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Galium aparine L. extract inhibits the proliferation of HT-29 cells and induces apoptosis

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ABSTRACT

Aims: *Galium Aparine L.* (*G. aparine*) has been traditionally used as a medicinal herb for the treatment of cancer, fever, hypertension. This study aimed to evaluate the potential anticancer effects of G. aparine in HT-29 colon cancer cells and to investigate the molecular mechanisms that may be involved in these effects.

Methods: The LC/Q-TOF/MS analysis was employed to conduct a qualitative phytochemical characterization of *G. aparine*. To evaluate the potential anticolorectal cancer effects of *G. aparine*, HT-29 cells were pretreated with *G. aparine*. Then, cell viability was examined by XTT assay, cell cycle distribution by flow cytometry and cell death by annexin V assay. In addition, the effect of *G. aparine* treatment on epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase- 1 (ERK-1), ERK-2, and apoptotic markers caspase-3 and Bcl-2-associated agonist of cell death (BAD) levels were investigated by ELISA method.

Results: Following treatment with *G. aparine*, HT-29 cells exhibit increased apoptosis and cell cycle inhibition at the G0/G1 phase, as evidenced by the Annexin V binding assay ($p<0.05$). Moreever, *G. aparine* treatment significantly decreased EGFR and ERK-1 levels ($p<0.05$), while the reduction in ERK-2 level was not statistically significant ($p>0.05$). The apoptotic impact of *G. aparine* was additionally verified through ELISA assays evaluating the levels of BAD and caspase 3 ($p<0.05$).

Conclusion: Taken together, the results demonstrate that *G. aparine* extract exerts significant anti-colorectal cancer effects by promoting apoptosis and inhibiting EGFR/ERK signaling.

Keywords: Colorectal cancer, Galium aparine L., apoptosis, epidermal growth factor receptor

INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer-associated cause of mortality and the third most prevalent malignant cancer worldwide [1]. The incidence of CRC has been steadily increasing in recent years and is estimated to more than double by 2035, representing a major burden to society. Although chemotherapy in combination with surgery or radiotherapy is widely applied in the treatment of colorectal cancer, unfortunately, the 5-year overall survival after diagnosis is only about 60% [2]. Moreover, side effects such as nausea, vomiting and immunosuppression are common, and treatment resistance and metastasis are almost inevitable. Therefore, there is an unmet medical need for the development of novel anti-colorectal cancer therapies.

Galium aparine L. (*G. aparine*) is a wild, annual herbaceous plant belonging to the Rubiaceae family. Anatolia is home to 101 species of the genus *Galium*, which is a worldwide genus with over 1000 species [3]. *Galium* species have historically been known as "yoghurt herbs" in Anatolia because of their use in milk coagulation for

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cheesemaking. *G. aparine* is utilized in traditional medicine for the treatment of lymph edema, gout, epilepsy, hypertension, and cancer. Scientifically, various bioactive compounds have been discovered in *Galium* plants, such as flavonoids, tannins, iridoids, anthraquinone and vitamin C [4]. Previous pharmacological research has shown that *Galium sp.* has antibacterial, antioxidant, hepatoprotective, anti-inflammatory, immunomodulatory and antiproliferative effects [4,5]. Moreover, Hurtwell reported that *Galium verum* has historically been employed throughout Europe and North America to treat malignant ulcers and breast cancer [6]. Although numerous in vitro studies have reported antiproliferative effects of *Galium* species in various cancers [5,7,8], attempts to address the underlying mechanisms have remained limited. In addition, there has been no study to date on the effect of *G. aparine* on CRC.

The epidermal growth factor receptor (EGFR) and its downstream signalling pathways play a role in the formation and development of many human tumours, in particular CRC [9,10]. Disruption of the regulation of the EGFR signaling pathway, which is involved in the growth and proliferation of normal cells, can cause neoplastic cell growth, proliferation, and metastasis. [11]. Overexpression of EGFR or overactivation of EGFR pathways has been detected in CRC [12]. Concomitantly, a number of studies have suggested a correlation between EGFR overexpression in cancer and tumour grade (poor differentiation) or reduced survival [13,14]. In addition, ERK is an essential mediator of many EGFR-mediated cellular processes. Notably, resistance to EGFR kinase inhibitors has been reported to be mediated by aberrant ERK activation [15]. Furthermore, by measuring single cell ERK dynamics in CRC organoids, EGFR was shown to amplify oncogenic MAPK signaling [16]. For this reason, blocking the EGFR and its biochemical pathway has been of great interest in recent years.

