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Original Article

HERBAL POWER: ANTIMICROBIAL, ANTIOXIDANT AND ANTICHOLINESTERASE PROPERTIES OF *CITRULLUS COLOCYNTHIS*

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ABSTRACT

Citrullus colocynthis (L.) Schrad. is a biologically rich plant widely used in traditional medicine. This study aimed to investigate the antimicrobial, antioxidant and anticholinesterase potential and phenolic/flavonoid contents of *C. colocynthis*. Antimicrobial tests using ethanol and methanol extracts revealed that it exhibited strong antimicrobial effects especially against pathogens such as *Staphylococcus aureus*, *Candida albicans*, *C. krusei* and *Acinetobacter baumannii*. In addition, antioxidant activity tests showed that ethanol extract had high antioxidant capacity, while methanol extract was moderately effective. Phenolic and flavonoid content analyses showed that ethanol extract contained higher phenolic and flavonoid components. In addition, anticholinesterase activity tests showed that *C. colocynthis* extract exhibited a significant anticholinesterase effect, which could potentially be useful in the treatment of neurological diseases. These findings indicate that *C. colocynthis* is a potent natural resource with potential therapeutic benefits and its antimicrobial, antioxidant and anticholinesterase properties are of considerable importance in biomedical applications.

KEYWORDS: Antialzeihmer, Antimicrobial, Antioxidant, *Citrullus colocynthis*, Medicinal plants.

1. Introduction

Since ancient times, people have relied on natural products obtained from plants against various diseases [1]. These medicinal plants, one of the cornerstones of traditional medicine, are rich in bioactive substances and attract attention with their therapeutic effects [2]. At the same time, they are an important natural source for the discovery of new drugs used in the treatment of diseases in modern medicine [3,4]. Scientific studies have revealed that different plant species have many biological properties such as antimicrobial, antioxidant, anticancer, antiproliferative, hepatoprotective, antiaging, antiallergic and DNA protective [5-9]. These properties support the versatile use potential of herbal ingredients in the field of health.

Citrullus colocynthis (L.) Schrad. is a medicinally important plant belonging to the Cucurbitaceae family [10]. This plant, which grows widely in desert regions, has a high nutraceutical potential and is widely used in medical and pharmaceutical fields [11]. Known as "bitter apple" among the public, this plant is also known by different names such as colocynth, bitter cucumber, bitter melon, egusi melon and bitter vine of Sodom [12,13]. *C. colocynthis*, which has been used in traditional medicine for many years, has been reported to be effective in the treatment of various diseases such as cold, cough, bronchitis, asthma, diabetes, jaundice, dysentery and cancer [13]. This plant, which has been evaluated for medicinal purposes in different cultures

throughout history, has been reported to have significant potential in the field of health thanks to its bioactive components [13,14].

In this study, antioxidant, antimicrobial, antiallergic, total phenolic and total flavonoid contents of *C. colocynthis* were determined.

2. Materials and Methods

C. colocynthis plant samples were collected from Duhok region of Iraq. Collected plant samples are kept in the herbarium of Biology Department of Faculty of Science, Zakho University. For experimental studies, plant samples were first dried and powdered. Then, 30 grams of prepared powder samples were weighed and extracted with 250 mL of ethanol in Soxhlet apparatus at 50 °C for approximately 6 hours. After extraction, solvents were removed using rotary evaporator and the obtained extract was stored at +4 °C until experiments were performed.

2.1. Antimicrobial activity tests

In this study, antibacterial and antifungal activities of *C. colocynthis* plant extract were determined using agar dilution method. Bacterial strains used in the study were *Staphylococcus aureus* ATCC 29213, *S. aureus* MRSA ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606. Fungal strains used were *Candida albicans* ATCC 10231, *C. krusei* ATCC 34135 and *C. glabrata* ATCC 90030. Bacterial strains were precultured in Müller Hinton Broth and fungal strains were precultured in RPMI 1640 Broth before the experiment. The plant extract was tested by preparing stock solutions in the concentration range of 12.5-800 µg/mL. The lowest concentrations that inhibited the growth of microorganisms were determined and the results obtained were reported in µg/mL [15-18].

