and compare the cytotoxicity between its flowering and fruiting periods.

Key words: *Erica manipuliflora*, cytotoxicity, HepG2, HUVEC

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Introduction

The genus *Erica* L. (Ericaceae) is represented by more than 700 species worldwide. In Turkey, it comprises five species (*E. arborea*, *E. bocquetii*, *E. manipuliflora*, *E. sicula*, *E. spiculifolia*). *E. manipuliflora* Salisb. is commonly distributed in all coasts of Turkey (Davis, 1978; Güner et al., 2012). These species are commonly known as "funda", "püren" or "süpürge çalısı" in Turkey (Baytop, 1999). In traditional Turkish medicine, the aerial parts of *E. arborea* and *E. manipuliflora* are used as diuretic and astringent and in the treatment of urinary infections and the infusion (5%) of *E. arborea* as a weight-loss measure. Additionally, the decoction

of *E. manipuliflora* is used as weight-loss medicine, diuretic and in the treatment of diabetes, in Mugla (Turkey) and Western Mediterranean Region in Turkey (Başer et al., 1986; Baytop, 1999; Tuzlacı & Eryaşar Aymaz, 2001; Fakir et al., 2009; Sağıroglu et al., 2013). The species of this genus contain flavonoids, anthocyanidols, coumarins and triterpenic compounds (Ballester et al., 1975; Bennini et al., 1993; Chulia et al., 1995; Crowden & Jarman, 1976; Mendez, 1978; Vieitez et al., 1972.). Cytotoxic, anticarcinogenic, antiulcer, and antimicrobial activities of some *Erica* species were reported (Carballeira, 1982; Toro Sainz et al., 1987; Reyes Ruiz et al., 1996; Oddo et al., 1999; Martin-Cordero et al., 2001).

In a study, the inorganic compounds of 4 species (E. *arborea, E. manipuliflora, E. bocquetii* and *E. sicula* subsp. *libanotica*) were investigated and in these four *Erica* species the concentrations of Cd, As and Pb were found lower than the permissible levels as stated by WHO (World Health Organization) (Güvenç et al., 2007).

Küpeli Akkol et al. (2008) investigated the antiinflamatory and antinociceptive activities of 4 *Erica* species, one of them was *E. manipuliflora* and remarked that, the ethyl acetate extracts of *Erica manipuliflora* exhibited notable inhibition against carrageenan-induced (29.2-35.1%) and Prostaglandin E_2 (PGE_2)-induced (6.2-34.1%) hind paw edema as well as 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema models in mice and also that, this extract was found to exhibit remarkable antinociceptive activity in p-benzoquinone-induced abdominal constriction test at a dose of 100mg/kg (36.3%).

In a project to investigate the antioxidant activities of 4 *Erica* species grown in Turkey, their total fenolic contents and their antioxidant activities by DPPH and TBA assays were determined. The ethyl acetate extracts of all species were the most active ones. *E. manipuliflora* was determined as the most active species among all of them (Güvenç A., 2007).

The volatile constituents of *E. manipuliflora* were determined by Tzitsa et al. from Dirfis and Taygetos mountains (Greece). The major compounds of the essential oil from Dirfis mountain were heptacosane (19.9%), benzyl salicylate (9.9%), nonacosane (8.9%) and caryophyllene oxide (8.7%) and from Taygetos mountain l-octen-3-ol(16.2%), nonanal (9.8%), n-octanol (7.7%) and β-caryophyllene (7.5%) (Tzitsa et al., 2000).

Because of the minority of the studies on this species and lack of a study on the cytotoxicity of *E. manipuliflora* and a comparison between its flowering and fruiting periods, it is aimed to improve the knowledge about this plant, to determine the cytotoxicity potentials of different extracts from this species.

Materials and methods

Plant material

The aerial parts of *Erica manipuliflora* in flowering period were collected from A2(A) Istanbul – Burgaz Adası (Turkey) and in fruiting period from Istanbul – Kınalı Ada (Turkey), in November 2013 and identified by Dr. Pharm. Bahar Gürdal. The voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE 110340; 110341).

