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Original Article:

Chemical Composition, Antiradical Capacity and Antimicrobial Activity of Hydroalcoholic Extracts of *Ficus johannis* Boiss

Zahra Ebrahimnezhad[1], Hamid Beyzaei[2], Sadegh Keshtegar[3], Mehdi Dehghani[4*]

1. Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Zabol, Zabol, Iran.
2. Department of Chemistry, Faculty of Science, University of Zabol, Zabol, Iran.
3. Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran.
4. Department of Biology, Faculty of Science, University of Zabol, Zabol, Iran.

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*** Corresponding author:**

Mehdi Dehghani, MD.

Address: Department of Biology, Faculty of Science, University of Zabol, Zabol, Iran

E-mail:
dehghanimehdi55@uoz.ac.ir

Abstract

Introduction: *Ficus johannis* Boiss. is a shrubby species in the family Moraceae, exclusively found in Iran, Afghanistan, and Pakistan. This research studies the total phenolic content, flavonoid concentrations, antioxidant capacity, and antimicrobial activity of this biologically unknown species.

Materials and Methods: The Leaves and fruits of the plant were gathered from the northern slope of Taftan Mountain in Sistan and Baluchestan, in the summer of 2022. After washing, drying, grinding, and obtaining hydroalcoholic extracts of the fruits and leaves, quantifying the phenolic and flavonoid concentrations was done spectrophotometrically utilizing the Folin-Ciocalteu and aluminum chloride colorimetric techniques, respectively. The anti-radical activity of the organs was assayed *via* the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) protocol and their antimicrobial effects were assessed using the broth microdilution and streak plate techniques against six bacterial, and three fungal pathogens including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Bacillus cereus*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Candida albicans*.

Results: The phenolic compounds were found to be evenly distributed in both leaf and fruit extracts (around 43 mg GAE/g dry extract) while flavonoids were detected in significantly higher concentrations in the fruit extract (14.62 *vs* 9.57 mg QrE/g dry extract). The leaf extract was better at scavenging free radicals compared to the fruit extract (IC_{50} 93.79 *vs* 239.62 µg/ml, respectively), while both extracts showed higher IC_{50} values than the positive control. The leaf extract showed better inhibitory effects on the tested microorganisms compared to the fruit extract. The leaf extract was effective against all tested bacteria, whereas, among the investigated fungi, only *Aspergillus fumigatus* was vulnerable to it. Conversely, the fruit extract was able to prevent the growth of all investigated fungal strains, but only two bacterial strains (*Streptococcus pyogenes* and *Klebsiella pneumonia*) were affected by it. Moreover, both lea and fruit extracts showed the best antimicrobial activity against *Aspergillus fumigatus* with MIC values of 128 and 512, repectively.

Conclusion: The *Ficus johannis* fruits and leaves are great sources of phenolic and flavonoid compounds with moderate anti-radical capacity. Moreover, the fruit extract mainly contains antifungal ingredients while the leaf extract chiefly includes antibacterial agents.

Keywords: Anti-bacterial agents, Antifungal agents, Antioxidants, *Ficus johannis*, plant extract

1. Introduction

Plants have been used for centuries as a source of medicine owing to their antimicrobial properties. The antimicrobial activity of plants is believed to be due to the existence of various secondary metabolites, especially phenolic compounds and flavonoids, which are responsible to a large extent, for plants' antioxidant properties. Studies have demonstrated that these secondary metabolites possess diverse antimicrobial properties that can combat bacteria, fungi, viruses, and parasites [1, 2].

There are many sources of natural antimicrobial agents such as plants, microbes, animals, algae, and mushrooms. However, most attention has been given to plant extracts that are rich in polyphenols [3, 4].

The use of plant-based antimicrobials is becoming increasingly popular due to the rise in antibiotic-resistant bacteria, considered worse than terrorism, and global change in cost and consequences. Plant-based antimicrobials offer a natural alternative that can be used in place of synthetic antibiotics. However, further research is required to fully decipher the mechanisms behind the antimicrobial activity of plants and their potential use in clinical settings.

The process of testing plant extracts to determine their phenolic and flavonoid contents, antioxidant properties, and the ability to hinder microbial growth is seen as a promising method for identifying potential molecules to treat specific illnesses [5].