2.2. Antioxidant tests

Total antioxidant (TAS) and total oxidant (TOS) levels of *C. colocynthis* plant extract were determined using Rel Assay kits. The protocol provided by the manufacturer of the kits was followed in the analyses. TAS values were expressed as mmol Trolox equivalent/L, while TOS values were expressed as µmol hydrogen peroxide equivalent/L [19,20]. Oxidative stress index (OSI) is a parameter that shows the extent to which oxidant compounds are suppressed by antioxidant compounds. In the OSI calculation, firstly the units of TOS and TAS values were equalized, and then the ratio of the TOS value to the TAS value was taken and expressed as a percentage [21].

2.3. Anticholinesterase tests

Anticholinesterase activity of ethanol extract of *C. colocynthis* plant was determined using Ellman method [22]. In the study, plant extract was prepared at different concentrations in the range of 200-3.125 µg/mL using distilled water. For analysis, 130 µL of 0.1 M (pH=8) phosphate buffer, 10 µL of stock solution and 20 µL of enzyme solution (acetylcholinesteras (AchE) or butyrylcholinesterase (BChE)) were added to the microplate wells, respectively. The mixture was incubated at 25 °C in the dark for 10 min. After the incubation period, 20 µL of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) solution and 20 µL of substrate (acetylcholine iodide or butyrylcholine iodide) were added to each well and the absorbance of the reaction was measured at 412 nm wavelength. Experiments were performed in three parallel repetitions and the results obtained were expressed in µg/mL by calculating IC50 values.

2.4. Total Phenolic and Total Flavonoid Analyses

A 1 mL stock solution was prepared from the ethanol extract of *C. colocynthis* plant. In order to determine the amount of phenolic compounds, 1 mL of Folin-Ciocalteu reagent (1:9, v/v) was added to the prepared solution and mixed. Then, 0.75 mL of 1% Na_2CO_3 was added to the mixture and incubated for 2 hours at room temperature. At the end of the incubation period, the absorbance value was measured at 760 nm and the total phenolic content (TPC) was calculated in mg/g using the calibration curve of the gallic acid standard solution [23].

The total flavonoid content (TFC) of the ethanol extract of the plant was determined using the aluminum chloride test. For the analysis, 0.1 mL of 10% $Al(NO_3)_3$, 0.1 mL of 1 M NH_4CH_3COO, 4.3 mL of methanol, 0.5 mL of quercetin and 0.5 mL of plant extract were mixed. After the obtained solution was incubated for approximately 40 minutes, the absorbance value was measured at 415 nm. The total flavonoid content was calculated and expressed in mg/g [23].

3. Results and Discussion

3.1. Antimicrobial activity

Today, the origin of many diseases is microorganisms. The effects of drugs used to combat microorganisms are insufficient due to resistant microorganisms. In this context, the discovery of new antimicrobial drugs has become inevitable [24]. Many medicinal plants show strong antimicrobial activity against various bacterial and fungal species thanks to the bioactive components they contain, and these properties make them an important source for natural treatment alternatives and new drug discoveries [25,26]. In our study, the lowest extract concentration of *C. colocynthis* that prevented the growth of test microorganisms was determined. The findings are shown in Table 1.

Table 1. Antimicrobial potential of *Citrullus colocynthis*

Microorganisms	Solvent	
	Ethanol	Methanol
S. aureus	50	100
S. aureus MRSA	50	100
E. faecalis	100	200
E. coli	100	100
P. aeruginosa	200	400
A. baumannii	50	50
C. glabrata	100	100
C. albicans	50	100
C. krusei	50	100

*50, 100, 200 and 400 µg/mL are the lowest extract concentrations that inhibit the growth of microorganisms.

In the literature, *C. colocynthis* is reported to be infected with *Enterococcus faecalis, Staphylococcus*

