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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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ABSTRACT

Ethnopharmacological relevance: Galium species have been traditionally used for its anti-cancer, anti-oxidant, 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 G1 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*.

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, alkanes, 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; Deliorman et al., 2001; Yang et al., 2009; Jian et al., 2010). Moreover, it is

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[4] Carried out phytochemical characterization of GA extract by LC/Q-TOF/MS analysis.

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known that some *Galium* specieses 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-amino-actinomycin 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 cm^2 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% CO_2. 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: N_2 gas flow rate, 14.0 L/min; N_2 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 7890 A 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 of 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 1×10^4 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% CO_2 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-sulfophenyl)-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 1×10^6 cells/well in 2 mL culture medium and incubated for 24 h at 37 °C in a CO_2 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 5×10^5 cells/well and incubated for 24 h at 37 °C in a CO_2 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 400*g* for 5 min After 5 min, 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