



Active Phytochemical Detecting, Antioxidant, Cytotoxic, Apoptotic Activities of Ethyl Acetate and Methanol Extracts of *Galium aparine* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Author ÖSA designed the experiment, performed the statistical analysis and wrote the protocol. Author TAÇ designed the study, managed the literature searches and wrote of the manuscript. Author BK designed the HPLC analyses of the extracts. Author FK interpreted and wrote the HPLC analysis. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to detect active phytochemicals in ethyl acetate (EAE) and methanol (ME) extracts of *Galium aparine* L. and determine in vitro antioxidant, cytotoxic and apoptotic activities of these extracts.

The phytochemicals present in the plant were determined using standard methods and HPLC.

EAE and ME of *Galium aparine* arial parts were tested for antioxidant (by using DPPH radical scavenging, H₂O₂ scavenging and metal chelating assays), cytotoxic (by using MTT assay on

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MCF-7 breast cancer and Caco-2 colon cancer cells), and apoptotic activity (DNA diffusion assay on MCF-7 breast cancer and Caco-2 colon cancer cells). Human peripheral blood lymphocytes were used as control cells.

As a result of phytochemical screening of EAE and ME of *Galium aparine* arial parts, phenols, tannins, alkaloids, anthraquinones and saponins were detected. Furthermore, rutin, a flavonoid phytochemical was found in both of the extracts by HPLC analysis. EAE and ME have cytotoxic and apoptotic activity on human MCF-7 breast cancer and Caco-2 colon cancer cells. These activities may depend on polarity of solvents used in the extraction and on concentration of the extracts. EAE and ME showed strong DPPH[•] radical scavenging, very low H₂O₂ scavenging and metal chelation activity. Besides, EAE showed higher cytotoxic and apoptotic effect on human peripheral lymphocytes compared to ME. The *G. aparine* EAE and ME led cytotoxicity and apoptosis in human MCF-7 breast cancer and Caco-2 colon cancer cells.

Keywords: Apoptosis; Caco-2 cells; cytotoxicity; DPPH radical scavenging activity; *G. aparine* L.; MCF-7 cell; phytochemical screening.

ABBREVIATIONS

GA: *Galium aparine*; **EAE:** Ethyl Acetate Extract; **ME:** Methanol Extract.

1. INTRODUCTION

In recent years, plant derived natural products such as flavonoids, terpenoids, steroids among others have received considerable attention due to their different pharmacological properties, including antioxidant and antitumor activity [1,2]. Plants were used for a long time as a source of anticancer agents, and 60% of currently used anticancer agents are derived from natural sources [3,4]. Medicinal plants have a significant role in cancer treatment. Therefore, many researchers investigate extensively the mechanism of interaction between phytochemicals and cancer cells [5,6]. Phytochemicals may both modulate cell signalling pathways thereby inhibiting cancer development or progression and induce apoptosis in malignant cells [7].

Determination of cytotoxicity, commonly used to evaluate the biological activity of natural products, is helpful to confirm whether plant extracts have potential anti-neoplastic properties [8], and induction of DNA damage in cancer cells is a well recognized therapeutic strategy for cancer treatment [9]. Many chemopreventive agents have been associated with anti-proliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells [10].

The medicinal plant genus *Galium* L. (Rubiaceae) is represented in Turkey by 101

species in 10 sections [11]. *Galium* species are traditionally used to coagulate milk because of an enzyme in their composition and, therefore, this plant is known as "yoğurt herb" [12]. *Galium aparine*, *Galium cruciata* and *Galium verum* are used as diuretics, choleric agents, against diarrhea and in the treatment of some stomach complaints, gout and epilepsy in folk medicine [13,14]. Iridoids, anthraquinones, triterpenic saponins, naphthalene derivatives, flavonoids, lignan bis-glucosides and alkaloids were isolated from *Galium* species previously [15-17]. *Galium aparine* L. is a fast-growing herbaceous annual plant from the family Rubiaceae [18]. This plant is used in Turkish folk medicine as diuretics, choleric agents and against diarrhea [14]. Furthermore, *Galium aparine* (Fig. 1) is used as anticancer medicinal plant in Europe, Africa and Australia [19].

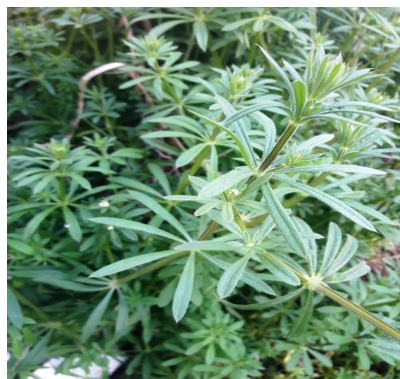


Fig. 1. *Galium aparine* L. (Photo by Tülay Aşkın Çelik)

Although phytochemical contents, antioxidant, antimicrobial/antifungal, antiparasitic, antifeedant and insecticidal properties of various *Galium* species are known, no data have been reported previously on the phytochemical contents and anticancer activities of *Galium aparine* L. Therefore, we investigated phytochemical components, potential antioxidant activity and anticancer activities on MCF-7 breast cancer and Caco-2 colon cancer cells of ethyl acetate and methanol extracts from the aerial parts crude extracts of *Galium aparine* L. in this study.

2. MATERIALS AND METHODS

2.1 Plant Collection

Aerial of the plant *Galium aparine* L. (GA) were identified and collected in the spring season at 160 m above sea level in Adnan Menderes University, Central Campus, Aydın, Turkey (N. 37°85'228" E.027°851'34"). A voucher specimen was deposited in the Herbarium of the Adnan Menderes University (AYDN 874). Fresh aerial parts of plant were washed 2-3 times with water and dried at room temperature and stored at room temperature in closed containers in the dark until used.