aureus, S. epidermidis, Listeria monocytogenes, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Vibrio parahaemolyticus, Vibrio alginolyticus, Candida albicans, Candida glabrata, Candida kreusei and *Candida parapsilosis* [27]. In a different study, it was reported that aqueous and methanol extracts of *C. colocynthis* were effective against *Bacillus subtilis, Staphylococcus aureus, Streptococcus faecalis, Streptococcus pyogenes, Escherichia coli, Klebseilla pneumoniae, Proteus mirabilis, Salmonella typhi, Vibrio cholerae, Aspergillus fumigatus, Mucor* sp. and *Penicillium* sp. [28]. In our study, ethanol and methanol extracts of *C. colocynthis* were used. As a result of the study, it was determined that ethanol extracts generally exhibited stronger antimicrobial activity compared to methanol extracts. Ethanol extract at 50 µg/mL and methanol extract at 100 µg/mL were effective against *S. aureus, S. aureus* MRSA, *C. albicans* and *C. krusei*. Both ethanol and methanol extracts at 100 µg/mL were effective against *E. coli* and *C. glabrata*. Ethanol extract at 100 µg/mL and methanol extract at 200 µg/mL were effective against *E. faecalis*. Ethanol extract at 200 µg/mL and methanol extract at 400 µg/mL were effective against *P. aeruginosa*. Both ethanol and methanol extract at 50 µg/mL were effective against *A. baumannii*. In this context, it was determined in our study that *C. colocynthis* was effective at concentrations of 50-400 µg/mL. As a result, it was determined that *C. colocynthis* has antimicrobial potential. This antimicrobial effect is due to the bioactive components of the plant, and it is known that compounds such as flavonoids, alkaloids, terpenes and phenolic acids damage the cell walls of pathogenic microorganisms and inhibit their biosynthetic pathways [29,30]. In addition, some studies have reported that the antibacterial and antifungal effects of *C. colocynthis* are also effective on microorganisms resistant to traditional treatment methods, which increases the importance of the plant as a potential source in the pharmaceutical field. Similarly, in our study, it was determined that ethanol and methanol extracts were effective against important pathogens, especially *S. aureus, Candida albicans* and *Pseudomonas aeruginosa*. These findings reveal the potential of *C. colocynthis* as a natural treatment alternative that can be used in the treatment of resistant infections in modern medicine.

3.2. Antioxidant activity

Oxidative stress is a condition that causes cellular damage as a result of the accumulation of free radicals and reactive oxygen species (ROS) in the body. Normally, the body has antioxidant systems to neutralize these harmful components [31]. However, excessive free radical production can have negative effects on cellular structures such as lipid peroxidation, protein denaturation, and DNA damage, which can lead to the development of many diseases such as aging, cancer, cardiovascular diseases, and neurological disorders [32]. Plants exhibit strong antioxidant properties with bioactive components such as phenolic compounds, flavonoids, alkaloids, and terpenoids [33]. These compounds can help protect cells from oxidative damage by neutralizing free radicals [34]. Plant antioxidants, in addition to their direct free radical scavenging ability, also provide more comprehensive protection by increasing the activity of the body's own antioxidant enzymes [35]. Therefore, the antioxidant potential of plants has become an important area of research in modern medicine in the treatment of diseases caused by oxidative stress [36]. In our study, the antioxidant potential of ethanol and methanol extracts of *C. colocynthis* was determined. The obtained findings are shown in Table 2.

Table 2. TAS, TOS, OSI, TPC and TFC values of *Citrullus colocynthis*

	Ethanol	Methanol
TAS (mmol/L)	8.750±0.147	7.453±0.153
TOS (µmol /L)	10.678±0.083	10.951±0.078
OSI (TOS/(TAS*10))	0.122±0.004	0.147±0.003
TPC (mg/g)	92.74±1.47	86.41±1.38
TFC (mg/g)	149.63±2.37	112.43±2.18

In the literature, the antioxidant potential of *C. colocynthis* has been reported using different methods [37-39]. In our study, TAS, TOS and OSI values were determined using Rel Assay kits. The antioxidant potential of *C. colocynthis* has not been reported with Rel Assay kits in the literature. It was detected for the first time in our study. TAS, TOS and OSI values of different plant species were reported using Rel Assay kits. In this context, TAS values of *Mentha longifolia, Anthemis cotula, Hypericum spectabile, Arum dioscoridis, Glaucium alakirensis* and *Alcea kurdica* were reported as 6.094, 7.625, 9.306, 6.486, 3.496 and 3.298 mmol/L. TOS values were reported as 14.050, 11.247, 13.065, 13.578, 2.204 and 8.312 µmol/L. OSI values were reported as 0.231, 0.148, 0.140, 0.209, 0.063 and 0.252 [40-45].