A vast number of plants that are commonly utilized in pharmaceuticals have been screened for chemical composition and antimicrobial properties around the world, however many more remain to be considered in the future. Currently, due to the expanding rise of multidrug-resistant pathogens, the disclosure of modern and natural antimicrobial compounds is profoundly acknowledged [6].

Ficus (Latin for fig tree) stands as the biggest genus in the Moraceae family encompassing over 800 distinguished species [7], found primarily in tropical and subtropical regions of America, Africa, and notably, India and Malaysia, with a limited number in temperate regions [8-10]. Various species of *Ficus* are used traditionally to treat different disorders including cancer, skin problems, diabetes, gum bleeding, digestive tract diseases et cetera [11, 12]. *Ficus* species are also utilized both as indoor and outdoor ornamental plants and as a source of food due to their high nutritional value worldwide [12]. *Ficus* is represented by four species in Iran [13]. *Ficus johannis* Boiss. is a profusely branched large shrub or a small tree that grows up to 3-(4) m. The species is closely related to *F. carica* with 3-5-lobed leaves and lateral nerves running parallel in the leaf blade, and non-cauliflorous hypanthodia borne on young branches in leaf axils or behind the leaves. However, it differs from *F. carica* by shorter petiole, non-spathulate leaf lobes, and nearly glabrescent to scabrous leaves. *Ficus johannis* is found in three countries Iran, Afghanistan, and Pakistan. In Iran, the plant is commonly found in the western, central, and southern regions [14]. The leaves of the plant are used in traditional Iranian medicine as a remedy for diabetes. It has also been shown that ethanolic extract of the leaves of *F. johannis* exhibits favorable properties for managing diabetes by enhancing insulin sensitivity, promoting GLUT-4 translocation and carbohydrate metabolism, and restraining lipid synthesis in rats [15]. In addition, the fruits are edible and the leaves are grazed by animals and the sap is used in Pakistan to turn milk into cheese due to the presence of a cysteine protease with milk-clotting activity in it [10, 16]. The preliminary analysis of the leaf extract from *Ficus johannis* sub sp. *Afghanistanica* (Warb.) Browicz revealed the existence of carbohydrates, phenolic compounds, and tannins, indicating its potential for more detailed studies [17]. There are no other studies available in the literature that address the biological properties of this unexplored plant species.

The objective of this study is to measure the levels of phenolic and flavonoid, evaluate antioxidant potential, and assess antimicrobial activity of fruit and leaf hydroethanolic extracts of *Ficus johannis* against selected bacterial and fungal pathogens.

2. Materials and Methods

Plant sampling

In this descriptive cross-sectional study, the fruits and leaves of *Ficus johannis* Boiss. (Mountain Fig) were gathered in July 2022 at an altitude of 1733 m from Cheshmeh Musa on the northern slope of Taftan, Tamin village, Taftan city, Sistan and Baluchestan, Iran (Figure 1). The samples were taken from three plant individuals and were mixed together to capture the variation in the plant population. The scientific name of the plant was confirmed as *Ficus johannis* subsp. afghanistanica (Warb.) Browicz by the last author and the UOZH-1506 voucher code was assigned to a herbarium specimen preserved in the University of Zabol Herbarium. *Ficus johannis* encompasses two subspecies in Iran, *Ficus johannis* subsp. *afghanistanica* possesses larger leaves and fruits compared to *Ficus johannis* subsp. *Johannis*.

Figure 1. A. the geographical locality of the population from which the samples were taken, B-C. the habitat and habit of *Ficus johannis,* D-E. the fruits and leaves detailed morphology

The plant organs, including leaves and fruits, were dried under room conditions in the shade separately for two weeks after removing the waste materials. Finally, the organs were finely pulverized using an electronic mill and were sieved for uniformity.

Plant extracts preparation

To prepare the extracts, 3 grams of dried *Ficus johannis* fruit and leaf powders were immersed in 30 ml of a hydroalcoholic solvent comprising ethanol and water in a 1:1 ratio, in separate beakers. The solutions were subjected to continuous shaking on a shaker in a dark room at ambient temperature for 24h. The samples were then passed through a Whatman filter to remove any impurities using a vacuum pump and then maintained at 37°C for 48-72 hours to evaporate the solvent [5, 6].

