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Effects of *Galium aparine* extract on the cell viability, cell cycle and cell death in breast cancer cell lines

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\(*\) Designed the study, conducted statistical analysis and wrote the manuscript.

\(1\) Carried out XTT assay and conducted cell cycle by flow cytometry.

\(2\) Carried out GC-MS analysis and comparative analysis of volatile compounds.

\(3\) Carried out GC-MS analysis and comparative analysis of volatile compounds.

\(4\) Carried out phytochemical characterization of GA extract by LC/Q-TOF/MS analysis.

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**A B S T R A C T**

Ethnopharmacological relevance: Galium species have been traditionally used for its anti-cancer, anti-inflammatory, antimicrobial and cardioprotective effects in the folk medicine. *Galium aparine* (GA) is a typical climbing plant growing widespread in Anatolia.

**Aim of the study:** To investigate the potential anti-proliferative and apoptotic effect of GA methanol (MeOH) extract on MCF-7 and MDA-MB-231 human breast cancer cells and MCF-10A untransformed breast epithelial cells.

**Materials and methods:** First, the extract was characterized by both liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS) and gas chromatography-mass spectrometry (GC-MS) analyses. Then, cell viability and cell cycle distribution were investigated by XTT assay and PI staining by flow cytometry, respectively. Cell death was determined by Annexin V FITC/7-AAD staining.

**Results:** A total of 14 major phytochemicals were identified by LC/Q-TOF/MS and 34 volatile compounds were determined by GC-MS. The extract was cytotoxic in both breast cancer cell lines in a concentration and time dependent manner and showed GI\(_b\) block after 72 h extract treatment. However, it was not cytotoxic to MCF-10A breast epithelial cells. Flow cytometry analyses revealed that apoptosis was induced in MDA-MB-231 cells; however, necrosis was induced in MCF-7 cells.

**Conclusion:** Our study suggests that GA MeOH extract may have potential anti-cancer effects against breast cancer cells without impairing normal breast epithelial cells. Ability to induction of non-apoptotic cell death besides apoptotic cell death by this complex plant-derived mixture may enable the killing of apoptosis resistant breast cancer cells but further studies should be conducted to investigate the bioavailability and metabolism of it in vivo.

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1. **Introduction**

Plants have been used as a source of natural medicine since ancient times. Today, approximately 25–48% of drugs originate from plants or their synthetic derivatives and there has been growing interest in the plant world (Tuorkey, 2015). Phytochemicals are plant derivatives and more than 10,000 of them are used in cancer treatment as anti-cancer compounds. Furthermore, it is shown that they could enhance the efficacy of anti-cancer compounds and reduce their toxic effects (Tuorkey, 2015; Tan et al., 2011).

*Galium* genus belongs to Rubiaceae family; consist of 1000 species worldwide and is represented by 101 species in Turkey (Orhan et al., 2012; Guvenalp et al., 2006). *Galium aparine* L. (Cleavers, local name: Karoon) is a typical climbing plant growing widespread in Anatolia and known as “Yogurt herb” because of its usage in cheese manufactures (Ergun et al., 1999). Other usage areas of GA are treatment of lymph swellings, wounds, fever, hypertension, epilepsy disease and cancer (Orhan et al., 2012; Ergun et al., 1999; Ahmad and Javed, 2007). Reported active components of GA are anthraquinones, iridoids, alkalines, flavonoids, tannins, polyphenolic acids, and vitamin C (Bokhari et al., 2013). A number of *in vitro* studies was demonstrated the anti-proliferative effects of Galium specieses on leukemia cells, laryngeal carcinoma and head and neck cancer cell lines (Sener and Ergun, 1988; Delorme et al., 2001; Yang et al., 2009; Jian et al., 2010). Moreover, it is
known that some *Galium* species are traditionally used for treatment of cancerous ulcers or breast cancer in Europe and Northern America (Hartwell, 1971). However, its anti-proliferative effects on human breast cancer cells have not been reported before.

