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DNA Protective Assay and Some Biochemical Properties of *Galium* Species

Yener Tekeli[1*], Omer Kilic[1,2], Elif Esra Altuner[3**]
[1] Department of Pharmaceutical Techniques, Faculty of Pharmacy, University of Adiyaman, Adiyaman, Turkey
[2]Department of pharmacy professional sciences, Faculty of Pharmacy, Adiyaman, Turkey
[3]Program of Medical Laboratuary and Tecniques, Department of Medical services and Techniques, Europe Vocational School, Kocaeli Health and Technology University, Kocaeli, Turkey

✉: elifesraaltuner@gmail.com [1]0000-0003-1524-457X [2]0000-0003-3409-1572 [3]0000-0001-7663-6898

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ABSTRACT

Herbal-derived drugs prepared using various extracts obtained from different organs of plants that have been scientifically proven to be “medicinal” or directly from these plants are often used today as a method to prevent or treat diseases in humans and animals. This method, called phytotherapy, is a rational, evidence-based, and allopathic treatment method and deals with which active substance group is responsible for the biological effects. In this context, the phytotherapeutic effectiveness of *Galium* species, which are also used for medicinal purposes among the public, has been examined and proven in many studies. In this study, the biochemical efficacies of five different *Galium* species were measured and their antioxidant, antimicrobial, and DNA protective effects were tested. It was found that the tested *Galium* species showed remarkable biochemical efficacies. The results were also compared with the results of some other studies in the literature.

Keywords: Antimicrobial; Antioxidant; DNA; *Galium* types; Phytotherapy; Protective assay.

DNA Koruyucu Testi ve *Galium* Türlerinin Bazı Biyokimyasal Özellikleri

ÖZ

Bilimsel olarak “tıbbi” olduğu kanıtlanmış bitkilerin farklı organlarından elde edilen çeşitli ekstraktlar veya doğrudan bu bitkilerden elde edilen çeşitli ekstraktlar kullanılarak hazırlanan bitkisel kökenli ilaçlar, günümüzde insanlarda ve hayvanlarda hastalıkların önlenmesi veya tedavisinde bir yöntem olarak sıklıkla kullanılmaktadır. Fitoterapi adı verilen bu yöntem akılcı, kanıta dayalı, allopatik bir tedavi yöntemi olup, biyolojik etkilerden hangi etken madde grubunun sorumlu olduğunu ele almaktadır. Bu bağlamda halk arasında tıbbi amaçlı da kullanılan *Galium* türlerinin fitoterapötik etkinliği birçok çalışmada incelenmiş ve kanıtlanmıştır. Bu çalışmada beş farklı *Galium* türünün biyokimyasal etkinlikleri ölçülmüş, antioksidan, antimikrobiyal ve DNA koruyucu etkileri test edilmiştir. Test edilen Galium türlerinin dikkate değer biyokimyasal etkinlik gösterdiği tespit edildi. Sonuçlar ayrıca literatürdeki diğer bazı çalışmaların sonuçlarıyla da karşılaştırıldı.

Anahtar Kelimeler: Antimikrobiyal; Antioksidan; DNA; *Galium* türleri; Fitoterapi; Koruyucu tahlil.

INTRODUCTION

Medicinal herbs are a source of healing, and many modern medicines used today are made from active compounds derived from these herbs. They are not only used in drugs but also widely consumed by the public in the treatment of many diseases, especially infectious diseases [1]. Traditional herbal medicine, which is also called phytotherapy is a phenomenon that has been developed by people through trial and error and is as old as human history. In addition, herbal remedies are the most widely used complementary or adjunctive therapy tools around the world and have an important place, especially in developing countries [2,3]. A sedentary lifestyle and wrong eating habits, occurring especially with the developing technology, increase oxidative stress-related diseases because of free radicals [4]. Free radicals are molecules/atoms that contain one or more unpaired electrons and are highly active [5]. Free radicals, both reactive nitrogen species (RNS) and reactive oxygen species (ROS), can be derived from both endogenous and exogenous sources. Here, the harmful effects of oxidative stress can be defined as the negative effects of these ROS (reactive oxygen species) [6-7]. Reactive oxygen species (ROS), such as 1O2 [singlet oxygen], OH. (hydroxyl radicals), are natural by-products of cellular metabolism. Increasing ROS in cells can lead to the induction of oxidative stress. The excessive cellular level of ROS damages macromolecules, including DNA. This means increased oxidative stress that causes cell damage, which means cell death. Cell death is

