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Cytotoxicity assay of Türkiye's rare endemic *Helianthemum germanicopolitanum* Bornm. plant extract on HT-29 cell line

Türkiye'nin nadir endemiği Helianthemum germanicopolitanum Bornm. bitki ekstraktının HT-29 hücre hattı üzerindeki sitotoksisite analizi

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ABSTRACT

Aim: In this study, the cytotoxic potential of the endemic *Helianthemum germanicopolitanum* Bornm. plant against colon cancer is investigated.

Materials and Methods: This study pioneers the investigation of medicinal applications of the *H. germanicopolitanum* plant, specifically targeting the HT-29 human colon cancer cell line. The phytochemical profile of the aerial parts of the plant, especially the flavonoid content, was analyzed using High Performance Liquid Chromatography (HPLC). Cytotoxic effects were then evaluated by WST-1 assays on the HT-29 cell line; this revealed time- and dose-dependent inhibition of cancer cell growth.

Results: These results also highlight the need for comprehensive research into *H. germanicopolitanum*'s unique flavonoid composition and its broader implications in cancer treatment.

Conclusion: These results also highlight the need for comprehensive research into *H. germaicopolitanum*'s unique flavonoid composition and its broader effects in cancer treatment.

Keywords: *Helianthemum germanicopolitanum*, colon cancer, HT-29 cell line, flavonoids, cytotoxicity.

ÖZ

Amaç: *Bu çalışmada endemik Helianthemum germanicopolitanum Bornm. bitkisinin kolon kanserine karşı sitotoksik potansiyeli araştırılmıştır.*

Gereç ve Yöntem: *Bu çalışma, özellikle HT-29 insan kolon kanseri hücre hattını hedef alarak H. germanicopolitanum bitkisinin tıbbi uygulamalarının araştırılmasına öncülük etmektedir. Bitkinin topraküstü kısımlarının fitokimyasal profili, özellikle de flavonoid içeriği Yüksek Performanslı Sıvı Kromatografisi (HPLC) kullanılarak analiz edilmiştir. Sitotoksik etkiler daha sonra HT-29 hücre hattı üzerinde WST-1 deneyleri ile değerlendirilmiştir; bu, kanser hücresi büyümesinin zamana ve doza bağlı inhibisyonunu ortaya çıkarmıştır.*

Bulgular: *Flavonoid profillerinin ve sitotoksisitenin karşılaştırmalı analizleri, benzer türler üzerindeki mevcut literatüre göre yapılmıştır. Bu çalışmanın bulguları, H. germanicopolitanum'un kolon kanseri tedavisinde terapötik uygulamaları olan biyoaktif bileşiklerin kaynağı olarak tıbbi açıdan potansiyelinin belirlenmesine ışık tutmaktadır.*

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***Sonuç**: Bu sonuçlar ayrıca, H. germanicopolitanum bitkisinin eşsiz flavonoid bileşimi ve kanser tedavisindeki daha geniş etkileri hakkında kapsamlı araştırmalara duyulan ihtiyacı vurgulamaktadır.*

***Anahtar Sözcükler:** Helianthemum germanicopolitanum, kolon kanseri, HT-29 hücre hattı, flavonoidler, sitotoksisite.*

INTRODUCTION

Cancer is the cause of one in six deaths worldwide in 2018 (1). The last parts of the large intestine other than the rectum is called the colon, and its cancer is called colon cancer, and all cancers of the large intestine are called colorectal cancer (2). Colorectal cancer is the third most common type of cancer worldwide and the second leading cause of cancer-related deaths worldwide, accounting for approximately 10% of all cancer cases (3). Incidence and mortality rates in colon cancer show large geographical differences, with the highest incidence rates in Europe, Australia, and New Zealand, while the highest mortality rates were observed in Eastern Europe (3).

The relationship between humans and plants dates to 1.2 million years ago (4). Considering fossil records, primitive man's use of plants as medicine for treating diseases dates back at least 60,000 years ago (5). Medicinal plants are used in the treatment of various diseases around the world (6).

Plants are very important natural treasures in traditional medicine thanks to the phytochemicals in their composition (7). Medicinal plants, which have important roles in the fight against cancer, contain secondary metabolites such as alkaloids, tannins, flavonoids, pigments, and terpenoids, which do not have an active role in their growth (1). These compounds show anticancer properties by causing DNA repair, suppressing cancer-inducing enzymes, increasing immunity, producing anticancer enzymes, and inducing antioxidant activity (8).