Evaluation of total phenolic content

To assess the total phenolic content of the organs of *Ficus johannis*, the Folin Ciocaltio method [18] was employed. 0.5 gram of the powder of each plant organ was soaked in 3 ml of methanol and was placed on a shaker for 24 hours in in darkness at ambient temperature. Then, 0.5 ml of methanolic extract of each organ was blended with 2.5 ml Folin-ciocalteu reagent (0.2 M), shaken well, and left at room temperature for 5 minutes. Next, an2 ml sodium carbonate solution (7.5% w/v) was appended to the mixture. After two hours of resting in darkness, the solution's absorbance was recorded at 760 nm using a 6405 UV/Vis Jenway spectrophotometer against blank water. The same method was repeated with gallic acid solutions of 50, 100, and 200 µg /ml as a standard to create the standard curve, where gallic acid replaced the extract in the

previous step. The experiments were conducted in triplicate, and the results were presented as micrograms of gallic acid equivalent (GAE) per gram of dried extract (µg GAE/g DE).

Evaluation of total flavonoid content

Aluminum chloride reagent was utilized to estimate the flavonoid level of the hydroalcoholic extracts [19]. A mass of 0.5 grams of the powder of each plant organ was immersed in 3 ml of methanol and agitated in darkness at air temperature for 24 h. Subsequently, 0.5 ml of each extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% methanolic aluminum chloride ($AlCl_3.6H_2O$), 0.1 ml of 1 M sodium acetate (CH_3CO_2Na) and 2.8 ml of distilled water. Then, the absorbance of each mixture was read at 415 nm against distilled water after 40 minutes of incubation at ambient temperature. The same approach was employed to gauge the absorbance of quercetin at 50, 100, and 200 µg /ml as a standard, with quercetin replacing the extract in the previous step. All assays were performed in triplicate and the flavonoid content was calculated as micrograms of quercetin equivalent (QE) per gram of dried extract (µg GAE/g DE).

Antioxidant activity and IC50 determination

The 2,2-diphenyl-1-picrylhydrazyl ((DPPH) test was conducted to estimate the antioxidant properties of the fruit and leaves of *Ficus johannis* as described in our previously published [20, 21]. First, DPPH solution (0.004% w/v) and the desired plant organ mixtures at concentrations of 125, 250, 500, and 1000 µg/ml were provided utilizing methanol as the solvent. Next, 3 ml of DPPH solution was introduced to each concentration of plant organs. The mixtures were then left to rest in darkness at lab temperature for 30 minutes preceding the measurement of their absorbance at 517 nm. Besides, the DPPH solution absorbance was recorded as a control. Using the obtained absorbances, the inhibition percentage was computed for each concentration utilizing the following formula: I% = [(BA - SA)/(BA)] × 100, where BA and SA represent the blank and sample absorbances at 517 nm. The inhibition percentage was then plotted against the concentration to construct a graph; afterwards, the straight-line equation was attained as y = ax + b. The IC_{50} (half-maximal inhibitory concentration) was determined as a concentration at which y equals 50 (i.e., IC_{50} = -b/a).

Assessment of antimicrobial activity

A total of 9 microorganism strains were used to evaluate the antimicrobial properties of *Ficus johannis* leaves and fruits. The microorganisms in question consisted of both Gram-negative and Gram-positive bacteria as well as fungal and yeast strains including: *Pseudomonas aeruginosa* PTCC 1310, *Klebsiella pneumoniae* PTCC 1290, *Escherichia coli* PTCC 1399, *Staphylococcus epidermidis* PTCC 1435, *Streptococcus pyogenes* PTCC 1447, *Bacillus cereus* PTCC1665, *Aspergillus fumigatus* PTCC 5009, *Fusarium oxysporum* PTCC 5115, and *Candida albicans* PTCC 5027. All samples were purchased from the Persian Type Culture Collection (PTCC), Karaj, Iran.

Determination of the Minimum Inhibitory Concentration (MIC), Minimum Bacteriocidal Concentration (MBC), and Minimum fungicidal Concentration (MFC) of the extracts

The microdilution method was utilized to discover the MIC of the plant extracts in sterile 96-well plates, following the M07-A9 and M26-A CLSI standards [22]. Initially, the hydroethanolic extracts of *F. johannis* leaves and fruits were dissolved in dimethyl sulfoxide, creating an initial concentration of 40960 µg/ml for each sample. Two-fold serial dilutions were then performed in each column of the 96-well plate. Subsequently, 80 microliters of liquid medium and 100 microliters of diluted suspensions of bacteria or fungi were added to each well. Positive controls were employed during the experiment, using gentamicin and clotrimazole. The incubation process of the plates lasted for 24 hours in an incubator set at 37°C. Visible turbidity in wells was regarded as microorganism growth and the well with the lowest concentration showing no visible turbidity was reported as MIC.