Therefore, this study aimed to evaluate the cytotoxic effect of *G. aparine* extract on the HT-29 colon epithelial tumor line and the underlying mechanisms that cause this effect. For this, cell viability was examined by XTT assay, cell cycle distribution by flow cytometry and cell death by annexin V assay. In addition, the effect of *G. aparine* treatment on EGFR, ERK-1, ERK-2, and apoptotic markers, caspase-3 and BAD levels, was investigated by the ELISA method.

METHODS

This is a cell line study. There is no need for approval from the ethics committee for studies involving cell lines.

Preparation of *G. Aparine* Extracts

G. aparine. plants (CUFH-Voucher specimen no: AA 7390) were obtained from the Cumhuriyet University Herbarium of the Department of Biology (Cumhuriyet University, Sivas, Turkey). *G. aparine* methanolic extraction was carried out as described previously [4]. In brief, after the dried G. aparine was ground using a hammer mill to a particle size that would pass through a 200 µm sieve, the powdered plant material (100 g) was extracted in 500 mL of 65% methanol. The resulting extract was then stored in a light-free environment at room temperature for a duration of 2 weeks. The mixture was subsequently subjected to vacuum filtration machinery in order to produce a clear extract of *G. aparine*. Finally, rotational evaporation enabled the elimination of the ethanol in the extract, resulting in a concentrated *G. aparine* extract. Using a similar technique previously published, LC/QTOF/MS conducted phytochemical analysis of *G. aparine* [4].

Cell Culture, Reagents, and Cell Viability

The HT-29 (HTB38, ATCC, USA) cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 (Thermo Fisher Scientific, Altrincham, UK) with 1% L-glutamine, 10% Fetal Bovine Serum (FBS), and 1% penicillin/streptomycin. The cells were kept at a temperature of 37°C in a humidified environment with 5% CO_2. Before the treatment, *G. aparine* was dissolved in a medium and stock solutions were prepared.

The XTT test (Roche Diagnostic, MA, USA) was used to determine cell viability. Before adding *G. aparine*, these cells were cultured at a concentration of 10×10^3 cells per well and incubated overnight. Before *G. aparine* was added, cells were seeded in a 96-well cell culture plate with the concentration adjusted to 10×10^3 cells per well and incubated overnight for attachment. Cells were subsequently treated with *G. aparine* for 24 h in a dose-dependent manner (12, 25, 50, 100 and 200 µg/ml). Following incubation, 50 µL of the XTT mixture was administered to each well. A microplate reader (Thermo Fisher Scientific, Altrincham, UK) was then used to measure absorbance at 450 nm. A level of 100% viability was set at the average optical density (OD) number in the control cells [17]. Half maximum inhibitor concentration (IC_{50}) values were calculated using Graph Prism 7 software (GraphPad, Software, Inc., USA).

Cell Cycle Analysis

The Muse Cell Cycle Assay Kit was employed to determine cell cycle arrest in accordance with the

manufacturer's instructions. The cells treated with *G. aparine* and the cells not treated with *G. aparine* were collected by centrifugation at 300×g for 5 minutes. They were then fixed in chilled 70% ethanol using mild vortexing after one wash using pre-cooled PBS. Later, the cells were washed with PBS and subsequently suspended in MuseTM Cell Cycle Reagent (Part No. 4700-1495). Subsequently, they were subjected to a period of light exposure at room temperature for a duration of 30 minutes, prior to conducting analysis. Finally, the Muse Cell Analyzer (Millipore) was employed to evaluate the G0/G1, S, and G2/M phases of the cell cycle [18].

Annexin V Binding Assay

HT-29 cells seeded in 6-well plates were harvested after 24 h incubation with *G. aparine* and then resuspended with PBS and mixed with equal volumes of Annexin V & Dead Cell reagent according to the manufacturer's instructions. Finally, live, dead, early, and late apoptotic cells were determined using the Muse Cell Analyzer [19].