an important factor in homeostasis, pathology, and aging. Therefore, because cell death is the main reason for many diseases, such as cancer and coronary heart diseases, medical assays based on preventing cell death have gained a significant place in medical research in recent years [8]. In addition to these efforts of medicine, herbal medicine, which has an important place in alternative medicine, is also used for therapeutic purposes. Epidemiological data show that the long-term consumption of plants rich in secondary metabolites, especially polyphenols, protects against the development of cancer, diabetes, osteoporosis, and cardiovascular, and neurodegenerative diseases. Herbal remedies are defined as products that consist of a combination of substances entirely derived from plants and contain active ingredients [9]. Patients with chronic diseases tend to use herbal products and phytochemicals more along with their current medications compared to the general population. This trend, which involves the use of herbal products for therapeutic purposes, is also referred to as traditional medicine [10-15].

The Rubiaceae Family is herbaceous or woody, perennial, or annual plants and is represented in the world with approximately 500 genera and 6500 species. *Galium* L., belonging to the Rubiaceae family and is represented by 101 species (122 taxa), 61 of which are endemic to the flora of Turkey, is an important material for researchers due to its phytochemical properties [16]. The Rubiaceae is not only a family comprised of ornamental plants but also is used in traditional medicine to treat various diseases. These herbs are used for more than 70 medical indications, such as hepatitis, eczema, edema, cough, hypertension, diabetes, and impotence Studies have shown that most of these herbs have antimalarial, antimicrobial, antihypertensive, antidiabetic, antioxidant, and anti-inflammatory activities [17]. Asperuloside, which is a monoterpene and is known as one of the iridoid heterosides, is an important compound found in various species of the Rubiaceae family, including *Galium***.** This compound shows the physiological effects of both alkaloids and monoterpenes.

In addition, *Galium* is a popular medicinal herb due to its insecticide, hypotensive, sedative, antipyretic, antitussive, and wound healing properties. It also causes coagulation of milk owing to an enzyme in its content. For this reason, it is known as “yogurt grass” among the people. Moreover, *Galium* species are notable for their in vitro antimicrobial and antioxidant properties such as secondary metabolites, alkaloids, and flavonoids. Their rich phytochemical structures and antimicrobial properties are among the reasons that increase the importance of *Galium* species [16-19].

MATERIAL & METHOD

Sample collection and preparation

In this study, plant species were collected from different regions. *Galium murale* (L.) All. was collected from the vicinity of Geyiktas village (Keban-Elazığ), steppe areas, in May 2019 with collected number ÖK 5223. *Galium cassium* Boiss. was collected from north of Duydum village (Sanliurfa-Siverek), steppe rocky areas, in June 2019 with collected number ÖK 5318. *Galium humifusum* Bieb. were collected from east of the Sancak (Bingol) district, humid areas, in July 2018 with the collected number ÖK 5318. *Galium verum* L. subsp. glabrescens Ehrend. were collected from northeast of Sancak (Bingol) district, rocky humid areas, in June 2018 with collected number ÖK 5267. *Galium verum* L. subsp. *verum* was collected from the vicinity of Gokce village (Bingol), stony shrubby areas, in June 2018 with collected number ÖK 5252. All plants were identified by plant taxonomist Dr. Omer Kilic. Herbarium samples of plant samples are stored in Adiyaman University (Turkey) Pharmacy Faculty herbarium, Hacettepe University, and Yıldırımlı herbarium from Ankara (Turkey). Plants were collected, air-dried, pulverized, and stored in black airtight bags. The extraction of the plants was carried out based on the method described by several studies, with some modifications [20-24]. Briefly, the dried and powdered *Galium* species, about 30 g, were extracted using a magnetic stirrer at 45 °C in 250 mL of methanol and then filtered. The filtrates were first concentrated with a rotor evaporator (Buchi, Switzerland) at reduced pressure and 45°C, and then they were concentrated to dryness by using a water bath at 45°C.