The MBC and MFC were detected by culturing and incubating the contents of all transparent wells from the MIC determination stage at 37 °C for 24 hours. The MBC or MFC was reported as the lowest concentration of extracts that inhibited the growth of microorganisms.

Statistical evaluation

The experiments were conducted thrice to ensure the accuracy of the results, and the values represent the average of three independent experiments. Graphs were created using Excel software, and data analysis was performed using SPSS version 16.

3. Results

Table 1 displays the levels of phenolic and flavonoid compounds found in the hydroalcoholic extracts obtained from the leaves and fruits of *F. johannis*. The concentration of phenolic compounds in the leaf extract was insignificantly higher ($P < 0.05$) compared to that in the fruit extract, whereas the fruit extract showed a significantly higher level of flavonoid

content ($P < 0.05$). Also, the hydroalcoholic extract of the leaves demonstrated higher potency in scavenging free radicals ($P < 0.05$) compared to the fruit extract, with IC_{50} of 93.79 *vs* 239.62 µg/ml, respectively. However, the IC_{50} of both extracts was remarkably higher than the positive control, proving less antioxidant activity of the organs compared with vitamin C (Table 1).

The antimicrobial efficacy of the hydroalcoholic extracts obtained from the leaves and fruits of *F. johannis* was quantified using gentamicin and clotrimazole as positive drug controls for bacteria and fungi, respectively, (Table 2). The leaf extract exhibited more extensive inhibitory effects on tested microorgnisms compared to the fruit extract. The leaf extract was effective on all tested bacteria, while only *Aspergillus fumigatus* among the investigated fungi was sensitive to it. On the other hand, the fruit extract prevented the proliferation of all investigated fungal strains, but only two bacterial strains were susceptible to it (*Streptococcus pyogenes* and *Klebsiella pneumonia*).

Table 1. Total phenolic and flavonoid contents and antioxidant capacity of hydroalcoholic extracts of leaves and fruits of *F. johannis*

Samples	IC_{50} (µg/ml)	Total flavonoids content (mg quercetin/g)	Total phenolic content (mg gallic acid/g)
Leaf Ex	93.79	9.57	44.35
Fruit Ex	239.62	14.62	42.75
Vitamin C	3.94		

Table 2. The MIC, MBC, and MFC values of the hydroethanolic extracts of leaves and fruits of *F. johannis* and positive controls against tested microorganisms. ND= not detected.

Microbe strains		Extracts		Positive controls	
		Fruit	Leaves	Gentamicin	Clotrimazole
E. coli 1399	MIC[a]	ND	1024	8	-
	MBC[b]	ND	2048	8	-
P. aeruginosa 1310	MIC	ND	4096	0.063	-
	MBC	ND	4096	0.063	-
K. pneumoniae 1290	MIC	2048	4096	4	-
	MBC	2048	4096	4	-
B. cereus 1665	MIC	ND	1024	0.25	-
	MBC	ND	1024	4	-
S. pyogenes 1447	MIC	4096	2048	2	-
	MBC	4096	2048	2	-
S. epidermidis 1435	MIC	ND	4096	1	-
	MBC	ND	4096	2	-
C. albicans 5027	MIC	2048	ND	-	256
	MFC[c]	4096	ND	-	512
A. fumigatus 5009	MIC	512	128	-	32
	MFC	512	256	-	32
F. oxysporum 5115	MIC	2048	ND	-	256
	MFC	2048	ND	-	512

The most potent antimicrobial activity was observed against *Aspergillus fumigatus* with both extracts exhibiting the lowest MIC values, specifically 128 for the leaf extract and 512 for the fruit extract. As a result, the leaves and fruits of this plant can be used in the treatment of diseases caused by this strain. Moreover, the results of this study indicated the leaf extract serves as a source of antibacterial compounds, while the fruit extract acts as a source of antifungal agents.