Türkiye is a very rich country in terms of endemic plant species, and one-third of approximately 9,000 medicinal and aromatic plants are endemic (9). It is known that some species of *Helianthemum* Adans., which are widespread in our country, have anti-constipation and astringent effects, and in some countries, species of this genus are also used as anti-inflammatory, antiulcerogenic, wound healing, antimicrobial, cytotoxic and antidiabetic agents (10, 11). In addition, some *Helianthemum* species contain phytochemicals of high medical importance and rich antioxidants, confirming their use in the treatment of various human diseases (12, 13).

Türkiye is home to 4 genera (*Cistus*, *Fumana*, *Tuberaria*, *Helianthemum*) and 37 taxa of Cistaceae, of which about ∼19% are endemic. *Helianthemum* is represented by 19 taxa in Turkey, 4 of which are endemic (14), giving an endemism rate of 21, which ranks first in Turkish Cistaceae species. *Helianthemum germanicopolitanum* Bornm., whose vernacular name is 'özgegüngülü' (14), is a rare endemic plant native to Çankırı province and grows in gypsum/marly areas (15). There are studies in the literature emphasizing the medical importance of *H. germanicopolitanum*, which is endemic in Türkiye, in diseases such as diuretics, constipation, and hemorrhoids (16, 17, 18).

Helianthemum germanicopolitanum, which is less popular because its growing area is limited to Çankırı province (Turkey), is an endangered plant. The fact that studies revealing the effects of *H. germanicopolitanum*, whose medical importance has been emphasized in several studies, on any cancer are not included in the literature causes our research to gain momentum. Therefore, this study aimed to investigate the cytotoxic effect of the plant by determining the active compounds of the aerial parts of endemic *H. germanicopolitanum*, which may be useful in the treatment of colon cancer. Local medical sources use the plant material as an exploration to cure various diseases and situations (etc. scar regeneration and replenishment), transforming such plants from nature to modern medicine needs dosing with toxicity tests then *in vitro* and *in vivo* respectively. In this study, we investigated the first step to understand the potential of the plant for medical use.

Local sources, observations, and limited literature reported the anorectal usage of this plant material. To support this claim, we started our study with the human colon cancer cell line of HT-29. This cell line may mimic neovascularization, hemorrhoids, and anal fissures. Such disorders can be classified as gastrointestinal tract invasions. Characteristics of

HT-29 may be implemented from the cecum to the anus as one. According to our current knowledge, this study is the first to investigate colorectal medical aspects of specimen usage.

MATERIALS and METHODS

Plant Material

Helianthemum germanicopolitanum is a suffruticose, perennial herb, with erect flowering stems up to 30 cm tall (Figure-1a). The leaves are elliptic or oblong, and stellate-tomentose. The inflorescence is branched or simple, and laxly 3-6 flowered. Corolla is yellow. Flowering time is in June (19). The habitat of the species is gypsum-rich soils (Figure-1b).

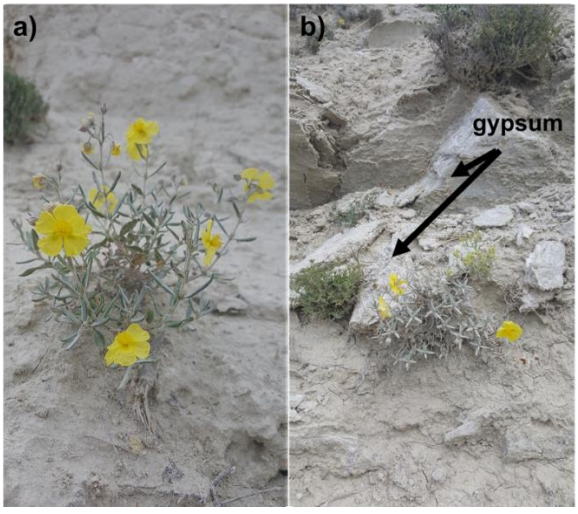


Figure-1. (a) *Helianthemum germanicopolitanum* habit (from Çankırı province), (b) Gypsum habitat where the plants grow (Photos by A. Kayabaş Avşar)

H. germanicopolitanum was collected from gypsum habitats on Çankırı-Korgun road (Coordinates: 40°38’55.1"N, 33°36’29.7"E, Çankırı, Turkey) in May-June 2021. Samples were identified taxonomically according to Flora of Turkey and the East Aegean Islands (19). The identification of plant species was made by the author (A. Kayabaş Avşar).