Antioxidant Activity Assays

Total Phenolic Content (Folin-Ciocalteu Method)

The total phenolic content of methanolic extracts was determined using gallic acid (Sigma-Aldrich) by the Folin–Ciocalteau method as a standard and expressed as mg of gallic acid equivalents (GAE) curve [20–23].

The stock solution prepared as 2 mg/mL was diluted 4 times. Then, 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of 7.5% Na_2CO_3 solution were added to each. After keeping for 45 minutes in darkroom conditions, absorbances were read at 760 nm wavelength in a spectrophotometer. The experiments were performed in triplicate.

Scavenging Activity of DPPH Free Radicals

DPPH free scavenging assay, which is one of the most popular and frequently used methods among antioxidant experiments, was evaluated using Cary 60 UV-Vis Spectrophotometer. The performed DPPH radical scavenging assay for the determination of *Galium* species was based on the previous studies, with some modifications [24-30]. Briefly, 0.1 mM of DPPH radical in methanolic solution was prepared, and then 0,5 mL of this solution was mixed with 2 mL of different

concentrations (2-0,125 mg/mL) of *Galium* species solutions [25]. After 30 min incubation in dark and at room temperature, to evaluate the reduction in DPPH radicals, absorbance was measured at 517 nm, and the inhibition % values expressing the reduction in radicals were calculated as below;

$$I\% = ((A_{DPPH} - A_S)/A_{DPPH}) \times 100$$

where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution. Synthetic antioxidants BHA and BHT were used as the positive control. The concentration indicating that the initial DPPH concentration decreased by 50% was calculated from the graph by plotting the percentage of inhibition against the sample concentrations. This value is called IC_{50} and the lowest IC_{50} value represents the highest antioxidant value. Each measurement was performed in triplicate [10,26,27].

Antibacterial Activity

To evaluate antimicrobial activities, the microdilution experiment was applied using a 96-well plate. The antibacterial activities of *Galium* species were tested on two Gram-negative and two Gram-positive bacteria. The antibacterial properties were assessed using microdilution sensitivity tests conducted in nutritional broth [22]. The *Galium* species were interpreted in terms of the activities of antimicrobial effects against four pathogenic bacterial strains, including Gram-positive (*E.faecalis* ATCC 29212), *S.aureus* ATCC 29213and Gram-negative bacteria (*E. coli* ATCC 25912, *P. aeruginosa* ATCC 10231). The antimicrobial analyses were carried out by modifying [28, 29]. In summary, DMSO (Sigma-Aldrich) was used to create solutions of Galium species up to 5 mg/mL, and the samples were diluted seven times and examined at eight different concentrations. Each microplate well received 100 µL of bacterial strain, adjusted for density using a McFarland densitometer, so that the bacteria were roughly 106 CFU/mL. Ceftriaxone and ampicillin were used as standard antibiotic agents, and the same procedures were also applied to them. Microplates were incubated for 24 hours at 37°C to measure antibacterial activity, then optical intensities were estimated at 600 nm (OD600) by using an Elisa reader (Bio-Tek Instruments, Inc.; Winooski, USA). Three MIC evaluations were conducted for every species of microorganism. The 24-hour incubation period ended with the determination of minimal inhibitory concentrations, or MICs. Standard antimicrobial drugs were used as the positive control in control tests, while unvaccinated medium was used as the negative control. The circumstances of the species under investigation were maintained throughout.