EGFR, Erk-1, Erk-2, BAD and Caspase-3 Expression Analyses

The human ELISA kits of EGFR (BT Lab, catalog #E0313Hu), ERK-1 (BT Lab, catalog #E4263Hu), ERK-2 (BT Lab, catalog #E4264Hu), Bcl-2 antagonist of cell death (BAD) (BT Lab, catalog #E4102Hu) and caspase 3 (BTLab, catalog # E4804Hu) were used to examine in *G. aparine* and untreated HT-29 cells. The levels of EGFR, ERK-1, ERK-2, BAD, and caspase-3 in cell lysates were measured following the manufacturer's instructions.

Statistical Analysis

The results of the laboratory were expressed as mean±standard error. A one-way ANOVA followed by Tukey's post hoc test was used to evaluate the effects of different concentrations of *G. aparine* on cell viability. The results obtained from the EGFR, ERK-1, ERK-2, BAD, and caspase-3 levels tests were tested using an independent sample t test. $p < 0.05$ was determined as significant.

RESULTS

G. aparine Treatment Suppressed the Proliferation of HT-29 Cells

The phytochemical profile of *G. aparine* extract was characterized by LC-MS/MS analysis, and the identified compounds with their retention times and m/z (mass-to-charge ratio) values are presented in Table 1. Firstly, the inhibitory effect of *G. aparine* on cell proliferation was assessed in HT-29 cells. Figure 1 demonstrates that *G. aparine* treatment effectively inhibited the growth of HT-29 cells at all doses, in comparison to untreated cells ($p < 0.001$). The IC_{50} value of *G. aparine* in HT-29 cells was determined to be 21.51 µg/mL for 24 h.

Table 1. *Phytochemical compounds identified in methanolic extract of G. aparine by LC-Q-TOF-MS.*

Peak No	Retention Time (min)	Molecular Formula	m/z	Compound
1	1.733	$C_7H_{12}O_6$	191,0555	Quinic acid
2	2.135	CHO	147,0287	Citramalic acid
3	3.206	$C_{13}H_{16}O_{10}$	331,0672	1-O-Galloyl-beta-D-glucose
4	3.844	$C_{10}H_8O_4$	193,0505	5,7-Dihydroxy-4-Methylcoumarin
5	3.875	$C_{16}H_{22}O_{11}$	389,1077	Monotropein
6	7.356	$C_{16}H_{18}O_9$	353,0881	Chlorogenic acid
8	11.238	$C_{16}H_{18}O_9$	353,0871	Chlorogenic acid isomer
9	12.309	$C_{33}H_{40}O_{21}$	771,1986	Quercetin Derivative
10	14.384	$C_{16}H_{18}O_8$	337,0933	p-coumaroyl quinic acid
11	19.805	$C_{27}H_{30}O_{16}$	609,1466	Rutin
12	25.427	$C_{15}H_{10}O_6$	285,0405	Luteolin
13	25.762	$C_{11}H_{12}O_4$	207,0659	Dimethylcaffeic acid
14	26.601	$C_{15}H_{10}O_5$	271,0599	Apigenin

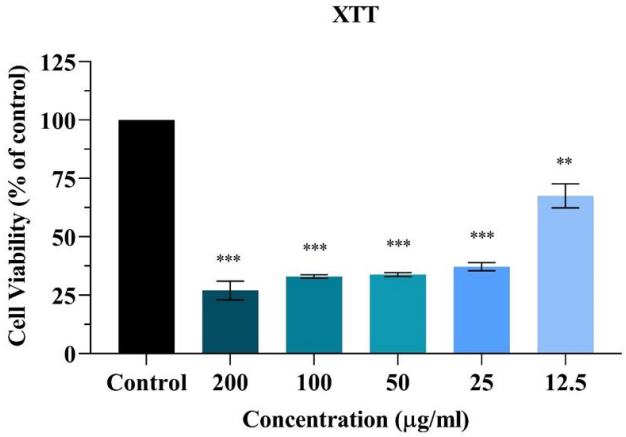


Figure 1. *The effect of G. aparine on HT-29 cell viability. The data are given as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to the control group.*

G. Aparine Induced G0/G1 Phase Cell Cycle Arrest in HT-29 Cells

The next step was to conduct the cell cycle experiment on HT-29 cells to determine whether *G. aparine* had any influence on cell cycle arrest. As demonstrated in Figure 2, HT-29 cells were able to be arrested in the G0/G1 phase after being treated with *G. aparine* for 24 h.