Preparation of extracts

The dried and powdered aerial parts of the species in flowering and fruiting periods were successively macerated with petroleum ether (FLM1PE and FRM1PE), acetone (FLM1Ac and FRM1Ac) and ethanol (FLM1EtOH and FRM1EtOH), with stirring for 1 day. Furthermore, two portions of the aerial parts were individually macerated with ethanol (FLM2 and FRM2) and water (FLM3 and FRM3), with stirring for 1 day. An infusion was prepared from another portion with boiled water (I1). The extract was stored at ± 4°C after preparation.

Biological Assays

Cell lines, culture conditions and treatments: Roswell Park Memorial Institute Medium 1640 (RPMI-1640) with glutamine, 10% FCS and penicillin / streptomycin mixture was used for the human liver carcinoma cells (HepG2; HB-8065). Primary human umbilical vein endothelial cells (HUVEC; umbilical veins of patients, ethiknumber: #131/08) were isolated from umbilical cords by digestion with 0.01% collagenase A solution (Roche) and grown in Endothelial Cell Growth Medium with supplement mix (Promocell; C-39215) containing 10% FCS, penicillin (100 U/mL) / streptomycin (100 mg/mL) mixture, and kanamycine (50 mg/mL). Umbilical cords were obtained with the consent of patients (permission by the local ethics committee). All cells were grown at 37°C and 5% CO_2 in a humidified cell incubator. The culture medium was changed every 2 days.

The monolayer cells grown to 75–85% confluence were detached with trypsin-ethylenediamine tetraacetic acid to make single cell suspensions and the viable cells were determined using the trypan blue exclusion test and diluted with medium to give a final density of 10^5 cells/mL. The passage number range for HepG2 cell lines was maintained between 21 and 26. The passage number for primary HUVEC cells was maintained between 3 and 5. The extracts were initially dissolved in dimethylsulfoxide (DMSO) and the stock solution of each extract was prepared at the concentration of 20 mg/mL. Further, the stock solutions were diluted in the medium to obtain final concentrations of 0.01-0.15 mg/mL. 100 µL of cell suspension per well were seeded into 96-well plates at plating density of 10^4 cells/well for the HepG2 cells and $2x10^4$ cells/well for HUVEC and incubated to allow for cell attachment at 37°C and 5% CO_2 for 24 h. After 24 h, the medium was aspirated and the cells were treated with 100 µL serial concentrations of all extracts. The plates were incubated at 37°C and 5% CO_2 for 24 h. The medium without samples (negative control); 20% ethanol (positive control) and with 1% and 2% DMSO (solvent) served as controls. When controlling cell viability in the presence of solvent, concentrations of up to 0.75% DMSO were shown to be nontoxic. For each extract, all concentrations were tested n=2 or n=3 in quadruplicate. After 24 h, the cells in each well were quantified by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) test (Mosmann, 1983; Kiemer et al., 2002; Diesel et al., 2011; Kessler et al., 2013).

Cytotoxicity studies: For MTT tests, the medium was aspirated and the cells were then incubated with 150 µL MTT (0.5 mg/mL in medium) solution for 3 h. After the removing of the MTT solutions, the formed formazan crystals were solubilised in 80 µL of DMSO and then the absorbance was measured at 550 nm and at 690 nm as control wavelength using a microplate reader.

The cell viability in treated cells compared to that of negative control cells was calculated. Then, the half maximal inhibitory concentration (IC_{50}) was expressed as the sample concentration that caused an inhibition of 50% in cytotoxicity in the cells calculated. The percentage cell viability was calculated with respect to solvent control as follows (LIT):

$$\% \text{ Cell viability} = \text{Abs}_{\text{Extract}} / \text{Abs}_{\text{Solvent Control}} \times 100$$

The results were expressed IC_{50} compared to the negative control.

Results and discussion

The cytotoxicity of the extracts was investigated against the HepG2 cells and HUVEC cells. As a first step, the extracts were tested against HepG2 cell line. Then the extracts, which showed good cytotoxic activity, were applied on HUVEC cells for the aim to determine the effectiveness on the healthy human cells.