DNA Damage Protection Activity

Fresh LB medium containing ampicillin (100 µg/mL) was inoculated with glycerol stocks of *E. coli* Dh5α cells harboring pET21a plasmid (100 µL) were added to 10 mL of Luria-Bertani (LB) Broth containing ampicillin (100 µg/mL) and cultured for overnight at 37°C with shaking at 220 rpm. Cells were collected by centrifugation, and plasmid DNA was extracted using a plasmid DNA isolation kit (K0502, Thermo Fisher Scientific™) according to the manufacturer's recommendations and stored at 4°C until it was used. DNA protective activity of methanol extracts of each of the examined *Galium* taxa was studied by using pET21a plasmid (Novagen, USA). Oxidative DNA damage was induced using an ultraviolet (UV)/H_2O_2-radical system and checked on a 0.8% agarose gel electrophoresis as previously described by Verma et al. 2015. The reaction was realized in a total volume of 15 µL containing 5 µL plasmid DNA (30 ng/µL), 5 µL of the lyophilized plant extract dissolved in ddH2O (25 µg/mL), and 5 µL of 3% H_2O_2. The mixture was then placed directly on a UV transilluminator (300 nm) for 15 min under room conditions. The negative control contained only plasmid DNA and was not exposed to (UV)/H_2O_2. 5 µL of 6X loading dye (Thermo Scientific) was added to the reaction mixtures, and then the mixtures were loaded on 0.8% agarose gel containing 5 µL of 10mg/ml ethidium bromide (Sigma-Aldrich). Electrophoresis was carried out at 90 V for 1 hour. The gel was visualized via the Quantum ST5 Gel Documentation system.

RESULTS and DISCUSSION

In this study, the polyphenol contents as equivalent to gallic acid of *Galium* species were examined, and the results are presented in Table 1. These results showed that *Galium verum* subsp. *verum* (0.4257 mgGAE/mL) had the highest concentration of phenolic substance equivalent to gallic acid. It was followed by *Galium verum* subsp. *Glabrescens* (0.3455 mgGAE/mL) and *Galium cassium* (0.2754 mgGAE/mL). *Galium murale* on the other hand, had the least phenolic substance content with 0.2680 mgGAE / mL. Phenolic compounds have reducing properties, and therefore they show a strong antioxidant effect [24]. From this point of view, it can be said that *Galium verum* subsp. *verum* has more antioxidant effects than others. The results of the free radical scavenging effect method showed this clearly (Table 1). The free radical scavenging capacity of galium extracts was measured by the DPPH method. This method is based on the retention of DPPH radicals in the solvent environment by antioxidant molecules. The following reaction occurs between the DPPH radical and the antioxidant compounds. This method also works on H atom transfer.

$DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}$

$DPPH^{\cdot}$ is a stable free radical that possesses a deep purple color and a strong absorption around 517 nm (AH) [30]. After the *Galium* solutions prepared in methanol were diluted serially, a decrease in absorbance of 0.1 mM DPPH free radical solution, prepared on it, was determined at 517 nm. Percentages of inhibition of free radicals of each *Galium* species were calculated

depending on the change in absorbance values, and IC_{50} values were determined based on the inhibition values

Table 1. The total phenolic concentrations, IC_{50} value and percentages of free radical scavenging of Galium species.

	mgGAE/ mL $x10^{-4}$	IC_{50}	Inhibition %
Galium murale (L.) All	0.2688±0.21	6.06±0.12	50.39
Galium humifusum Bieb	0.2724±0.15	5.27±0.19	51.09
Galium cassium Boiss.	0.2754±0.17	4.04±0.22	58.40
Galium verum L. subsp. *Verum*	0.4257±0.10	2.26±0.16	69.08
Galium verum L. subsp. *glabrescens* Ehrend	0.3455±0.16	2.37±0.20	66.52
BHA	---	0.047±0.13	89.56
BHT	---	0.42±0.13	78.60

All *Galium* species showed noteworthy antioxidant properties, with described variability in IC_{50} values. Free radicals are well-known reasons for DNA damage, and this kind of damage to the DNA can make a cell cancerous [31]. DNA damage protective assays performed with natural resources having potential antioxidants and bioactive components are employed as in vitro models to ascertain how harmful radicals cause DNA production [32]. Results of this study revealed that every extract exhibited DNA damage protecting ability against free radicals produced by the UV/H_2O_2-radical system (Fig 1). In lane 2, which was exposed to UV and H_2O_2 without any plant extract, there was not a single DNA band. All the methanol extracts of the examined *Galium* taxa effectively protected the DNA from the hydroxyl radicals generated by UV and H_2O_2. Between lanes 3 and 7, where methanolic extracts were added to DNA exposed to UV and $H2O_2$, there was obvious protection against DNA damage. Many studies are proving the effectiveness of DNA protection [33-36].