G. Aparine Treatment Caused Apoptosis of HT-29 Cells

The Annexin V binding assay demonstrated that compared to control cells, the proportion of early and late apoptotic cells was significantly increased by *G. aparine* treatment (Figure 3). In comparison with the untreated cells, both the percentage of early apoptotic cell population (0.67 ± 1.21%) and the percentage of late apoptotic cell population (1.89 ± 1.43%) were higher in

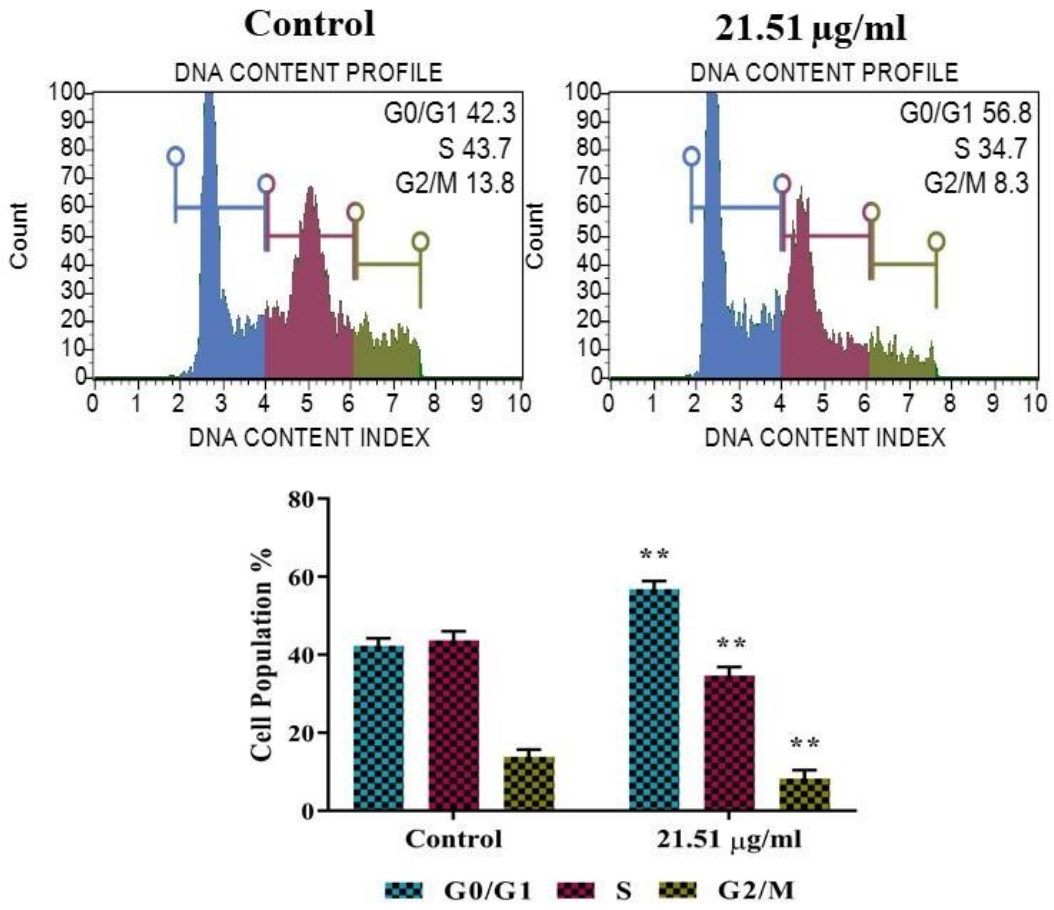


Figure 2. *The effect of G. aparine on cell cycle progression in HT-29 cells. Each experiment was performed three times. The data are given as mean ± SEM. **p < 0.01 as compared to the control group. The percentage of cells in the G0/G1 phase in the control group was 42.3 ± 1.9%, but in the GA-treated groups, it reached to 56.8 ± 2.1%. (p < 0.01). G. aparine treatment also decreased the G2/M (from 13.8 ± 1.9% to 8.3 ± 2.1%) and S (from 43.7 ± 2.3% to 34.7 ± 2.2%) population in G. aparine -treated cells compared to the control cells (p < 0.01).*

G. aparine -treated cells (8.75 ± 2.12% and 20.51 ± 1.22%, respectively) ($p < 0.01$). The ELISA assay was employed to further confirm the induction of apoptosis by *G. aparine* treatment through the evaluation of pro-

respectively) ($p < 0.05$). Similarly, *G. aparine* treatment also led to a decrease in ERK-2 levels, but this reduction did not reach statistical significance (from 1258.87 ± 42.80 to 1073.54 ± 15.75) ($p > 0.05$).

