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The Antimicrobial Activity of *Citrus limon* L. against Foodborne Pathogens and Its Anti-Oxidant and Antibiofilm Properties

Research Article

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Abstract

The objective of this study is to develop a new and effective antibacterial agent against food pathogens that poses a major threat to human health and to investigate the antioxidant activity of this plant. Methanol, ethanol, and aqueous extracts were analyzed for antimicrobial potency. Eight different microorganisms were used in the study, one of which was yeast. These microorganisms are food pathogens. Antimicrobial activity testing was performed using a disc diffusion method. Another test for antimicrobial activity is the minimum inhibitory level. Antioxidant activity was conducted using 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•) and 1,1-diphenyl-2-picrylhydrazyl (DPPH•). *Citrus limon* ethanol extract had a maximum inhibiting zone against *Escherichia coli* (12 mm). In the ABTS method, the highest scavenging activity was obtained from an ethanol extract (58.3 percent). The methanol extract (95.4%) led to the largest DPPH scavenging activity. Consequently, *Citrus limon* extracts have antimicrobial, antioxidant, and anti-biofilm potential against foodborne pathogens.

Key Words: Citrus, Food pathogen, Antimicrobial activity, Antioxidant activity, Antibiofilm activity, TLC.

Introduction

Microbial damage to food is caused by the uncontrolled growth of undesirable microorganisms. In European countries, over 23 million people get sick every year as a result of eating contaminated food (1). Although chemical antimicrobial agents have been used in food processing for decades to reduce the effect of microbial contamination, the demand for natural food ingredients and additives; Including natural antimicrobial compounds, is now on an upward trend (2, 3, 4). Herbal drugs are natural whereas in contrast, treatment provided by chemically synthesized drugs has side effects on our body.

Excess drug use has resulted in bacterial resistance (5). As there is an increase in antibiotic resistance to some bacteria, there is always research for an alternative drug (6, 7). The issue of microbial resistance to existing antibiotics can become attractive to conventional medicine (8). The issue of resistance of bacteria to antibacterial drugs is one of the most significant global challenges. Furthermore, plant-derived antibacterials are attractive because they often lack the many side effects associated with synthetic antibacterials. New antibacterial components from natural sources are therefore being investigated (9).

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Moreover, bacterial biofilms are surface-associated microbial communities contained within an autogenerative exopolysaccharide matrix (10). Biofilms are responsible for about 65% of all bacterial infections in humans (11). Infection caused by a microorganism is further complicated by its ability to produce biofilms, thereby reducing antimicrobial penetration (12, 13, 14).

Over the past few years, consumer desire for natural ingredients and preservative-free foods has increased the popularity of natural antimicrobials (15). To this end, the effectiveness of medicinal and aromatic plants and their extracts for food safety and preservation purposes was assessed. The majority of their preservative properties are due to their essential oils (EOs) and other secondary components of plant metabolite (16). EOs have well-known properties, like antimicrobial and antioxidant properties. Herbal medicine has gained a great deal of attention and awareness as a substitute for the treatment or prevention of health-related disorders (17). Today, around 80% of the world's population relies on local herbal drugs (18). Antimicrobially active herbs and spices have been widely used traditionally and commercially to increase the shelf life and safety of food (19, 20). According to the World Health Organization (WHO), medicinal plants are the most important source of drug supplies. Therefore, these plants should be studied for a better understanding of their properties, safety and efficacy (21).

The genus *Citrus* belongs to the family Rutaceae which comprises about 140 genera and 1300 species and, for example, *Citrus limon* (Lemon) is amongst the important species of the genus *Citrus* (22). *Citrus* is one of the most significant commercial and industrial agricultural activities in the World (23). Turkey is one of the most important nations among