2.2 Preparation of Ethyl Acetate and Methanol Extract of *Galium aparine* Aerial Parts

The dried plants were milled to a fine powder with a blender (200 g) and then ethyl acetate and methanol were used to extract the *Galium aparine*. For 200 g plant material two liters of solvent was used. After completing extraction (at room temperature for approximately 12 h, until the solvent became colorless) with ethyl acetate and filtration, the plant material was dried and subjected to the second extraction with methanol [20,21]. The extracts were evaporated and yielded 0,813 g and 1.136 g dried mass, respectively.

2.3 Preliminary Phytochemical Analysis

Phytochemical analyses were carried out according to Ravishankara, 2002 and Dominguez, 1973 [22,23]. The details of the tests as are follows:

2.3.1 Detection of phenols

Ethyl acetate and methanol extracts prepared in ethanol were spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the

spots and was exposed to ammonia vapors. Blue coloration of the spots indicates the presence of phenols.

2.3.2 Detection of tannins

To 2-3 ml of methanolic extract, 10% alcoholic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicates the presence of tannins in the extracts.

2.3.3 Detection of alkaloids

A drop of extracts prepared in methanol was spotted in a small piece of pre-coated TLC plate and the plate was sprayed with Dragendorff's reagent. Orange coloration of the spot indicates the presence of alkaloids.

2.3.4 Detection of anthraquinones

About 50 mg of the extracts was heated with 10% ferric chloride solution and 1 ml of concentrated hydrochloric acid. Extracts were cooled, filtered and the filtrates were shaken with diethyl ether. The ether extracts were further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicates the presence of anthraquinones.

2.3.5 Detection of saponins

10 mg of the extracts were mixed with hot water and the mixtures were shaken for 30s. The formation of stable foam indicates the presence of saponins.

2.3.6 Detection of flavonoids

To 2-3 ml of the extracts prepared in methanol, a piece of magnesium ribbon and 1 ml of concentrated hydrochloric acid were added. Pink-red or red coloration of the solution indicates the presence of flavonoids.

2.4 HPLC Analysis of Extracts

HPLC analysis of extracts were performed using Agilent 110 series, DAD detector (diode array detection), EC 250/4.6 NUCLEOSIL 100-10 C-18 colon and at 25°C. Injection volume was 0.5 ml and detection at 282 and 350 nm.

A: 0.1 % formic acid, water

B: 0.1% formic acid, acetonitrile were used as solvents and analysis was performed with isocratic mode. Neohesperidin, uvaol, rutin ve β -sitosterol were used as standards in analysis.

2.5 Determination of Total Phenolic Content

Total phenolic compound contents in extracts were determined by the Folin-Ciocalteu method [24]. 100 and 300 µl of properly diluted extract solutions were mixed with 1 ml of FC reagent. The reagent was pre-diluted, 10 times, with distilled water. After standing for 3 min at room temperature, 3 ml of (2% w/v) sodium carbonate solution were added. The solutions were mixed and allowed to stand for 2 h at room temperature. Then, the absorbance was measured at 760 nm, using a UV-visible spectrophotometer (Shimadzu PharmaSpec UV-1700, Japan). A calibration curve was prepared, using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l). Results were expressed as mg gallic acid equivalents/100 g of sample.

2.6 Determination of Antioxidant Activity

2.6.1 DPPH free radical scavenging assay

Assay for DPPH free radical scavenging potential is based on the scavenging activity of stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). The free radical scavenging activity of ethyl acetate and methanol extracts of *Galium aparine* were tested to see if they would bleach the stable DPPH radical so the free radical scavenging activity could be measured [25]. One milliliter of 0.1 mM DPPH methanol solution was added to 3 ml of various concentrations (100, 200 and 300 µg/ml) of extracts in methanol.

After shaking the mixture vigorously it was left to sit in dark at room temperature. After 30 min, the absorbance of mixture was measured at $\lambda=517$ nm. All the tests were carried out three times. Rutin (50 and 100 µg/ml), a citrus flavonoid glycoside, was used as a standard, and the Shimadzu PharmaSpec UV-1700 UV-Visible spectrophotometer was used for measurement. Finally, the DPPH radical scavenging activity of extracts was calculated using the following equation:

$$\text{Scavenging capacity \%} = 100 - \left[\frac{\text{Ab of sample} - \text{Ab of blank}}{\text{Ab of control}} \times 100 \right]$$

where Ab control is the absorbance of DPPH solution without extracts.

The amount of the extract concentrations that inhibits 50% of DPPH radical (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

2.6.2 H₂O₂ scavenging assay

Hydrogen peroxide scavenging activities of ethyl acetate and methanol extracts from *Galium aparine* were determined the method described by Ruch et al. [26]. A solution of H₂O₂ (40 mM) was prepared in phosphate buffer (pH 7.4). Reaction mixtures contained 40 mM of H₂O₂ and different concentrations of extracts, and absorbance values were measured after 10 min using wavelength of 230 nm. Ascorbic acid was used as the standard.

2.6.3 Metal ion chelating ability

The chelation of ferrous ions by extracts was estimated by method of Dinis et al. [27]. Briefly, 50 µl of 2 mM FeCl₂ was added to 1 ml of different concentrations of the extract (100, 200 and 300 µg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated as:

$$\left[\frac{A_0 - A_s}{A_s} \right] \times 100$$

where A₀ was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na₂EDTA was used as positive control (standard).

2.7 Cell Culture and Cytotoxicity Assay

In cytotoxicity assay Human breast cancer cells (MCF-7) (ATCC HTB-22) and Human colon cancer cells (Caco-2) (ATCC HTB-37) and human peripheral lymphocytes were used. Stock cells of these cell lines were cultured in DMEM supplemented with 10% FBS for MCF-7 cells and 20% for Caco-2 cells, penicillin-streptomycin under humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were trypsinized and cytotoxicity assays were carried out in 24 well-plates.