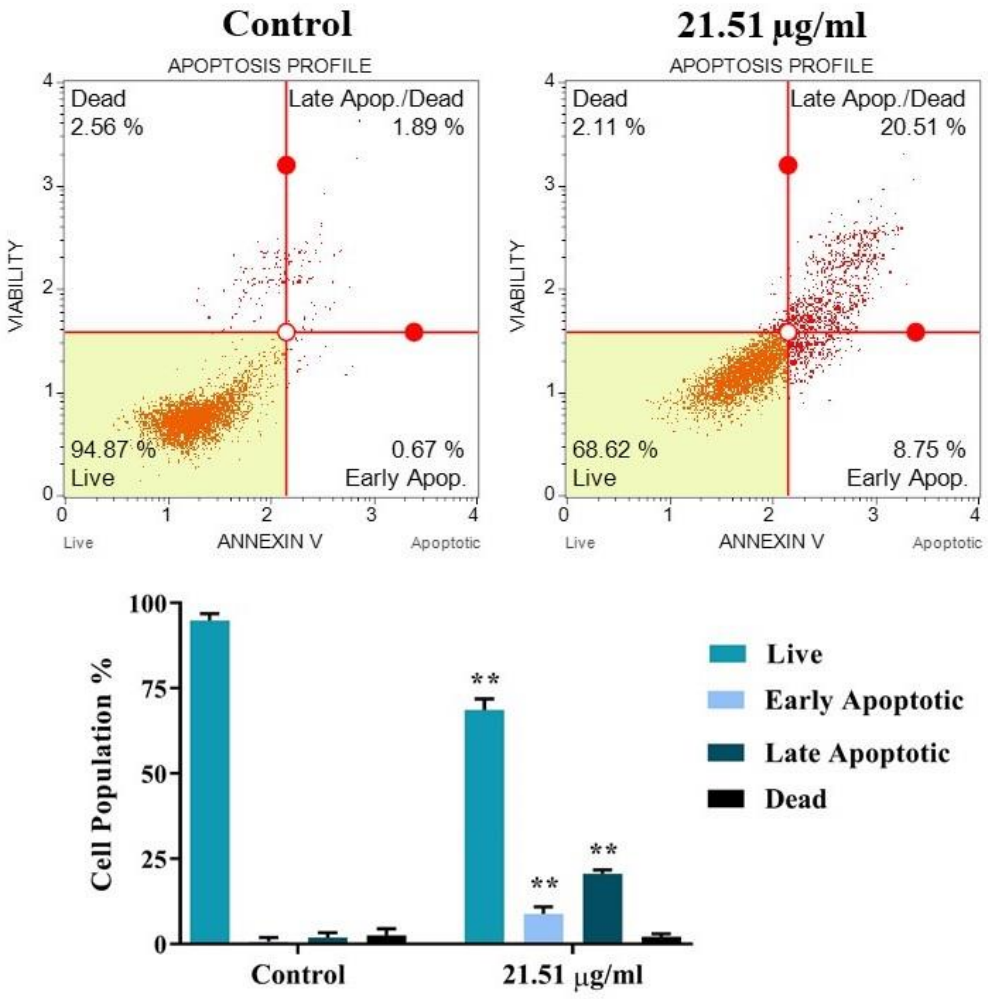


Figure 3. *G. aparine induced apoptosis of HT-29 cells as determined by Annexin V staining. The data are given as mean ± SEM. **p <0.01 vs. untreated HT-29 cells and G. aparine-treated groups*

apoptotic proteins, including caspase 3 and BAD, in HT-29 cells (Figure 4). As presented in Figure 4, treatment with GA for 24 h markedly increased BAD expression (from 493.75 ± 7.15 to 885.65 ± 4.40) and caspase 3 expression (from 4.47 ± 0.09 to 10.21 ± 0.01; $p < 0.01$).