Citrus growers. The genus *Citrus* consists of evergreen plants, shrubs or trees (3-15 m in height). Their leaves are leathery, egg-shaped or elliptical. A few have spikes. The flowers grow individually in the axilla of the leaves. Each flower has 5 petals, white or red. The fruit is a citrus berry. *Citrus* is naturally occurring in warm and mild climate regions, mainly in the Mediterranean region. They are typically susceptible to frost (24). One of the most widely known and used species in the genus *Citrus* is the lemon—*Citrus limon* (L.) Burm. f. (Latin synonyms: *C.*x *limonia*, *C. limonum*). *Citrus* fruits have been used by people for centuries for agricultural, medical and herbal purposes (25). Several pharmacological properties have been ascribed to different members of *Citrus* species. These properties are anticancer (26, 27, 28, 29, 30), antifungal (31), anti-typhoid (29), anti-oxidant (32), antiulcer (33), hypolipidemic (34). Carvone and limonene have a broad range of anti-fungal and anti-microbial activities (35, 36). No studies with *Citrus* lemon flowers were found in the literature. As part of this study, various *Citrus* flower extracts were studied for antimicrobial, antioxidant and antibiofilms activities. This research aims to contribute to the lack of knowledge about the antimicrobial, antioxidant and antibiofilm activities of *Citrus limon* flowers.

Material and Methods

Microorganisms and Cultivation

In this work, *Citrus limon* flowers were singly tested against food pathogens such as *Bacillus subtilis* RSKK245, *Staphylococcus aureus* RSKK2392, *Salmonella typhimurium* RSKK19, *Enterococcus faecalis* ATCC8093, *Escherichia coli* ATCC11229, *Listeria monocytogenes* ATCC7644, *Yersinia enterocolitica* NCTC11174, and *Candida albicans* RSKK02029. The bacteria were grown for 24 hours, at 37˚C in Mueller Hinton Broth (Merck). *C. albicans* was grown for 24-48 hours, at 30°C in Sabouraud Dextrose Broth (Merck). These pathogens were provided from different culture collections. These strains of bacteria and *C. albicans* were obtained from ATCC (American Type Culture Collection, USA), RSKK (Refik Saydam National Type Culture Collection, Turkey), or NCTC (National Collection of Type Cultures).

Plant material

Citrus limon L. flowers were obtained from different local herbalists in the Mugla region, Turkey in 2019. The taxonomic identification of the plant was performed by Dr. Olcay CEYLAN (voucher number: 1300) from Mugla Sitki Kocman University, Turkey and a specimen was stored in the herbarium. The identity of this specimen was applied by the Flora of Turkey (37).

The flowers were cleaned thoroughly two to three times with flowing and sterile distilled water. These materials were dried by air. All of the samples were stocked at room temperature. Then, the plant parts pulverized in a disruptive mill. Then the plant parts were stocked at 4°C until needed for assay.

Plant Extraction

The dried matter (50 g of flower) was extracted with organic solvent (250 ml of ethanol, methanol, and water) using the Soxhlet device (8 hours). The materials have evaporated. Subsequently, the materials were stored in matte cylinders. These vials were stored and refrigerated for use (300 mg/ml).

Antimicrobial Activity Assay

In this study, the determination of Kirby-Bauer was applied for antibacterial activities (38). Bacteria were stored in plates of MHA at 37˚C. Microbiological cultures adjusted to 0.5 McFarland. Incubation of the bacteria took place at 37˚C over a 24-hour period. The yeast incubation was at 30˚C during 24 hours. The assessment of antibacterial activity was based on measuring the diameter of the inhibition zones around the disks after 24 hours. Ethanol, methanol, water, chloramphenicol (30 µg) and nystatine (100 µg) were used as controls in this study. As well, the extract concentration was used at 300 mg/ml.

Minimum Inhibitory Concentration Assay (MIC)

The MIC for plant extracts was measured using the broth dilution assay. The MIC values were reported as the lowest concentration of herbal extracts. The broth dilution test was performed according to the literature (39, 40, 41). We have used final concentrations of the extract in our work. These concentrations were in the range of 13000 µg/ml to 1625 µg/ml (13000; 6500; 3250; and 1625 µg/ml).

Determination of Non-Enzymatic Antioxidant Activity

Two methods have been used for non-enzymatic antioxidant activity experiments. The non-enzymatic antioxidant activity was investigated with the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Stable DPPH was used to determine free radical sweep activities for the extracts. The extract (0.1ml) was added to the methanol DPPH mixture (0.1mM). This solution was incubated for 30 minutes, then the absorption of the solution was measured by spectrophotometry. Methanol was blank. Methanol with DPPH solution was utilized as a control variable (42). The free radical recovery capability expressed as a percentage (%) was calculated from the formula.