MCF-7 and Caco-2 cells were plated 5x10⁴ cells/plate in 24-well plate and incubated for 24 h, during which a partial monolayer formed. After that 100, 200 and 300 µg/ml concentrations of ethyl acetate and methanol extracts were added for treatment. Human peripheral lymphocytes were isolated according to Boyum [28] and they were also treated with 100, 200 and 300 µg/ml concentrations of ethyl acetate and methanol extracts. Control cells received only maintenance

medium. Furthermore, anticancer agents Farmorubicin (40 µg/ml) and Campto irinotecan (40 µg/ml) were used for MCF-7 and Caco-2 cells, respectively. 0.1% As a solvent control, DMSO was used. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 48 h. At the end of 48 h cellular viability was determined by MTT assay [29].

2.8 DNA Diffusion Assay

To evaluate the type of cell death DNA diffusion assay was performed following the protocol described by Singh [30]. Agarose pre-coated slides were made by spreading 50 µl of 1% 3:1 high resolution agarose on each slide and drying them at room temperature. Microgels were made on agarose-precoated slides by mixing extract-treated cells with 50 µl 0.6% high resolution agarose and pipetting this mixture onto the slide. The gel was immediately covered with a 24x50 mm cover glass. The slides were coded and allowed solidify for 5 min at room temperature. The cover glasses were removed from the second layer of microgel, and 200 µl of 2% SFR agarose was layered to make a third layer and immediately covered with a 24x50 mm cover glass. After 2 min, cover glasses were removed and slides were immersed in a freshly made lysing solution (1,24 M NaCl, 1 mM tetra sodium EDTA, 5 mM Tris-HCl, 0.01 sodium lauryl sarcosine, 0.2% DMSO freshly added, 0.3 N NaOH freshly added) for 10 min at room temperature. After lysis, the slides were twice immersed in a neutralizing solution (50% ethanol, 1 mg/ml spermine, 20 mM Tris-HCl, pH 7.4) for 30 min at room temperature. The slides were air dried and stored at room temperature. The slides were stained with 50 µl of 1 µM YOYO-1 and covered with a cover slip. 1000 cells per slide were analysed. The cells which were undergoing apoptosis or necrosis were distinguished from normal cells. Apoptotic cell nuclei have a hazy or undefined outline without any clear boundaries due to nucleosomal- sized DNA diffusing into the

agarose. Necrotic cell nuclei are bigger and poorly defined. They have clear, defined outer boundary of the DNA halo and a relatively homogeneous halo appearance [30].

2.9 Statistical Analysis

Each experiment was carried out triplicate. Values are expressed as means ± SD and analyzed by One Way ANOVA (SPSS 11.5 software package program). Statistically significant difference was considered at the level of p<0.05. The normality of variables was evaluated using the Kolmogorov – Smirnov Z test. The statistical differences between the control and treatment groups were carried out using the non-parametric Mann Whitney Test (for independent samples). The correlations between different variables were determined using the Spearman Rank Correlation Test.

3. RESULTS

3.1 Phytochemicals in Extracts

As a result of phytochemical screening, it was shown that phenols, tannins, alkaloids, anthroquinones and saponins were present in the ethyl acetate extract, and phenols, tannins and anthroquinones were present in methanol extract (Table 1).

3.2 Total Phenolic Content

The total phenolic contents of the ethyl acetate and methanol extracts determined by using the Folin-Ciocalteu's reagent, which is expressed in terms of gallic acid equivalent. The total phenolic contents were presented as mg of GAE/g of extract (Table 2). The total phenolic contents in extracts were 50.00± 0,036mg GAE/g in ethyl acetate extract, and 41.00 ± 0,280 mg GAE/g in methanol extract. Phenol concentration in ethyl acetate extract was found higher than in methanol extract.

Table 1. Phytochemical tests revealed the presence of following compounds tested in the *Galium aparine*

Compounds	Tests	EAE	ME
Phenols	Phosphomolybdic acid test	+	+
Tannins	Braemer's test	+	+
Alkaloids	Dragondroff's test	+	-
Flavonoids	Shinoda's test	-	-
Anthraquinones	Bornträger test	++	++
Saponins	Frothing test	+	-

(EAE: Ethyl acetate extract; ME: Methanol extract) (- absent; + present; ++ abundant)

Table 2. Total phenolic contents in the plant extracts expressed in terms of gallic acid equivalent (mg of GA/g of extract)

Extract	mg of GA/g of extract
EAE	50.00 ± 0,036
ME	41.00 ± 0,280

Each value is the average of three analyses ± standard deviation. EAE: Ethyl acetate extract; ME: Methanol extract

3.3 HPLC Analysis Results

HPLC analysis results of *Galium aparine* L. ethyl acetate and methanol extracts are present in Figs. 2 and 3. According to analyses, 598.2 ppm rutin was detected in 1 ml ethyl acetate extract,

and 597.7 ppm rutin was detected in 1ml methanol extract. Neohesperidin, uvaol and β -sitosterol were not detected in ethyl acetate and methanol extracts.

3.4 Antioxidant Activity

3.4.1 DPPH scavenging activity

The antioxidant activity of ethyl acetate and methanol extracts obtained from *Galium aparine* was determined by using a methanol solution of DPPH reagent. DPPH is very stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and