The whole plant samples were taken individually from their natural habitat, cleaned of soil and dirt using a fine paintbrush or by gently blowing on the sample followed by a wash with distilled water.

Plant Extraction

The aerial parts of the *H. germanicopolitanum* were used in the study. The roots of this perennial plant were not included in the analysis because they contain large amounts of cellulose due to their woody structure. Since the plant is an endemic and rare species, all aerial parts were analyzed as a single piece, considering the protection of the flora in nature. To increase the surface area before extraction and thus increase the extraction efficiency, the plant material was dried and made as homogeneous and small-sized particles as possible.

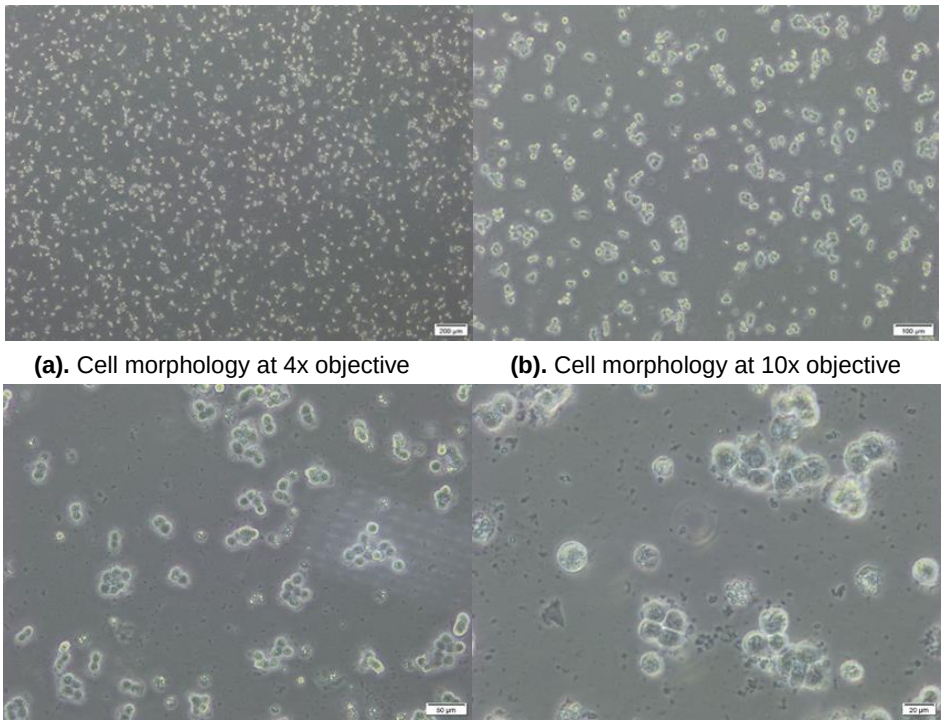
The extraction efficiency depends on how much and how long the plant material is in contact with the solvent and the choice of the appropriate solvent for extraction. Extraction time and temperature are important parameters during the extraction of plant particles with increased surface area. As extraction time, 20 g of plant material and 400 ml of methanol were extracted at 50^{o}C using the soxhlet device the first siphon was completed in the 20th minute and the next 4 siphons were completed at 15-minute intervals and the extraction process was carried out at the most appropriate time and temperature. After the extraction process was finished, the solvent was removed from the rotary evaporator system. The extract obtained was powdered with the help of a lyophilizer device and made ready for the next experimental procedures (20).

High Purity Liquid Chromatography (HPLC) Analysis

HPLC-DAD analysis was performed with a Thermo Ultimate 3000 HPLC (Thermo Scientific, Dionex, Bremen, Germany). The system components consisted of a pump, an autosampler, a column oven, and a diode array detector. An ODS RP C18 column (250 x 4.6 mm, 5 µm, Thermo Scientific, Bremen, Germany) was used for all separations. Liquid chromatography separations were performed using the following solvents: A-Ultrapure water: H_3PO_4 (0.2%) and B-acetonitrile. Before the last injection, the column was equilibrated for 3 min at initial conditions. The flow rate was 0.8 mL/min, and the column temperature was set at 30 °C. Detection was performed at 205 nm and the UV spectra of all samples were scanned between 190-400 nm (21).