In the current study, we investigated the potential anti-proliferative and apoptotic effect of GA MeOH extract on MCF-7 and MDA-MB-231 human breast cancer cells and MCF-10A untransformed breast epithelial cells. First, we characterized the GA extract by both liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS) and gas chromatography–mass spectrometry (GC-MS) analyses. The cell viability and cell cycle distribution were investigated by XTT assay and PI staining by flow cytometry, respectively. Cell death was determined by annexin V and 7-aminomycin D double staining.

### 2. Materials and methods

#### 2.1. Cell lines

Human MCF-10A untransformed breast epithelial cells were obtained from U.K’s Health Protection Agency. Human breast cancer cells (MCF-7 and MDA-MB-231) were purchased from Interlab Cell Line Collection (Genova, Italy). MCF-10A cells were cultured in DMEM F12 and human breast cancer cells were cultured in RPMI 1640 in 75 cm² polystyrene flasks (Corning Life Sciences, Tewksbury, MA). Both cell culture medium were supplemented with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO₂. Cell-culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

#### 2.2. Plant material

Dried plant material (*Galium aparine* L., voucher specimen no: 112) was purchased from a pharmaceutical company (Naturin Natural Products Ltd., Izmir, Turkey). The dried plant was powdered to a homogeneous size in a mill, sieved through a 40-mesh sieve. The powder (26 g) was extracted in 200 mL 99.7% (v/v) MeOH and ultrasonically extracted for 30 min, and then cooled at room temperature. 70% ethanol was added to compensate for the lost weight. The final concentration of extract was 130.0 mg/mL and stored at 4 °C. The final dilutions were made immediately before use. The MeOH solution was centrifuged at 15,000 rpm for 10 min, and the supernatants were transferred to an auto sampler vial for LC/Q-TOF/MS and GC-MS analyses.

#### 2.3. Phytochemical analysis by LC/Q-TOF/MS

GA MeOH extract was filtered through a 0.45 μm membrane filter, and then directly analyzed by LC/Q-TOF-MS (G6550A, Agilent Technologies) with a dual electrospray ionization (ESI) source. LC separations were carried out on a 100 × 3.0 mm Poroshell 120 EC-C18 (2.7 μm) column. A dual eluent system of 5 mM ammonium acetate (A) and methanol (B) was used. The flow rate was maintained at 0.6 mL min⁻¹ and the gradient as follows: 0 min (5% B), 0.5 min (5% B), 25 min (95% B), 28 min (95% B), 28.1 (5% B) and 33 min (5% B). The column temperature was 40 °C and the injection volume was 1 μL. The acquisition parameters were as follows: N₂ gas flow rate, 14.0 L/min; N₂ temperature, 175 °C; nebulizer, 45 psig; and fragmentor voltage, 3000 V. The mass range was recorded from m/z 100 to 1500 in both positive and negative modes.

#### 2.4. Volatile compound analysis by GC-MS

Qualitative analysis of the extracted volatiles was performed with an Agilent Technologies 7890A gas chromatography (GC) system hyphenated to a 5975C mass spectrometer (MS) operated in the electron-ionization (EI) mode. The chromatographic column was an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Oven temperature was initially 40 °C with 5 min hold, then heated to 280 °C at rate of 5 °C min⁻¹ and held for 5 min Helium gas (99.999%) was used as the carrier gas at constant flow rate 1.5 mL min⁻¹ and injector temperature was 250 °C. The extract was injected in the splitless mode with a 1 μL injection volume. MS were recorded at 70 eV ionization energy in full scan mode in the 35–550 amu range. The ionization source and the transfer line temperatures were 230 and 290 °C, respectively. Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST).