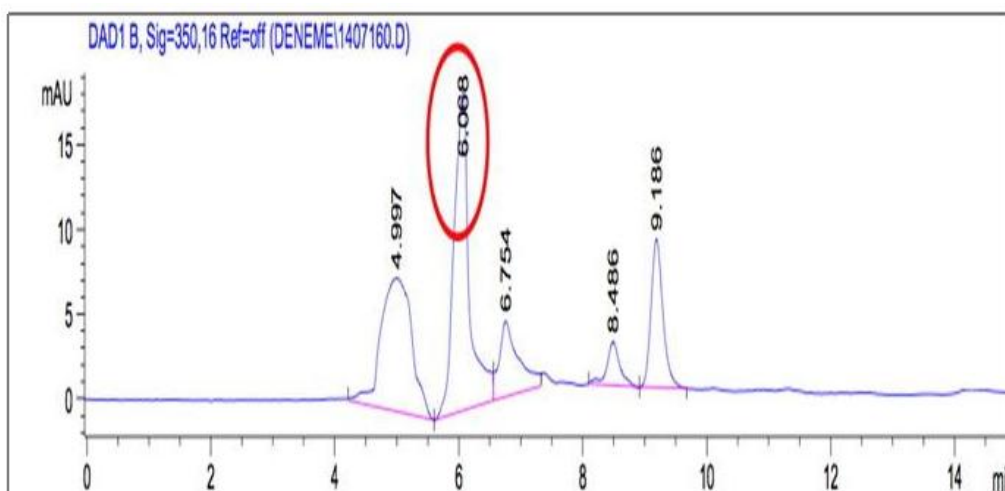


Fig. 2. HPLC spectrum of *Galium aparine* ethyl acetate extract

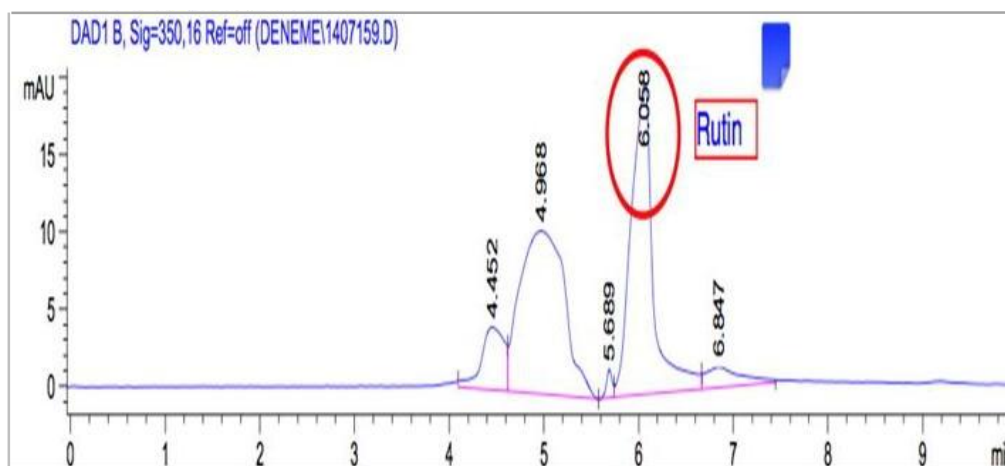


Fig. 3. HPLC spectrum of *Galium aparine* methanol extract

enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colorless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), and result in a decrease in absorbance at 517 nm band [31]. The results of the scavenging ability of the ethyl acetate and methanol extract and the standard rutin phytochemical expressed in percentage can be seen in Table 3. Extracts showed a concentration-dependent radical scavenging activity. Compared to ethyl acetate extract, methanol extract showed a stronger DPPH radical scavenging activity. The methanol extract exhibited a high activity (97.70%) at 300 µg/ml concentration which is better than that of flavonoid rutin (95.80%). The ethyl acetate extract showed a moderate activity.

3.5 H₂O₂ Radical Scavenging Activity

H₂O₂ scavenging activities (%) of different concentrations of extracts are shown in Table 3. It can be seen from the plotted curves that the percentage of H₂O₂ scavenger increased with the concentration of the ethyl acetate and methanol extracts. The ethyl acetate and methanol extracts exhibited a low activity at tested concentrations (100, 200 and 300 µg/ml), which is comparable with that of ascorbic acid.

3.6 Metal Ion Chelating Ability

Metal Ion Chelating potential of *G. aparine* ethyl acetate and methanol extracts were determined in the concentration range 100-300 µg/ml and results are depicted in Table 3. Although EDTA, which was used as a standard in the experiment, showed a high ion metal chelating potential, methanol extract showed a very low chelating activity (5.90%, 7.34% and 7.53%, respectively) and ethyl acetate extract showed no chelating activity.

3.7 In vitro Cytotoxicity Assay

The cytotoxic effects of *Galium aparine* ethyl acetate and methanol extracts on MCF-7 and Caco-2 cells are shown in Table 4. Ethyl acetate extract exhibited concentration-dependent cytotoxic effect on MCF-7 cell line (34.35%, 43.27% and 49.30%, respectively). Although cytotoxic effect of ethyl acetate extract was

significantly high compared to controls, this effect was significantly low at 200 and 300 µg/ml concentrations in comparison with the 200 and 300 µg/ml concentrations of methanol extract, and especially with anticancer drug Farmorubicin (98.48%). Cytotoxic effect of methanol extract is increased concentration dependently. The highest cytotoxic effect of methanol extract on MCF-7 cells was found as 71.14% at 300 µg/ml concentration. Although this value is lower than the effect of 40 µg/ml concentration of anticancer drug Farmorubicin (98.48%), it is statistically significant in comparison with control groups ($p < 0.05$). Ethyl acetate extract of *Galium aparine* has significant cytotoxic effect on Caco-2 line in comparison with solvent control. Standard anticancer drug Campto at 40 µg/ml concentration demonstrated 83.46% cytotoxic effect on Caco-2 cells (Table 4). Although cytotoxic effect of ethyl acetate extract was significantly high in comparison with solvent control, this effect was significantly low compared to the effect of the anticancer drug Campto (83.46%). Methanol extract of *Galium aparine* has no significant cytotoxic effect on Caco-2 line at 100, 200, and 300 µg/ml concentrations when compared with ethyl acetate extracts and anticancer drug Campto. Ethyl acetate extract of *Galium aparine* showed higher cytotoxic effect than the methanol extracts (Table 4).