The Effect of *G. Aparine* on EGFR, ERK-1, and ERK-2 Levels in HT-29 Cells

EGFR, ERK-1 and ERK-2 ELISA kits were used to evaluate the effect of *G. aparine* on their levels in HT-29 cells (Figure 5). As shown in Figure 5, both EGFR and ERK-1 levels decreased in the *G. aparine*-treatment group compared to the control group (from 58.81 ± 2.74 to 46.07 ± 0.44; from 2087.5 ± 8.15 to 1464.43 ± 105.17,

DISCUSSION

This study examined the anti-cancer effects of *G. aparine* MeOH extract and its mechanisms of action in HT-29, a common colon cancer cell line. Even though *G. aparine* has been reported to be the focus of numerous anticancer studies, as far as we know, no research has been undertaken on HT-29 cells. Our investigation demonstrated that *G. aparine* inhibited cell growth in HT-29 cells by causing cell cycle arrest. Furthermore, *G. aparine* induced apoptosis and decreased EGFR, ERK-1, and ERK-2 levels in HT-29 cells.

As with many other cancers, the standard treatment of CRC usually involves cytotoxic drugs, which unfortunately have strong limitations due to side effects and resistance to chemotherapy. Additionally, it is induced cell cycle arrest in G1 phase [7]. Consistently, in the current research, we found that *G. aparine* treatment suppressed the proliferation of HT-29 cells in a dose-dependent manner, with an IC_{50} of 21.51 µg/mL. Here,

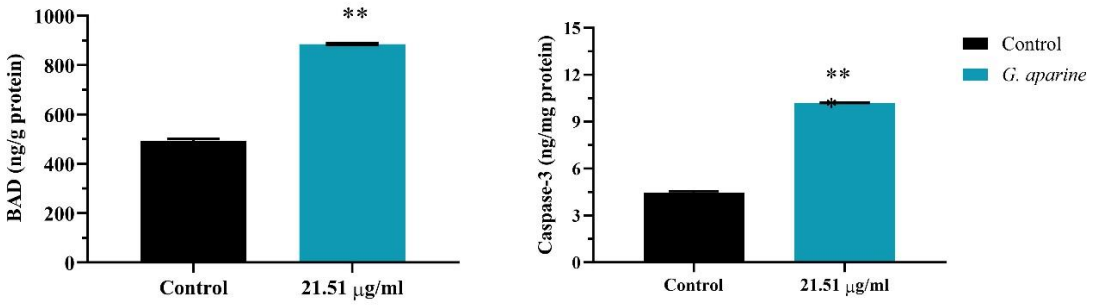


Figure 4. *Effect of G. aparine treatment on apoptotic protein expression in HT-29 cells after 24 h, as determined by ELISA. BAD and caspase 3 protein levels were significantly increased following G. aparine treatment. Values are presented as mean ± SEM. **p < 0.01 versus control group.*

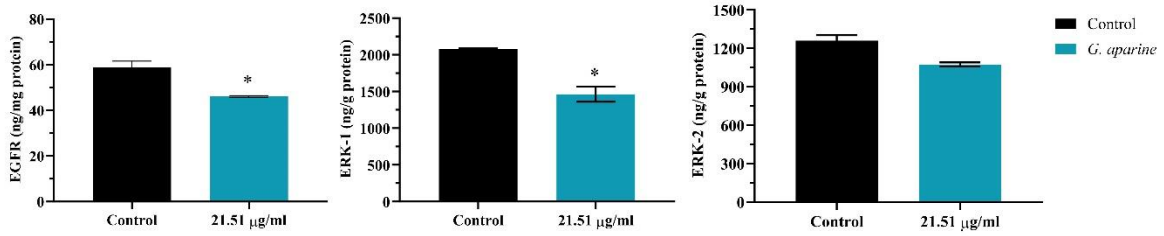


Figure 5. *The effect of G. aparine on EGFR, ERK-1, and ERK-2 levels of HT-29 cells. The data are given as mean ± SEM. *p < 0.05 as compared to the control group.*

important to consider the financial impact that an increase in the incidence of CRC will have on society [20]. These highlight the need for alternative treatment approaches.

Phytotherapy, defined as the use of plants or a mixture of plant extracts in the treatment of diseases, has been widely used since ancient times. There is currently a great interest in the potential efficacy of medicinal plants against various types of cancer, including CRC [21]. Both in vitro and in vivo studies have shown that medicinal plants have anticolorectal cancer activity through molecules that induce cell cycle arrest and apoptosis [22,23].