#### 2.5. XTT cell viability assay

Cells were seeded at 1 × 10⁴ cells/well, in a final volume of 200 μL, in 96-well flat-bottomed microtiter plates. After 24 h incubation, MCF-10A, MCF-7 and MDA-MB-231 cells were exposed to increasing concentrations of GA extract (100–700 μg/mL). Plates were incubated at 37 °C in a 5% CO₂ incubator for 24, 48, and 72 h. Cell culture media was not refreshed during this time. At the end of incubation, 100 μL of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) was added to each well, and plates were incubated at 37 °C for another 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, Miami, FL).

#### 2.6. Cell cycle analysis

Cell cycle distributions were analyzed by measuring the DNA fragments that were stained with propidium iodide (PI) by Cell Cycle Phase determination Kit (Cayman Chemical, USA). Cells were seeded in six-well plates at a density of 1 × 10⁶ cells/well in 2 mL culture medium and incubated for 24 h at 37 °C in a CO₂ incubator. Then, cells were treated with 250 μg/mL GA extract for 72 h. At the end of the treatment, cells were centrifugated and cell pellets were washed twice with cold phosphate-buffered saline (PBS). Then the cells were fixed and permeabilized for 2 h by adding 1 mL fixative. After centrifugation, the fixatives were decanted and the cell pellets were resuspended in 0.5 mL of staining solution containing 200 μL of DNase-free RNase (Sigma-Aldrich Co) and PI then incubated for 30 min at room temperature in the dark. Finally, flow cytometry was employed to measure cell cycle using Accuri™ C6 flow cytometer (BD Biosciences, USA).

#### 2.7. Evaluation of apoptosis and necrosis

Evaluation of either apoptosis or necrosis was done by Multi-Parameter Apoptosis Assay Kit (Cayman Chemical, USA). The breast cancer cells were grown in six-well plates at a density of 5 × 10⁵ cells/well and incubated for 24 h at 37 °C in a CO₂ incubator. Then, cells were exposed to 250 μg/mL of GA extract for 72 h. After 72 h treatment, cell pellets were suspended in 2 mL binding buffer and centrifuged at 400g for 5 min. Then, cells were stained with 250 μL of annexin V (AN) and 7-amino-actinomycin D (7AAD) staining solution mix for 10 min at room temperature in the dark. Then, cells with AN⁻/7AAD⁻, AN⁻/7AAD⁻, AN⁺/7AAD⁻ and AN⁻/7AAD⁺ which have been found to
correspond to live cells, early apoptotic cells, late apoptotic cells and necrotic cells, respectively, were determined by Accuri™ C6 flow cytometer (BD Biosciences, USA) and analyzed with its own software.

2.8. Statistical Analysis

All statistical analyses and graphs were performed with GraphPad Prism 5 (Graphpad software, La Jolla, CA, USA). The results of the study were expressed as mean ± S.D. and data was analyzed by using one way analysis of variance test (ANOVA) followed by Dunnett’s t-test for multiple comparisons. Values with P < 0.05 were considered as significant.

3. Results

3.1. Phytochemical characterization of GA extract by LC/Q-TOF/MS analysis

Qualitative phytochemical analysis of GA MeOH extract was performed to determine the biologically active compounds. Under the present chromatographic and MS conditions, a total of 14 phytochemicals; carminomycinone, pyridoxine, normetanephrine, spectinomycin, coumarin, chlorogenic acid, monotropein, cinnamic acid, pantothenic acid, esculetin, quercetin, p-Coumaric acid, quercetin and luteolin were identified. Table 1 shows the names, molecular formulas and retention times of the most abundant identified compounds.

3.2. Volatile compound analysis of GA extract by GC-MS

Volatile compounds identified in the GA MeOH extract sample by GC-MS are shown in Table 2. The analysis of GA volatile compounds revealed a total of 34 identified compounds. The major classes included aldehyde compounds and fatty acids. The major two peaks in the MS data were obtained from palmitic acid and dihydromaltol indicating that they are the most abundant compounds among all the identified volatiles in GA extract.