Ethyl acetate extract showed cytotoxic effect on human peripheral blood lymphocytes, which was used as control cells in the experiment (Table 4). This effect was found higher than the effects of anticancer drugs Farmorubicin and Campto. Methanol extract showed no significant cytotoxic effect on human peripheral blood lymphocytes, which was used as control cells in experiments. However, anticancer drugs Farmorubicin and Campto showed significantly higher cytotoxicity on lymphocytes compared to methanol extracts (Table 4). Ethyl acetate extract showed higher cytotoxic effect on lymphocytes compared to the methanol extract (Table 4).

3.8 Apoptotic Effect of Extracts

Apoptotic effects of *Galium aparine* ethyl acetate and methanol extracts on MCF-7 and Caco-2 cells were determined by the DNA Diffusion Assay. Results can be seen in Table 5 and Fig. 4. *Galium aparine* ethyl acetate and methanol extracts exerted apoptotic effects on MCF-7 and Caco-2 cells lines after 48 h. Ethyl acetate extract significantly induced apoptosis in MCF-7 cells in comparison with controls. This effect increased concentration-dependently. Although

apoptotic effect of the extract was significantly higher in comparison with controls, this effect was lower than the effect of the methanol extract. Anticancer drug Farmorubicin (40 µg/ml) significantly induced apoptosis in MCF-7 cells, when compared with controls and methanol and ethyl acetate extracts. The methanol extract was found to be a better apoptosis-inducing agent in MCF-7 cells than ethyl acetate extract. An increasing apoptotic effect was observed with the highest extract concentration of the methanol extract (300 µg/ml) on MCF-7 cells (Table 5).

Apoptosis was more efficiently induced by ethyl acetate extract in Caco-2 cells compared to control cells and the difference was statistically significant. This effect showed a concentration-dependent increase and the apoptotic effect was found significantly higher in Caco-2 cells compared to MCF-7 cells (Table 5, Fig. 5). However, methanol extract induced moderate

apoptotic effect on Caco-2 cells when compared to ethyl acetate extract. The apoptotic effects on these cells were statistically significant at 200 and 300 µg/ml concentrations compared with controls.

Ethyl acetate extract induced moderate apoptosis in human lymphocytes and this effect was found statistically significant when compared to controls. Apoptotic effect of the extract on lymphocytes was comparable to the effect of anticancer drugs such as Farmorubicin and Campto. Methanol extract did not show significant apoptotic effect on human lymphocytes (Table 5, Fig. 6).

Mild effects of necrotic effects of the ethyl acetate and methanol extracts on MCF-7, Caco-2 cells, and lymphocytes were observed and the differences were not found statistically significant (Table 5).

Table 3. Antioxidant (DPPH scavenging) activity, H₂O₂ scavenging ve metal chelating activity of *Galium aparine* presented as percentage of ethyl acetate and methanol extracts

Concentrations	DPPH scavenging activity (%±SD)	H ₂ O ₂ scavenging activity (%±SD)	Metal chelating activity (%±SD)
100 µg/ml EAE	25.10± 0.015	---	---
200 µg/ml EAE	37.24±0.008	2.06±0.001	---
300 µg/ml EAE	52.30±0.001*	8.79±0.003	---
100 µg/ml ME	39.46±0.001	3.82±0.002	5.90±0.001
200 µg/ml ME	68.20±0.001*	5.15±0.002	7.34±0.003
300 µg/ml ME	97.70±0.003*	7.80±0.001	7.53±0.008
Rutin	95.80±0.000	---	---
Ascorbic acid	---	43.90 ± 0.005	---
EDTA (50 µg/ml) ^{xxx}	---	---	77.56± 0.330*

* $p < 0.05$; EAE: Ethyl acetate extract; ME: Methanol extract. Each value is the average of three analyses ± standard deviation

^x Standard in DPPH scavenging assay; ^{xx} Standard in H₂O₂ scavenging assay; ^{xxx} Standard in metal chelating assay

Table 4. Cytotoxicity activity of *Galium aparine* L. ethyl acetate and methanol against MCF-7 and Caco-2 Cell Line and human peripheral lymphocytes at different concentrations by MTT assay

Groups	Concentrations	Cytotoxicity (% ± SD)		
		MCF-7	Caco-2	Lymphocytes
Solvent Control (DMSO)	% 0.1	2.86±0.008	2.30±0.021	2.17±0.010
Farmorubicin	40 µg/ml	98.48±0.009*	---	19.38±0.065*
Campto	40 µg/ml	---	83.46±0.036*	27.93±0.040*
EAE	100 µg/ml	34.35±0.030*	56.69±0.011*	17.37±0.183*
	200 µg/ml	43.27±0.181*	61.42±0.016*	37.18±1.523*
	300 µg/ml	49.30±0.062*	68.50±0.012*	39.72±1.491*
ME	100 µg/ml	34.30±0.063*	22.05±0.041*	4.35±0.188
	200 µg/ml	55.67±0.131*	26.77±0.004*	5.73±0.396
	300 µg/ml	71.14±0.136*	31.50±0.078*	8.32±0.495

* $p < 0.05$; EAE: Ethyl acetate extract; ME: Methanol extract

Table 5. Apoptotic effect of *Galium aparine* L. ethyl acetate and methanol extracts on MCF-7 and Caco-2 cells