Cell Culture and Treatment

HT-29 cells, a colon cancer cell line, were obtained from Ege University Faculty of Medicine, Department of Histology and Embryology. Dulbecco’s Modified Eagle’s Medium (DMEM) and Fetal Bovine Serum (FBS) (Gibco, USA) were purchased. Culture conditions are validated morphologically as seen in the Figure-2.



(a). Cell morphology at 4x objective **(b).** Cell morphology at 10x objective

(c). Cell morphology at 20x objective **(d).** Cell morphology at 40x objective

Figure-2. Cell morphology of HT-29 on culture conditions on 4x (a), 10x (b), 20x (c) and 40x (d) respectively.

HT-29 cholangiocarcinoma cells frozen at P/17 were thawed and plated with Dulbecco's Modified Eagle's medium prepared by adding 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin and 1% L-Glutamine and grown at 37°C in an incubator with 5% CO_2. Cells were seeded in 25 cm^2 cell culture flasks for 24 hours. Afterward, the cells were passaged and seeded into 75 cm^2 flasks.

Colon cancer cells were divided into control groups and dose-treated groups. HT-29 cells in all groups were grown in a complete medium. HT-29 cells were treated with *H. germanicopolitanum* doses (0, 5, 10, 50, 100 and 1000 µg/ml) for 24, 48 and 72 hours respectively and stored in the incubator. Cell cytotoxicity was evaluated by WST-1 assay in 96-well plates.

Cell Proliferation Assay

Cell proliferation experiments were performed. HT-29 colon cancer cells removed after passaging were seeded in 96 well plates at $8x10^3$ cells per well. In this study, cell cytotoxicity was measured with the Water-Soluble Tetrazolium-1 WST-1 Assay Kit (Cell Proliferation) (ab65475, Abcam, USA) kit according to the protocol determined by the manufacturer. The experiment was started by incubating the cells for 24 hours and allowing them to adhere to 96 well plates. When the cells reached 90% density, 10 µl WST-1 reagent was added to the cells in each 96 well according to the WST-1 kit (Assay Kit (Cell Proliferation) (ab65475, Abcam, USA) procedure, and the cells were incubated for 1 hour, the *H. germanicopolitanum* concentrations we prepared were added to the wells and left for 24, 48 and 72 hours of incubation. At 490 nm wavelength, readings were taken in 3 replicates (22).

As explained above, approximately $8x10^3$ HT-29 cells/mL were seeded in triplicate and 10 µl of WST-1 reagent was added. The cells were then incubated in the incubator for 1 hour and the absorbance at 490-520 nm was measured every 30 minutes.

RESULTS

To obtain cytotoxicity results we first investigated the HPLC profile of the plant and compared it with the literature, after that toxicity assay was investigated.

HPLC

The High-Performance Liquid Chromatography (HPLC) analysis of the *H. germanicopolitanum* extract has provided insightful findings,

demonstrating the existence of a diverse array of flavonoids (Figure-3). This conclusion is drawn from the distinctive peaks discerned in the analysis results. However, to gain a comprehensive understanding and ascertain the precise composition, a meticulous and in-depth investigation becomes imperative. Identifying the specific types of flavonoids within the extract requires a detailed examination, ensuring accuracy in characterizing the phytochemical profile of this plant.

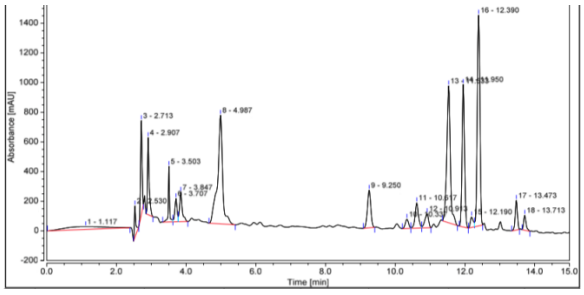


Figure-3. HPLC profile of *Helianthemum germanicopolitanum* extract.

Cytotoxicity

In the study, WST-1 analysis was performed to show the cytotoxic effects of the plant on the HT29 cell line, and when the results obtained were interpreted, it was found that the substance showed a time/dose-dependent effect. Increasing the concentration or prolonging the exposure time shows a more severe inhibition of cell growth. As shown in Figure-4 and Figure-5, the IC50 values of the substance were determined as 26.45, 37.81, and 36.21 µg/ml after 24, 48 and 72 hours of exposure.