3.3. Effect of GA extract on the viability of MCF-7 and MDA-MB-231 human breast cancer cells and MCF-10A breast epithelial cells

To evaluate the effect of GA MeOH extract on the viability of human breast cancer cells, MCF-7 and MDA-MD-231 cells were treated with increasing concentrations of the extract (100–700 μg/mL) for 24, 48, 72 h and then XTT assay was performed. GA extract decreased cell viability in a time- and concentration-dependent manner in both breast cancer cells as compared to untreated controls (p < 0.001). The highest cytotoxicity was observed at 72 h (Fig. 1A and B). The IC50 value of GA extract was 486 μg/mL and 503 μg/mL in MDA-MB-231 and MCF-7 cells at 72 h, respectively.

Effect of GA extract on the viability of MCF-10A was also evaluated to test the possible impairing effect of the extract on human non-cancerous breast epithelial cells. Even at the highest tested concentrations, GA extract was not cytotoxic to MCF-10A breast epithelial cells (IC50 > 1500 μg/mL)(Suppl. Fig. 1).

3.4. Cell cycle distribution after treatment with GA extract in human breast cancer cells

Cell cycle distributions of breast cancer cells after treatment with GA extract were evaluated by flow cytometry. At 72 h of treatment with the sub-toxic concentrations of GA extract (250 μg/mL) caused a reduction in the proportion of both breast cancer cells in S phase of cell cycle with simultaneous enhancement in the proportion of cells in G0/G1 phases (Fig. 2). The data indicates that the MeOH extract mediated growth inhibition of breast cancer cells is associated with G1 phase cell cycle arrest.
Fig. 1. Dose response effects of GA extract on the viability of MDA-MB-231 (A) and MCF-7 (B) cells at 24, 48 and 72 h. Cells were seeded at $1 \times 10^4$ cells/well and incubated at the indicated concentrations of GA extract. The results are expressed as the mean of 3 different experiments (± SD) (*P < 0.05 compared to untreated control.).

Fig. 2. Flow cytometric analysis of cell cycle distribution of MDA-MB-231 and MCF-7 cells before and after 250 μg/mL GA extract treatment at 72 h.
3.5. Induction of different cell death mechanisms by GA extract in breast cancer cells

Breast cancer cells were cultured under same conditions with cell viability assay in the presence of GA extract (250 μg/mL) for 72 h. Then, to investigate whether the inhibition of cell proliferation is related to apoptosis or necrosis, we stained cells with AN/7AAD and analyzed by flow cytometry. Results showed that treatment of MDA-MB-231 cells for 72 h with GA extract resulted in an increase of the AN+/7AAD− (corresponds to early apoptotic cells) and AN−/7AAD+ (corresponds to late apoptotic cells) cells (Fig. 3) (p < 0.001). However, in MCF-7 cells, treatment with GA extract for 72 h resulted in an increase of the AN−/7AAD+ (corresponds to necrotic cells) cells (Fig. 3) (p < 0.001).

4. Discussion

A great number of plant-derived phytochemical compounds and their synthetic derivatives are used in cancer treatment and many of them are under investigation for their potential use in cancer treatment. In the literature, some studies claim that the whole extract of a plant may be more beneficial than the isolated compounds through adsorption or metabolism of bioactive components. Moreover, diverse bioactive compounds within the extracts may have synergistic effects by inducing different signaling pathways. However, some other studies suggest that major components should be identified within the extract. Previous phytochemical investigations of different Galium species revealed the presence of iridoids, terpenoids, anthraquinones and flavonoids (Hartwell, 1971; Sener and Ergun, 1988; Tzakou et al., 1990; Deliorman et al., 2001; Yang et al., 2009; Jian et al., 2010). In the current study, phytochemical composition and volatile compounds of G. aparine MeOH extract were determined by highly sensitive methods LC/Q-TOF/MS and GC-MS, respectively. Data obtained from LC/Q-TOF/MS analysis revealed the presence of 14 major compounds (chlorogenic acid, coumarin, p-coumaric acid, cimicifugine, pyridoxine, normetanephrine, spectinomycin, monotropein, cinnamic acid, pantothentic acid, esculetin,
quercitrin, quercetin and luteolin) in the MeOH extract. Quercetin, luteolin, chlorogenic acid, coumarin and p-coumaric acid have previously been reported from G. aparine (Hartwell, 1971; Sener and Ergun, 1988, Yang et al., 2009; Tzakou, 1990), however, other 10 compounds determined by LC/Q-TOF/MS are being reported for the first time from G. aparine. According to GC/MS analysis, 34 volatile compounds were identified from the extract. The major component of the volatile compounds was palmitic acid (hexadecanoic acid). Beser et al. investigated the essential oil content of G. aparine oil by GC/MS and identified 72 compounds, but in low amounts. They also identified the major component of G. aparine oil as hexadecanoic acid (Beser et al., 2004).