Groups	Concentrations	Apoptosis and necrosis (% \pm SD)								
		MCF-7			Caco-2			Lymphocytes		
		Apoptotic cells	Necrotic cells	Total cells	Apoptotic cells	Necrotic cells	Total cells	Apoptotic cells	Necrotic cells	Total cells
Control	---	0.33 \pm 0.58	0.67 \pm 0.57	3000	0.33 \pm 0.58	0.67 \pm 0.58	3000	0.33 \pm 0.58	0.00 \pm 0.00	3000
Solvent Control (DMSO)	% 0.1	1.33 \pm 0.16	1.67 \pm 0.57	3000	0.67 \pm 0.15	0.33 \pm 0.16	3000	1.67 \pm 0.21	0.67 \pm 0.57	3000
Farmorubicin	40 μ g/ml	92.66 \pm 3.21*	1.67 \pm 0.28	3000	---	---	3000	10.33 \pm 1.02	1.33 \pm 1.15	3000
Campto	40 μ g/ml	---	---	3000	43.00 \pm 1.36*	12.67 \pm 1.52	3000	20.33 \pm 1.52*	4.67 \pm 0.57	3000
EAE	100 μ g/ml	21.00 \pm 1.00*	3.67 \pm 1.15	3000	30.00 \pm 1.73*	2.67 \pm 0.58	3000	15.00 \pm 1.00*	2.00 \pm 1.00	3000
	200 μ g/ml	25.00 \pm 1.00*	5.00 \pm 2.00	3000	46.67 \pm 2.31*	4.00 \pm 1.00	3000	17.67 \pm 1.52*	3.67 \pm 0.58	3000
	300 μ g/ml	38.00 \pm 1.15*	7.00 \pm 1.00	3000	50.33 \pm 3.05*	5.33 \pm 0.58	3000	18.33 \pm 1.52*	3.33 \pm 1.15	3000
ME	100 μ g/ml	28.33 \pm 1.15*	2.67 \pm 0.58	3000	13.00 \pm 0.15	8.00 \pm 3.46	3000	4.66 \pm 1.52	0.67 \pm 1.15	3000
	200 μ g/ml	52.00 \pm 1.64*	3.67 \pm 1.52	3000	16.67 \pm 1.15*	6.33 \pm 0.58	3000	5.00 \pm 1.00	2.67 \pm 0.57	3000
	300 μ g/ml	68.68 \pm 1.52*	3.00 \pm 1.00	3000	18.33 \pm 1.52*	6.67 \pm 0.58	3000	7.00 \pm 1.00	1.67 \pm 0.57	3000

* $p < 0.05$

EAE: Ethyl acetate extract; ME: Methanol extract

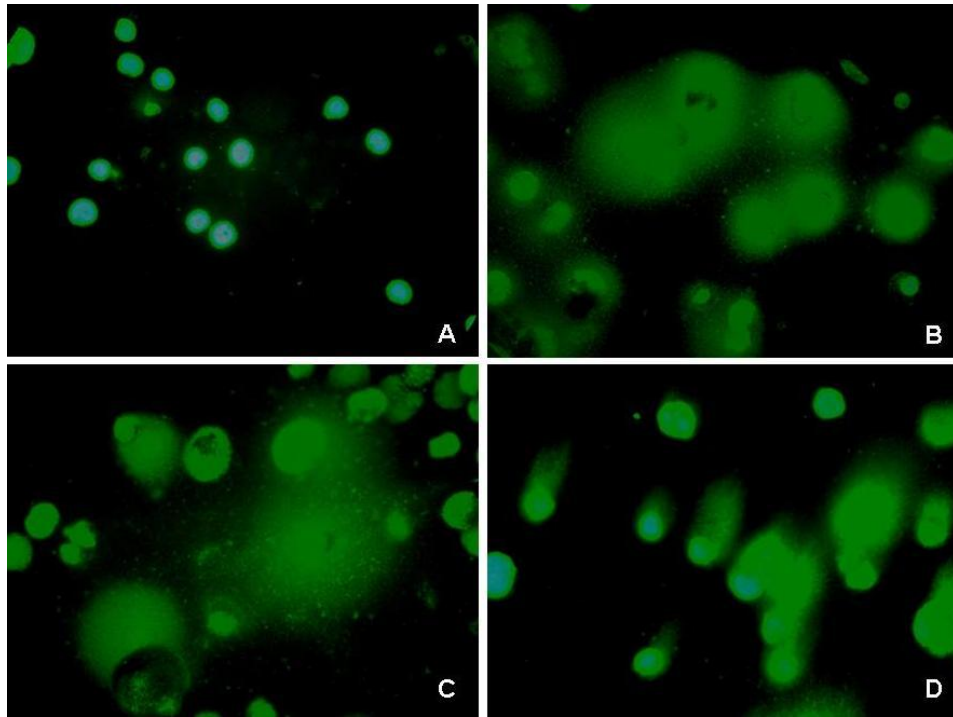


Fig. 4. A: MCF-7 control; B: 40 µg/ml Farmorubicin; C: 100 µg/ml ethyl acetate extract; D: 100 µg/ml methanol extract