Compared to these studies, the TAS value of the ethanol extract of *C. colocynthis* was determined to be higher than *M. longifolia, A. cotula, A. dioscoridis, G. alakirensis* and *A. kurdica*, and lower than *H. spectabile*. The TAS value of methanol extract of *C. colocynthis* was determined to be higher than *M. longifolia, A. dioscoridis, G. alakirensis* and *A. kurdica*, and lower than *A. cotula* and *H. spectabile*. The TAS value is an indicator of the totality of antioxidant compounds produced in natural products [46]. The high TAS values of *C. colocynthis* show that this plant is an important antioxidant source and its capacity to neutralize free radicals is significant compared to some other plants. However, when the TAS values of ethanol and methanol extracts were compared with different plants, it was observed that some plants were higher and some plants were lower. This result suggests that the content of antioxidant compounds of *C. colocynthis* may vary from plant to plant and these differences may depend on the extraction method and the properties of the solvent.

The TOS value is an indicator of the totality of oxidant compounds produced in natural products [46]. In our study, it was observed that the TOS values of both ethanol and methanol extracts of *C. colocynthis* were lower than *M. longifolia, A. cotula, H. spectabile* and *A. dioscoridis*, and higher than *G. alakirensis* and *A. kurdica*. Since the TOS value indicates the level of oxidant compounds in plants, a low value suggests that the plant has a balanced structure in terms of oxidant components. In our study, it was determined that the TOS values of ethanol and methanol extracts of *C. colocynthis* were lower than some plants and higher than some plants. This situation reveals that the potential of the plant to produce different oxidant compounds is shaped by

environmental factors and the genetic characteristics of the plant. Regulation of oxidant compounds can strengthen the plant's defense mechanisms, which can contribute to being more resistant to stress factors in the natural environment.

The OSI value shows how much oxidant compounds are suppressed by antioxidant compounds [46]. The OSI value of the ethanol extract of *C. colocynthis* used in our study was determined to be lower than *M. longifolia*, *A. cotula*, *H. spectabile*, *A. dioscoridis* and *A. kurdica*, and higher than *G. alakirensis*. The OSI value of the methanol extract of *C. colocynthis* was determined to be lower than *M. longifolia*, *A. cotula*, *A. dioscoridis*, *A. kurdica*, and higher than *H. spectabile* and *G. alakirensis*. In this context, it was determined that *C. colocynthis* has a significant potential in suppressing oxidant compounds. The OSI value is an important parameter because it shows the interaction of antioxidant and oxidant compounds and the suppression rate of this interaction on the body. In our study, the OSI values of ethanol and methanol extracts of *C. colocynthis* revealed that this plant has an important potential in suppressing oxidant compounds. While the OSI value of the ethanol extract was lower than many plants, it was observed that the methanol extract was lower than certain plants. This suggests that methanol is a more effective solvent and that the methanol extract of *C. colocynthis* may have a stronger oxidative balancing capacity. As a result, it was determined that *C. colocynthis* showed the potential to balance antioxidant and oxidant components with high TAS and appropriate TOS values. These properties suggest that the plant may be an important source that can be used both in preventing oxidative stress and strengthening natural defense mechanisms. However, further research is needed to support the validity of these findings in larger studies and to better understand the clinical and pharmaceutical potential of *C. colocynthis*.

3.3. Total phenolic and total flavonoid contents

Plants are rich in phenolic and flavonoid components, and these components play a role in the defense mechanisms of the plant and may have beneficial effects on health by exhibiting various biological activities [47]. In our study, total phenolic and total flavonoid contents of ethanol and methanol extracts of *C. colocynthis* were determined. The findings are shown in Table 2. As a result of the analyzes, the TPC values of ethanol and methanol extracts of *C. colocynthis* were determined as 92.74±1.47 and 86.41±1.38 mg/g, respectively. In addition, TFC values were determined as 149.63±2.37 and 112.43±2.18 mg/g, respectively. In the literature, the total phenolic content of *C. colocynthis* was reported as 5.39 mg/100 g and the total flavonoid content as 938.0 mg/100 g [48]. Compared to this study, the total phenolic content (TPC) value determined in our study was significantly higher, while the total flavonoid content (TFC) was determined at lower levels. This difference may be due to the differences in the extraction methods used, solvent type, plant growth conditions, developmental stage and analysis techniques. The amount of phenolic and flavonoid components may vary depending on the ecological factors (such as soil structure, water stress, duration of exposure to sunlight) where the plant grows [49]. In addition, the chemical polarity of the solvent used in the extraction method may affect the extraction efficiency of different phenolic compounds [50]. The higher TPC and TFC values of the ethanol extract compared to the methanol extract in our study may be attributed to the fact that ethanol can dissolve flavonoids and phenolic compounds more effectively.