There are also several studies investigating the anti-proliferative effects of Galium species on various cancer types. Amirghofran et al. reported that G. mite extract exhibited cytotoxic effects against human leukemia cells (Amirghofran et al., 2006). In another study, anti-cancer effect of G. verum aqueous extract was investigated on drug-sensitive and -resistant laryngeal carcinoma cell lines. The aqueous extract was found to be cytotoxic against all tested laryngeal carcinoma cell lines, but angiogenesis was not affected by G. verum aqueous extract (Schmidt et al., 2014a). Schmidt et al. studied the effect of G. verum aqueous extract on the motility of head and neck cancer cell lines and revealed that sublethal doses of extract acted as strong inhibitor of motility (Schmidt et al., 2014b). In our study, effect of GA MeOH extract on human breast cancer cell lines were investigated for the first time and it is found that the extract was cytotoxic in both breast cancer cell lines in a concentration and time dependent manner. However, the extract was not cytotoxic to MCF-10A human breast epithelial cells even at the highest concentrations suggesting the tumor specific effect of GA MeOH extract. Moreover, the effect of extract on cell cycle distribution was investigated. GA extract treated breast cancer cells showed G1 block after 72 h, as well as an increase in the G1 fraction. Sub G1 fraction was increased only in MDA-MB-231 cells exposed to GA extract indicating apoptotic effect.

Since evading apoptosis is one of the main properties of a cancer cell, the ultimate goal of cancer treatment is to induce cancer cell death selectively (Hanahan and Weinberg, 2000; Okada and Mak, 2004). Moreover, studies of chemotherapeutic agents used clinically reported that alternative cell death pathways are activated by these agents for complete tumor regression (Guerriero et al., 2008). Therefore, novel chemotherapeutic and anti-tumor agents should be able to induce apoptosis and alternative cell death pathways. After staining breast cancer cells with AN and 7AAD simultaneously, flow cytometry analysis was conducted in order to determine the cell death pathways activated by the extract. Results revealed that GA extract induced different types of cell death in different subtypes of breast cancer cell lines. Sublethal doses of extract induced apoptotic cell death in MDA-MB-231 cells, but necrosis was induced in MCF-7 cells having null caspase-3.

5. Conclusions

Galium species have been used in the folk medicine for its anti-cancer, antioxidant, anti-inflammatory, antimicrobial and cardioprotective effects for ages. Several in vitro studies have reported the antioxidant potential of G. aparine (Lindsey et al., 2002; Bozkhari et al., 2013) but, to the best of our knowledge, effect of GA MeOH extract on human breast cancer cells was not investigated before. This in vitro study suggests that G. aparine MeOH extract may have potential anti-cancer effects against breast cancer cells without impairing normal breast epithelial cells. Ability to induction of non-apoptotic cell death besides apoptotic cell death by this complex plant-derived mixture may enable the killing of apoptosis resistant breast cancer cells. Further studies should be conducted to investigate the bioavailability and metabolism of it in vivo.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.04.007.

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