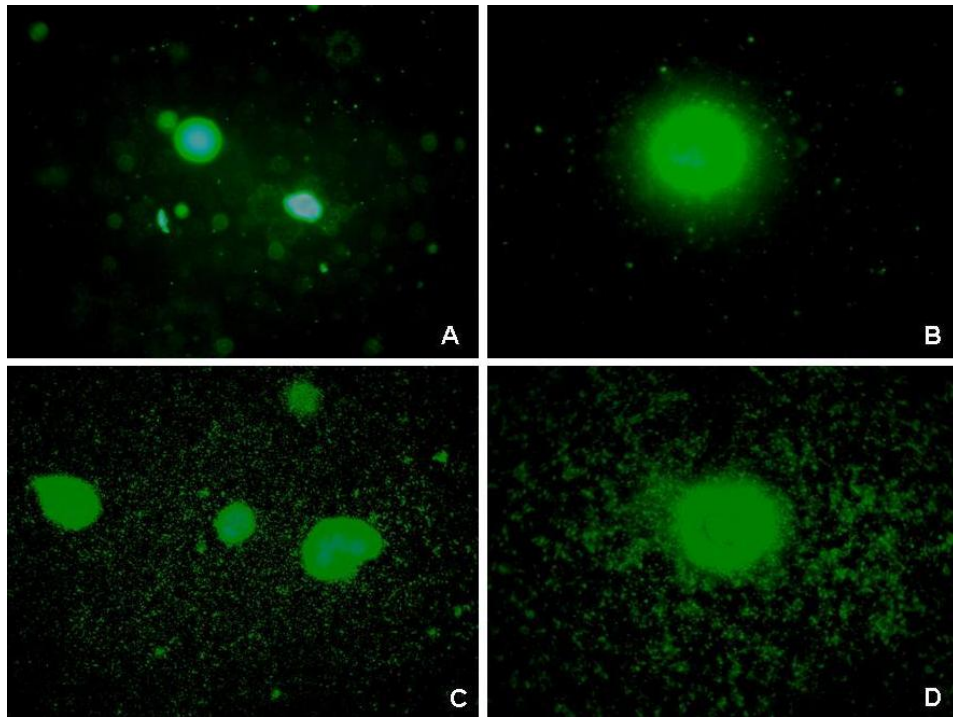
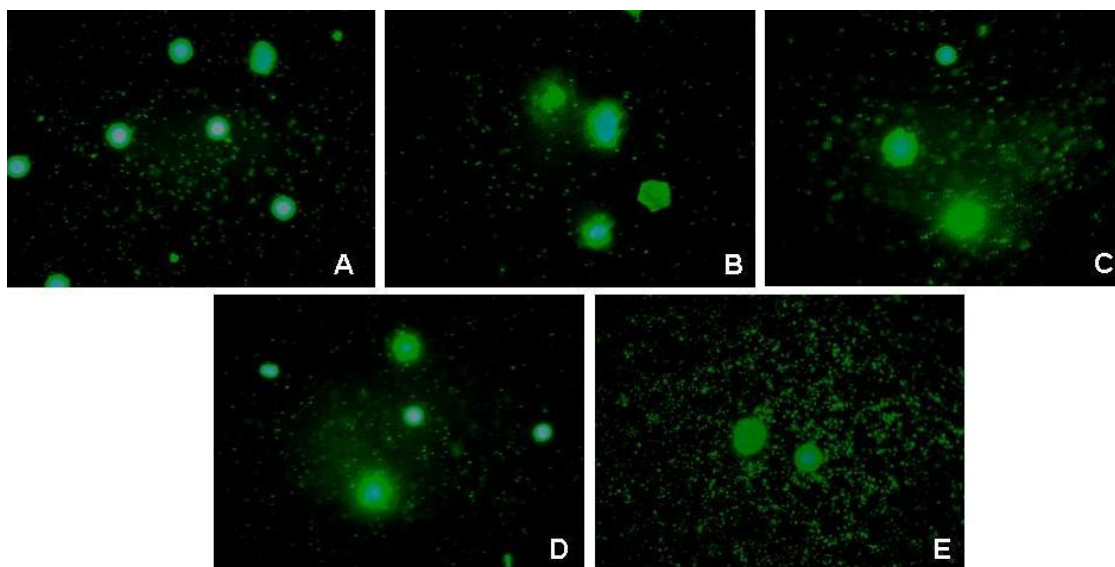


Fig. 5. A: Caco-2 control; B: 40 µg/ml Campto; C: 300 µg/ml ethyl acetate extract; D: 300 µg/ml methanol extract



**Fig. 6. A: Lymphocytes control; B: 40 µg/ml Farmorubicin; C: 40 µg/ml Campto
D: 200 µg/ml ethyl acetate extract; E: 200 µg/ml methanol extract**

4. DISCUSSION

Cancer is one of the most prominent diseases in humans. Because of the high death rate associated with cancer and because of serious side effects of chemotherapy and radiation therapy, many cancer patients seek alternative complementary treatment methods [32]. Natural products have played an important role in treating and preventing human diseases such as cancer, and these products have been derived from various source materials, especially medicinal plants. Hence, finding a new natural source with anticancer activities would aid in finding new tools for cancer therapy [33-35]. The importance of plants as antitumor agents with few side effects in modern medicine has been discussed in recent reviews and reports [36,37]. Hence, finding a new natural source with anticancer activities would aid in finding new tools for cancer therapy [33-35].

The chemical constituents of the plants or crude extracts are known to be biologically active ingredients. Some chemical constituents are considered as secondary metabolites components. They are directly responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer [38-40]. All these secondary metabolites showed antioxidant and antimicrobial properties through different mechanisms. Normally these secondary metabolites were isolated from the polar plant extract [41].

In this study, the phytochemical screening of ethyl acetate and methanol extracts from dried aerial parts of *Galium aparine* was performed. The results show that while phenols, tannins, alkaloids, anthroquinones, and saponins are present in the ethyl acetate extract and phenols, tannins and anthroquinones are present in the methanol extract. The results of HPLC analysis show the presence of 598.2 ppm flavonoid rutin in 1ml ethyl acetate extract and 597.7 ppm flavonoid rutin in 1ml methanol extract.

Flavonoids exhibited a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angiogenic, anticancer, and anti-allergic [42-45]. Flavonoids could not be determined in this study by phytochemical screening. Mean of ppm is "part per million by mass" and this unit is very small. Therefore, it is possible that the rutin component cannot be detected by phytochemical screening. Mean of ppm is "part per million by mass" and this unit is very small. Therefore, it is possible that the rutin component cannot be detected by phytochemical screening.

Saponins are other bioactive chemical constituents which are involved in plant disease resistance because of their antimicrobial activity [45]. Tannins are phenolic compounds and their derivatives are also considered as primary antioxidants or free radical scavengers [43]. These findings emphasize the importance of traditional knowledge in the use of plants for

medicinal purposes as well as for the pharmaceutical development. Several methods have been developed in order to measure the efficiency of antioxidants as pure compounds or in extract. These methods focus on different mechanisms of the antioxidant defense system that is scavenging active oxygen species and hydroxyl radicals, inhibiting lipid peroxidation, or chelating metal ions [46]. The total phenol contents of the crude extracts were reported as gallic acid equivalents. The Folin-Ciocalteu assay was employed to determine the total phenolic content in *Galium aparine* ethyl acetate and methanol extracts. Among two extracts, ethyl acetate extract contained the higher amount of phenolic compound ($50.00 \pm 0,036$ mg GAE/g) than the methanol extract ($41.00 \pm 0,280$ mg GAE/g).