The anticancer effect of *G. aparine* has been previously investigated in several types of cancer [7,24,25]. Schmidt et al. reported that a significant inhibition of the motility of head and neck cancer cells occurred when treated with an aqueous extract of Galium verum [24]. Also, Atmaca et al. showed that *G. aparine* methanol extract was cytotoxic in MDA-MB-231 and MCF-7 human breast cancer cells and we also found that *G. aparine* treatment in HT-29 cells resulted in cell cycle arrest at the G0/G1 phase. In the same study, Atmaca et al. also reported that *G. aparine* treatment induced apoptosis and necrosis in breast cancer cells. Again, we showed that the administration of *G. aparine* in the HT-29 cells resulted in the induction of apoptosis by enhancing the binding of Annexin V. In the current study, increased levels of the pro-apoptotic proteins BAD and caspase-3 were observed, supporting that *G. aparine* treatment promotes apoptosis in HT-29 cells. Previous studies have reported that plant extracts with anti-colorectal cancer properties induce apoptosis in cancer cells. Hence, our finding that treating HT-29 with *G. aparine* increased the levels of BAD and caspase-3 is in agreement with previous research observing that the anti-colorectal cancer activity of phytochemical molecules is associated with the induction of caspase-dependent apoptosis [26,27].

Accumulating evidence suggests that the EGFR and its related signaling pathways have a crucial role in the

growth and progression of CRC [28]. In addition, approximately 25-75% of patients with CRC have elevated EGFR levels, and there is a positive correlation between overexpression of EGFR and a poor prognosis [29]. For this reason, down-regulation of EGFR and other proteins in its downstream pathways represents an important approach in terms of anti-cancer effect. Currently, clinical trials targeting EGFR signalling include anti-EGFR monoclonal antibodies (e.g. ABX-EGF and cetuximab) and intrinsic tyrosine kinase inhibitors (e.g. erlotinib and gefitinib) [30]. The current investigation revealed that *G. aparine* treatment resulted in a reduction of EGFR, ERK-1, and ERK-2 levels in HT-29 cells. Our results are consistent with those of the other researcher who reported a significant decrease in ERK-1 and ERK-2 protein levels in MCF-7 and MDA-MB-231 cells exposed to *G. aparine* extract [25]. These observations support the premise that targeting and blocking EGFR can suppress cancer growth by inducing apoptosis.

The anti-colorectal cancer activity of *G. aparine* extract may be related to its phytochemicals such as quercetin, luteolin, apigenin and chlorogenic acid. Previous studies have reported that apigenin induces apoptosis by modulating the levels of caspases, BAX, Bcl-2, etc. in cancer cells [31], arrests the cell cycle at the G0/G1 checkpoint [32] and regulates the NF-κB/MAPK/ERK pathway in relation to the anticancer mechanism [33]. Bhatiya et al. showed that the administration of quercetin limited the growth of colon cancer cells via modulating the production of anti-aging proteins [34]. Similarly, Zhang et al. showed that quercetin promoted apoptosis through up-regulation of Bax in human colon cancer SW-620and CACO-2 cells [35]. Yoo and colleagues reported the anti-cancer activity of luteolin, a natural flavonoid, against colon tumours via autophagy and apoptosis [36]. Another flavonoid, chlorogenic acid, induced cell cycle arrest and apoptosis in HT-29 cells [37]. Overall, these observations provide a theoretical framework for the anti-colorectal effect of *G. aparine*, abundant in the aforementioned flavonoids, in the present study.

CONCLUSION

Overall, the present study reveals that administration of *G. aparine* methanol extract at a dosage of 21.51 µg/mL has anti-colorectal potential by inducing cell cycle arrest, suppression of EGFR, ERK-1 and ERK-2 expression, and eventually apoptosis in HT-29 cells. Our results suggest that *G. aparine* shows promise as an effective anticancer drug for treating CRC. In addition, in vivo investigations are needed to better understand the bioavailability and pharmacodynamic properties of *G. aparine* and its use as an integrated candidate for CRC.

ETHICAL DECLARATIONS

Ethics Committee Approval

Because the study doesn't contain any human or animal samples, Ethics Committee Approval is not applicable.

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Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Financial Disclosure

The authors declared that this study has received no financial support.

Referee Evaluation Process

Externally peer-reviewed.

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