Six extracts (FLM1EtOH, FLM2, FLM3, FRM1PE, FRM1EtOH, FRM2) exhibited more activity among other extracts. By the control of cell viability in the presence of solvent, concentrations of up to 0.75% DMSO were shown to be nontoxic. So, only FLM1EtOH, FRM1PE and FRM1EtOH extracts gave good results, but it can be realized that, the other three extracts showed a few activities besides the toxicity of DMSO. IC_{50} values of FLM1EtOH, FRM1PE and FRM1EtOH extracts were 0.08195 mg/mL, 0.1037 mg/mL and 0.05918 mg/mL on HepG2, respectively. Then, the cytotoxic extracts were investigated for their effectiveness on HUVEC. The FLM1EtOH exhibited cytotoxicity on HUVEC cells at the concentration of 0.1451 mg/mL, while the cytotoxic concentrations of the other extracts (FRM1PE and FRM1EtOH) were 0.171 mg/mL and 0.06279 mg/mL, respectively. These extracts were found nontoxic on HUVEC at the cytotoxic concentrations against HepG2 by using MTT method. The results of the cytotoxic tests were showed in Table 1.

Table 1. The IC_{50} values (mg/mL) of the extracts in MTT tests against HepG2 and HUVEC cell lines

Extracts	IC_{50} (HepG2; mg/mL)	IC_{50} (HUVEC; mg/mL)
FLM1PE	0,2663	*
FLM1Ac	4,427	*
FLM1EtOH	0,08195	0,1451
FLM2	0,1723	0,06504
FLM3	0,2602	*
I1	0,3522	*
FRM1PE	0,1037	0,171
FRM1Ac	0,2363	*
FRM1EtOH	0,05918	0,06279
FRM2	0,178	0,06704
FRM3	1,38	*
I2	0,4454	*
DMSO	0,4924	4,764

* have not been measured

There is an investigation, which informed on *in vitro* antimicrobial, antioxidant and cytotoxic activities of n-hexane, ethanol, methanol, ethyl acetate and aqueous extracts of the aerial parts of *Erica arborea* L. and *Erica bocquetii* P.F. Stevens (endemic species for Turkey) in literature. It was found that, any extracts of these *Erica* species showed a cytotoxic activity on brine shrimp (*Artemia salina*) (Kıvçak et al., 2013). Villareal et al. (2013) reported that lup-20(29)-en-3-one, the active component from the leaves of *E. multiflora* stimulates melanogenesis in B16 murine melanoma cells through the inhibition of phosphorylated extracellular signalregulated kinases 1 and 2 (ERK1/2) activation and showed this compound as possible therapeutic agent to address hypopigmentation disorders. Two pentacyclic triterpenoids (ursolic acid and alpha-amyrine), isolated from the methanolic extract of *E. andavalensis* aerial parts, have investigated for their cytotoxic potentials against renal adenocarcinoma (TK-10), breast adenocarcinoma (MCF-7) and melanoma (UACC-62) and ursolic acid exhibited the highest cytotoxicity (Martin-Cordero et al., 2001).

These existing studies give the first signals to the cytotoxic potential of *Erica* species, but there are still quite few studies about the cytotoxic potential of these species. Also, the investigations on *E. manipuliflora* are not so much. Beside of these, there is no study on the cytotoxicity of this species and on a comparison between the activities of its flowering and fruiting periods.

Upon analyzing the IC_{50} values, it can be seen that, the hexane extract (FRM1PE) of the plant in fruiting period was two times more cytotoxic than the hexane extract of the plant in flowering period (FLM1PE). The ethanol extracts (FLM1EtOH and FRM1EtOH), which obtained from the gradient maceration, were the most active two extracts among all extracts; but the cytotoxicity in fruiting period was higher than in flowering period. According to all, it can be realized that, this species contains cytotoxic non-polar compounds, especially in fruiting period, and the polar compounds, which exist the plant in fruiting period as well as in flowering period. Generally, it is designated that, the extracts in fruiting period showed higher cytotoxicity than the extracts in flowering period. The studies will be continued in this way and improved on the cytotoxic potential of the extracts and isolated compounds against various cancer cell lines.

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