In the present work, antioxidant activities of the extracts were evaluated by using the DPPH assay, H_2O_2 scavenging assay, and metal chelating assay. The results of the DPPH assay showed that there was a $97.70.19\% \pm 0.003\%$ antioxidant activity in the presence of $300 \mu\text{g/mL}$ *Galium aparine* methanol extract (Table 3). The results obtained in this investigation reveal that all the *Galium aparine* extracts are free radical scavengers and they can react with the DPPH radical, which might be attributed to their electron donating ability.

Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of the increase in the hydroxyl radicals in the cells. The H_2O_2 radical scavenging assay was also performed with the ethyl acetate and methanol extracts of the *Galium aparine*. Low H_2O_2 scavenging and metal chelating activities were recorded in *Galium aparine* ethyl acetate and methanol extracts (Table 3). Bokhari et al. [47] have also suggested that different fractions of methanol extract from *Galium aparine* possess antioxidant constituents, which can be utilized to scavenge or inhibit free radicals (DPPH, superoxide, hydroxyl and ABTS radicals). In addition, they have also suggested that there is a significant correlation between the radical scavenging activity and total phenolic content. However, there is no significant correlation between H_2O_2 scavenging activity and total phenolic content. Results of this study reveal that the phenolic compounds present in ethyl acetate and methanol extracts show high DPPH radical scavenging activity, but very low H_2O_2 scavenging and metal chelating activity.

Apoptosis or programmed cell death, a highly structured and orchestrated process, plays an important role in regulating the cell number for the growth and homeostasis of tissues by removing aged, damaged, and unwanted cells [48]. This self-destructive cellular process is critical for the organ development, tissue remodeling, immune regulation, and several disease conditions [49]. The homeostasis in eukaryotic cells is subjected to a delicate balance between survival and death signals originated from extracellular domain [50]. One of the salient characteristics of carcinogenesis is that the dividing tumor cells fail to initiate apoptosis following DNA damage [51]. Development of approaches that reinstall the apoptotic machinery selectively within tumor cells could be an effective measure of cancer control [52]. The primary mode of action of most anticancer drugs entails the induction of apoptosis in neoplastic cells. Likewise, a large number of natural agents have shown promising anticancer or cancer preventive properties by the induction of apoptotic pathways in transformed cells during the process of carcinogenesis [53-55].

In this present study, we focused on the antioxidant activity of *Galium aparine* ethyl acetate and methanol extracts in MCF-7 and Caco-2 cancer cell lines. Furthermore, the cytotoxic and apoptotic effects these extracts on MCF-7 and Caco-2 cancer cell lines were also examined. In this study, we demonstrated that *Galium aparine* ethyl acetate and methanol extracts had anticancer activity in two different human tumor cells such as MCF-7 and Caco-2 cancer cells by using MTT assay. The MTT assay results show that ethyl acetate and methanol extracts of *G. aparine* have cytotoxic and apoptotic effects on MCF-7 and Caco-2 cells. These effects are increased concentration dependently. The extent of apoptosis induction appeared to be greater on Caco-2 cancer cell line with ethyl acetate extract at $300 \mu\text{g/mL}$ compared to control cells (Table 5). Ethyl acetate extract had more cytotoxic and apoptotic effect on Caco-2 cells, while methanol extract had more cytotoxic and apoptotic effect on MCF-7 cells. Ethyl acetate extract also had significant cytotoxic and apoptotic effects on lymphocytes, which were used as control cells (Tables 4 and 5). The results suggest that *Galium aparine* extracts are capable of inhibiting cancer cell growth via apoptosis.

Reason of high cytotoxic effect of ethyl acetate extract on Caco-2 cells may be because of the

alkaloid and saponin content of the ethyl acetate extract, which was not found in methanol extract. Previous studies have reported that saponins from various plant materials can decrease the cell viability [56], suppress the proliferation of colon cancer cells and induce the apoptosis by modulating the signaling pathways [57] and regulating calpain-mediated glucose-regulated protein synthesis [58].

Many studies have suggested that the plant extracts have active phytochemicals with anticancer, oxidant, and apoptotic activities in cancer cells [58-63]. Phenolic compounds present in methanol extract may be more effective than ethyl acetate extract on MCF-7 cells and these phenolic compounds may act selectively on MCF-7. Therefore, the presence of phenolic and flavonoid compounds in the crude extracts is partly attributable to the anticancer activity of the crude extracts. Methanol extract and ethyl acetate extract of *Galium aparine* which contained phenols, tannins, alkaloids, anthroquinones, and saponins appeared to have anticancer activity in MCF-7 and Caco-2 cancer cell line (Tables 4 and 5). However, some other active anticancer constituents in the crude extracts may not have been identified under the current HPLC conditions. The identity of active phytochemicals responsible for the observed apoptosis-inducing effect is not known at the present time and additional studies are required to clarify this issue.

5. CONCLUSION

This is the first study conducted on contents of phytochemicals, and antioxidant, cytotoxic, and apoptotic effects of *Galium aparine* ethyl acetate and methanol extracts. Results of the present study demonstrate that both of the extracts have high total phenolic content and significantly high DPPH radical scavenging activity. Cytotoxic and apoptotic effects of the extracts show difference according to the type of the extract and the type of the cell. Although ethyl acetate showed more cytotoxic and apoptotic effects on Caco-2 cells compared to MCF-7 cells, cytotoxic and apoptotic effects methanol extract on MCF-7 are higher than on Caco-2 cells. Results of this study are important because these results can contribute to discover new compounds from plants, which can be used for breast and colon cancer treatment. However, further investigations are needed to identify the active components and establish the molecular mechanism of isolated compounds in each extracts in order to explain

their therapeutic efficacy in breast and colon cancer treatment.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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