Cell viability was determined by WST-1 analysis on the HT-29 cell line. At the end of the exposure period, it was observed that the plant showed an anti-proliferative effect on the cell line depending on time and dose. As a result of the analyses performed in previous studies Kılıç et al. evaluated the extracts of *H. ledifolium* varieties growing naturally in Turkey in terms of *in vitro* anti-trichomoniasis activity against *Trichomonas vaginalis* and reported that these extracts inhibited the proliferation of *T. vaginalis* dose-dependently from the 4th hour (23). In this study, the IC50 value of *H. glomeratum* was reported as 62.92 µg/mL (23), in their study with *H. oelandicum*, Ağca et al. investigated the inhibition activities of ethanol and aqueous solutions of plant extracts against α glucosidase for *in vitro* hypoglycemic activity determination and found IC50 values of 2.52±0.01 and 3.21±0.01 µg ml^{-1}, respectively (10). When these values were compared with the standard compound acarbose with IC50 value of 0.90±0.01 µg ml^{-1}, strong inhibition activities were found. As a result of all these evaluations, it was stated that the antioxidant, anti-inflammatory and hypoglycemic activity of the ethanol extract was higher than the aqueous extract (10), in other study investigating the wound healing ability of *H. canum*, Küpeli Akkol et al. reported that *H. canum* extract significantly reduced cell viability at doses higher than 156 µg/mL and had no toxic effect at low doses, but according to SRB assay results, it was toxic at high doses. In the RTCA test results, the IC50 value of *H. canum* extract at 24 hours was determined as 2.7 mg/mL (11). The results obtained from plants in this study are like previous studies / not like previous studies.

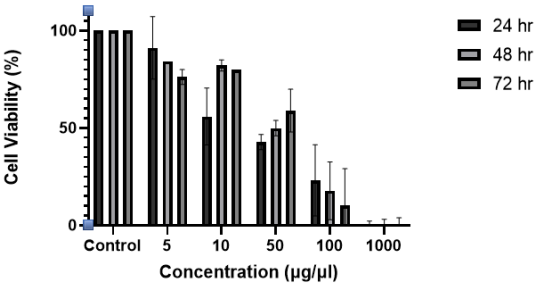


Figure-4. Cell proliferation measurements by WST-1 colorimetric method. Viability of HT-29 cells after 24, 48 and 72 hours of exposure to different concentrations.

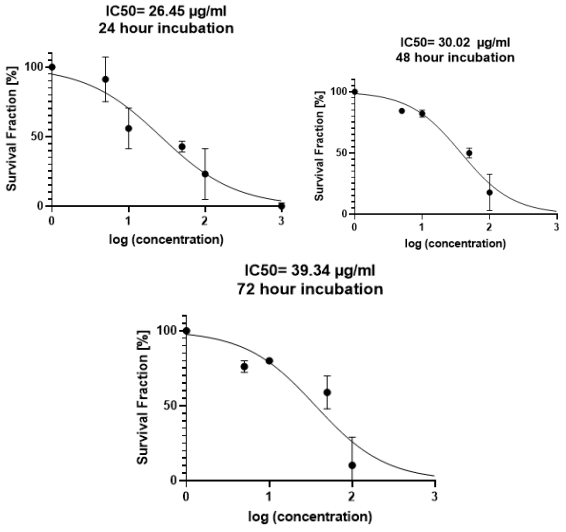


Figure-5. IC50 values were plotted in the GraphPad Prism program based on the data obtained from WST-1 analysis. HT-29 cell lines were exposed to 5-1000 µm/ml concentrations for 24, 48 and 72 hours and analyzed by WST-1 assay. The data obtained were normalized by comparison with the control group.

DISCUSSION

Members of the genus *Helianthemum* are known to be particularly native to the Mediterranean region and usually grow on sandy, stony, and calcareous soils. There are many different subspecies within this plant genus, each containing its specific flavonoid compounds (24, 25). *H. germanicopolitanum*, which we focused on in our study, is an endemic plant species specific to Çankırı and its surroundings. Local people use this plant in the form of ointment for the treatment of hemorrhoids and superficial injuries. However, comprehensive scientific studies on this plant are limited. Therefore, in our study, we investigated the cytotoxic effects of *H. germanicopolitanum* in detail. Our findings contribute to our understanding of the potential therapeutic effects of this special plant.