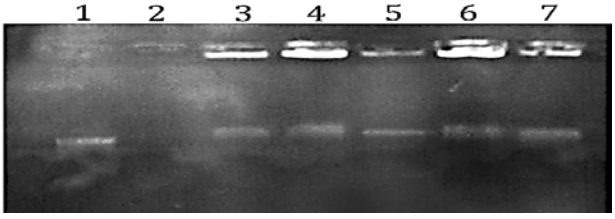

Fig 1. DNA damage protection activity of *Galium* taxa examined. 1: Supercoiled circular pET21a plasmid DNA, 2: pET21a plasmid DNA subjected to UV and H_2O_2, 3: *Galium verum* subsp. *verum*, 4: *Galium verum* subsp. *glabrescens*, 5: *Galium cassium*, 6*: Galium murale*, 7: *Galium humifusum.*

According to our findings, the extracts under investigation showed significant DNA protective action against free radical-induced DNA damage, suggesting that they may have anticancer potential.

Methanolic extract of *Galium* species was tested by the microdilution method against two Gram-Positive and two Gram-Negative bacterial strains from the ATCC Cell Biology Collection (*P. aeruginosa, E.faecalis, E. coli, S.aureus*). The MIC values of the extracts determined against bacterial strains ranged from 0.15625 to 5 mg/mL.

In general, it is seen that all species are effective against four bacteria, but they are especially effective against *P. aeruginosa* even at the lowest concentration (0.15625mg/mL). *P. aeruginosa* reproduces in humid media and leads to infection in sensitive patients. Therefore, it is one of the gram-negative bacteria that cause infection in the hospital environment. In this regard, it is very valuable that methanolic *Galium* extracts are effective against *P. aeruginosa* at low concentrations. Not only *P. aeruginosa*, but also *S. aureus* and *E. coli* can cause several diseases, especially hospital infections [37,40]. It is also important that methanolic *Galium* extracts are effective against *E. coli* at a concentration that can be considered low (Table 2). Against *S.aureus*, it is effective, albeit at higher concentrations. *E.faecalis* are microorganisms with low virulence, but they are important factors in the community and especially in hospital-caused infections33. It is also important that methanolic *Galium* extracts are effective against *E. coli* at a concentration that can be considered low (Table 2).

Table 2. Antibacterial activities of *Galium* species [mg/mL]

	P. aeruginosa	***E.faecalis***	***E. coli***	***S.aureus***
Galium murale (L.) All	0.1562	1.2500	1.2500	2.5000
Galium humifusum Bieb	0.1562	1.2500	1.2500	2.5000
Galium cassium Boiss.	0.1562	2.5000	0.3125	5.0000
Galium verum L. subsp. *Verum*	0.1562	1.2500	1.2500	2.5000
Galium verum L. subsp. *glabrescens* Ehrend	0.1562	2.5000	0.620	2.5000

Against *S.aureus*, it is effective, albeit at higher concentrations. *E.faecalis* are microorganisms with low virulence, but they are important factors in the community and especially in hospital-caused infections [40].

None of the compounds showed cytotoxic activity, which is active against gram-positive/gram-negative bacteria and yeast. However, every molecule exhibited a notable level of antioxidant action. Therefore, it can be said that the present study supports the value of the chemical and pharmacological effects of *Galium* species in many different fields. In addition, the *Galium* species tested in this study have not been studied in the literature [5,20,34]. This shows the originality of the study.

Statistical analysis

All tests were constructed by running standards or plant extracts of five different concentrations, in triplicate. Values are expressed as mean ± standard deviation of three replicate measurements. The results of ANOVA analysis show significant differences ($p<0.05$) in the means of total phenolic concentrations and free radical scavenging activities of the *Galium* species.

CONCLUSION

In this study, the biochemical activities of five different Galium species (*Galium murale, Galium humifusum, Galium Verum-Verum, Galium verum-glabrescens)* were measured and their antioxidant, antimicrobial, and DNA protective effects were tested. The tested Galium species were found to exhibit remarkable biochemical effects. The results of this study revealed that all samples indicated DNA damage safeguarding preventive action against deterioration of DNA caused by free radicals formed by the (UV)/H_2O_2-radical system.

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