4. Discussion

The results of this paper showed that the hydroalcoholic extracts derived from both leaves and fruits of *F. johannis* contained significant quantities of phenolic and flavonoid compounds. The leaf extract had almost the same amount of phenolic content as the fruits, while the fruit extract showed significantly higher flavonoid content than that of the leaves. This suggests that different parts of the plant may have varying levels of these compounds. Additionally, the

results revealed that the leaf extract had a significantly higher efficacy in neutralizing free radicals when compared to the fruit extract, suggesting stronger antioxidant properties in the leaf extract. In addition, the antimicrobial assay indicated that the fruit and leaf hydroalcoholic extracts contain significant antibacterial and antifungal ingredients. The fruit extract showed more efficacy against fungal strains while the leaf extract was more potent against bacterial strains with both extracts demonstrating the most significant results (lowest MIC) against *Aspergillus fumigatus*.

Phenolic and flavonoid compounds are known for their antioxidant properties which can help eliminate free radicals and safeguard the body against oxidative harm. These compounds have been shown to have various health benefits, including reducing inflammation and protecting against diseases such as cancer and heart disease [23]. Nowadays, there is an increasing inclination towards replacing synthetic antioxidants with natural alternatives, although the antioxidant activities of some natural antioxidants may be lower. Nevertheless, natural antioxidants can be a promising alternative to synthetic ones [24], especially those produced in plants. Thousands of plants have been investigated for biological properties, yet many more are still waiting to be screened, among them endemic plants are of vital importance. The literature includes only limited information regarding the phenolic and flavonoid contents, antioxidant properties, and antimicrobial capacity of *F. johannis*. According to the available literature, there is a preliminary analysis on the leaves phytochemicals of *Ficus johannis* Boiss. Sub sp. *Afghanistanica* from India that revealed the presence of various phytochemicals including carbohydrates, oils and fats, phenolic compounds, and tannins [17]. Also, an in vitro and in vivo study showed the significant antidiabetic effects of ethanolic extrct of *F. johannis* leaf extract and reported remarably higher amounts of phenolic (71.208 mgg^{-1} GAE) and flavonoid (26.38 mgg^{-1} QE) contents as well as considerably lower IC_{50} (33.81 µg/mL) compard to our data [15]. This contradiction may result from differnences in population genetics, climatic condition, or gathering time. Our data on phenolic and flavonoid contents are highly similar to exsiting data on Algerian varieties of *Ficus carica* L. [25], which is genetically closely related to *F. johannis*. An intermediate antibacrobial activity of the ethanolic extract of the leaves of *F. johannis* has been shown on *Aeromonas* species, with inhibition zone diameter of 9.38 -12,38 mm [26]. Moreover, ethanolic extract of the leaves of *F. johannis* was shown to have intermediate activity against *Candida albicans* [27].

The findings of this study are just the beginning of the journey toward examining and understanding other pharmaceutical and biological properties of *F. johannis*. However, it's crucial to acknowledge that there were certain limitations during this research, such as the inability to study other populations of this species occupying different geographical conditions. To overcome this weakness, we propose a wider study on various populations of *F. johannis* to discover the most powerful gynotypes. Furthermore, these outcomes were obtained under laboratory conditions, and it is necessary to conduct *in vivo* tests to verify the results. In addition, the lack of previous research studies on this species made the comparasions limited which will be automatically resolved as further studies are conducted.

5. Conclusion

Based on the findings of this study, it can be concluded that the hydroethanolic extracts of both the leaves and fruits of *Ficus johannis* contain substantial amounts of phenolic and flavonoid compounds, which are considered important for the medicinal and therapeutic properties of plants. The leaves of *Ficus johannis* showed moderate antioxidant and strong antibacterial effects, while the fruit had weak antioxidant but strong antifungal properties. In summary, the high concentration of phenolic and flavonoid compounds in *Ficus johannis* may contribute to its beneficial effects. It is concluded that the leaves and fruits of this species contain high levels of phenolic and flavonoid ingredients plus good antioxidants, and antifungal, and antibacterial effects, indicating their potential for medicinal and nutritional purposes. These results may be of interest to researchers seeking to develop new and efficient drugs using natural resources.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Author's contributions

Zahra Ebrahimnezhad conducted laboratory experiments, gathered the data, and wrote the manuscript draft. Hamid Beyzaei participated in

supplying experimental equipment, collecting data, and writing and editing the paper. Sadegh Keshtegar prepared plant materials and contributed to writing the primary version of the manuscript. Mehdi Dehghani contributed to the project concept, and composed and edited the manuscript. All authors have approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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