The root, stem, and leaves of the plant should be extracted separately to determine the flavonoids they contain. Flavonoids are components consisting of colored pigments with antioxidant properties found in plants. The main task of these components is to support the biological functions of the plant (26, 27).

The properties of flavonoids found in extracts obtained separately from the roots, stems, and leaves of plants of the genus *Helianthemum* have been studied in detail in the research (24, 28). Extracts obtained from these plants and fruits in various types include flavones such as apigenin, flavanones such as eriodictyol, hesperetin and naringenin, flavonols such as quercetin, kaempferol, myricetin and isorhamnetin, isoflavanoids such as genistein and daidzein, anthocyanins such as cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin, and flavonols such as epicatechin and proanthocyanidin (25).

In this first study on *H. germanicopolitanum*, the plant was extracted without separating the root, stem, and leaves. The graph we obtained after HPLC analysis (Figure 2) clearly shows that *H. germanicopolitanum* contains various flavonoids. However, specific analysis methods, such as NMR (Nuclear Magnetic Resonance Spectroscopy), need to be used to determine which flavonoids it contains (25, 29).

Flavonoids combat oxidative stress at the cellular level by reducing free radicals formed in the body. This contributes to maintaining a healthy cellular environment by protecting cells thanks to their antioxidant properties. Furthermore, flavonoids are known to reduce inflammation and exert anti-inflammatory effects (30, 31, 32).

Some types of flavonoids can regulate the cell cycle. They can inhibit the growth of cancerous tissue by stimulating apoptosis (programmed cell death) and inhibition of angiogenesis (formation of blood vessels). These properties emphasize the potential anti-cancer effects of flavonoids (33).

It is also known that flavonoids can regulate the gut microbiota. Some research suggests that flavonoids may have positive effects on stomach ulcers and inflammation. In addition, it is reported to have therapeutic and helpful properties for the digestive system by regulating intestinal motility. These diverse biological effects of flavonoids help us understand the potential positive health effects of these compounds from plant foods (34, 35, 36).

Terfassi et al. isolated a new type of flavonoid, 5,7,2′,4′,5′-pentahydroxyflavone 3-O-β-D-galactopyranoside and its derivatives from *H. getulum* Pomel. The structures of the extracts were determined using mass spectrometry and NMR techniques. Within the scope of this study, it was determined that 5 of the 13 flavonoids obtained from *H. getulum* Pomel were discovered for the first time in endemic plants of the genus Helianthemum. According to the results of the research, flavonoids are isolated by Siham Terfassia et al. were reported to have antidiabetic and antioxidant properties (37).

Plescia et al. carried out phytochemical analyses on *Helianthemum lippii*, an endemic plant native to Italy, and investigated the biological activities of the plant. According to the results of the studies, the extracts obtained were reported to have cytotoxic and antimicrobial properties (38).

Küpeli Akkol et al. investigated in detail the wound-healing mechanism of *H. canum* (L.) Baumg, which is known for its wound-healing properties. LC/MS-MS was used for phytochemical analysis of the extract obtained from the plant. Anti-inflammatory effects were evaluated through Interleukin 1, Interferon ɣ, and Interleukin 6 levels in fibroblast cells. In addition, histopathological analyses, collagenase, hyaluronidase, elastase enzyme inhibitors, and hydroxyproline estimation analyses were also performed. The results obtained show that the quinic and myricetin content of *H. canum* promotes wound healing by supporting

hydroxyproline production and wound contraction (11).

HT-29 cell line is a type of cancer derived from colon or colorectal adenocarcinoma cells lining the lining of the rectum, from which colorectal cancer originates. It is a model system frequently used in scientific research to understand the molecular mechanisms, biological properties, and potential treatment methods of colorectal cancer (39).

CONCLUSION

Our research reveals in detail that the *H. germanicopolitanum* plant species contains various flavonoids. Cytotoxicity studies of flavonoids in the extracts obtained because of the extraction procedures on colorectal cancer cell lines show that flavonoids offer anticarcinogenic effects by activating cell death pathways at certain concentrations. Our results are in line with the results of previous cytotoxic studies with flavonoids. These important findings emphasize the need for a more in-depth study of flavonoids in *H. germanicopolitanum* and the need for further research on these important compounds. The results obtained offer new insights into potential therapeutic applications and health effects, which may inspire future research.

Conflict of interest: No conflict of interest was declared between the authors.

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