In addition, the fact that the total flavonoid content reported in the literature is quite high, but was measured at lower levels in our study may also be due to differences in the analysis methods used. The aluminum chloride spectrophotometric method used to determine flavonoids may be more sensitive to some flavonoid compounds, and this may lead to variable results among studies [51].

In conclusion, these differences in phenolic and flavonoid content are important in understanding the biological activity and pharmacological potential of the plant. Future studies may provide more comprehensive information by optimizing the extraction parameters, detailed characterization of phenolic and flavonoid components, and investigating their effects on biological activities.

3.4. Anticholinesterase activity

The anticholinesterase activity of plants is important for the discovery of natural compounds that offer a potential treatment options, especially in the treatment of neurological diseases. These activities aim to improve neurological functions by inhibiting the acetylcholinesterase enzyme by the active compounds in herbal extracts and increasing acetylcholine levels [52,53]. In our study, acetylcholinesterase and butyrylcholinesterase activities of *C. colocynthis* were determined. In this context, the potential of the plant against neurodegenerative diseases such as Alzheimer's was determined. IC50 values of the obtained findings are shown in Table 3.

Table 3. Anti-AChE and anti-BChE values of *Citrullus colocynthis*

	AChE (µg/mL)	BChE (µg/mL)
Ethanol	14.96±1.16	31.00±0.96
Methanol	22.62±1.06	41.60±1.09
Galantamine	7.38±0.27	14.33±0.42

It has been reported in the literature that the acetylcholinesterase activity of the methanol extract of *C. colocynthis* is 131.01 µg/mL [54]. Compared to this study, the methanol extract of *C. colocynthis* used in our study exhibited higher acetylcholinesterase activity. There are several possible reasons for these differences. First of all, the biological activity of herbal extracts may vary depending on various such as growing conditions (soil structure, climate, geographical region), harvest time and extraction methods [50]. In addition, different solvents such as ethanol and methanol can dissolve and extract different components in herbal extracts. Ethanol is a solvent generally used to obtain extracts rich in polyphenols and flavonoids, and it is known that these compounds play a role in acetylcholinesterase inhibition [55]. In our study, the higher activity of the ethanol extract compared to the methanol extract suggests that ethanol may have extracted more effective compounds that may act as acetylcholinesterase inhibitors. However, when compared to the standard galantamine, both

extracts were found to have lower activity. This may be due to impurities, low specific activities or insufficiently high inhibitory concentrations of active compounds found in herbal extracts [56]. Therefore, fractionation and isolation studies are needed to make the extracts more effective. Future studies should include detailed analysis of the chemical profiles of the extracts, identification of the main active compounds and elucidation of the mechanisms of their inhibitory effects on acetylcholinesterase. In particular, identification of the phytochemical components underlying the higher activity of the ethanol extract compared to the methanol extract may help us better understand the therapeutic potential of the plant.

4. Conclusion

The findings of this study highlight the significant biological potential of *C. colocynthis* extracts, specifically those obtained through ethanol and methanol. Both extracts exhibited notable antimicrobial and antioxidant activities, with the ethanol extract showing a particularly strong antimicrobial effect. Additionally, both extracts demonstrated substantial acetylcholinesterase inhibitory activity, suggesting a potential role for *C. colocynthis* in the management of neurodegenerative diseases, such as Alzheimer's disease. The observed biological activities correlate with the high concentrations of phenolic compounds and flavonoids present in the extracts, which are likely responsible for their bioactivity. This study provides scientific support for the traditional use of *C. colocynth*is in herbal medicine and underscores its potential as a promising candidate for further pharmaceutical development. However, additional studies are required to evaluate the toxicity profile, pharmacokinetics, and clinical efficacy of *C. colocynthis* extracts before considering their therapeutic application.

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