The antioxidant activity of the flower extract was determined using ABTS [2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)], as described in (43). The radical sweep activity was determined within 15 minutes of incubation with extract. As documented, a 7 mM ABTS solution was mixed with a 2.4 mM potassium persulphate solution. In order to maintain the stability of the ABTS radical, the mixture was left in darkness for 12 to 16 hours. Subsequently, 10 µl of extract solution was added in 1 ml of stable ABTS radical. The absorbence of this mixture (extract solution + stable ABTS radical) incubated over 15 minutes was measured at a wavelength of 734 nm. The percentage ABTS ratios were determined by the absorbence values according to the formula given.

In studies of antioxidant activity, trolox has been used as a positive control of radical sweeping activities. Results were reported as mM Trolox equivalent (TE)/g dry weight. To obtain the standard Trolox curve (pH 7.4), different concentrations of solvents were prepared (0.25, 0.5, 1, 1.5, 2, 2.5 mM) in 5 mM saline phosphate buffer (PBS) and the absorbence of these samples was measured as described in (44).

Antibiofilm activity assay

Sterile 96-well polystyrene tissue culture plates were used as part of this study. Brain Heart Infusion Broth (100 µl), culture (50 µl) and plant extracts were placed in each well. Concentrations of plant extracts included 13000, 6500, 3250 µg/ml. All plaques were incubated at 37˚C for 24 hours. At the end of incubation, the plates were cleaned with phosphate buffered saline (PBS) three times. Once the nonadherent cells were removed, the plates were dried in the air. The purple crystal solution (% 0.4w/v) was added to the adhesive biofilm. Plates were incubated for about ten minutes. The absorbence measured at 570nm. The activities of antibiofilm were calculated using the literature formula (45).

Thin Layer Chromatography

Thin-layer chromatography was performed on the three extracts of *Citrus limon* (L) using a plate of alumina TLC whose surface was covered with silica gel. Thin-layer chromatography was carried out on slabs of silica gel 60F254 TLC (Merck, Germany, 1.05554.0001). The TLC plate was 10cm x 10cm, with a pencil mark approximately 2cm from the bottom edge of the original plate end. Prior to use, the plates used in TLC were activated at 120˚C during 10 min. The extraction samples (4 µL) were located on the chromatographic plates. The mixture of ethanol: hexane (90:10, v/v) was used as the moving phase (50 ml). The plates were developed vertically at room temperature (20˚C) to a distance of 7.5 cm in a mobile phase glass cabinet and dried for 24 hours at room temperature (20˚C) hood.

Stains of separate compounds were detected with a vanillin ethanol solution (1g vanillin, 100 ml 95% ethanol and 10 ml 95% sulphuric acid (VI)). The plate was heated to 100˚C until the coloured stains were visible. Stains were detected through a UV transilluminator (CAMAG; Swiss; λ = 295 nm).

The area of a point in the chromatogram matching the band was noted. The Rf value was calculated according to the formula.

Statistical analysis

In this study, the means of the activities were calculated with excel 2016.

Results

Antimicrobial activity studies were performed using a disc diffusion method. In this study, the highest inhibitory area was determined from the ethanol extract of the flower against *Escherichia coli.* This area is 12mm. *Staphylococcus aureus* was not affected by the flower extracts. The flower extracts were highly efficient against *Candida*. Ethanol extract produced a high inhibition area for *Candida albic*ans (11 mm). However, the inhibitory zone of the nystatin antibiotic was 7 mm (Table 1).

Table 1. Antimicrobial activities of *Citrus limon* flower extracts (300 mg/ml)

Microorganisms	Inhibition zone diameters (mm)			Antibiotics		Solvents		
	EE	ME	AE	C	N	E	M	A
Bacillus subtilis RSKK245	-	-	8	12	nt	-	-	-
Staphylococcus aureus RSKK2392	-	-	-	15	nt	-	-	-
Salmonella typhimurium RSKK19	8	7	7	22	nt	-	-	-
Enterococcus faecalis ATCC8093	8	-	8	22	nt	-	-	-
Escherichia coli ATCC11229	12	11	9	22	nt	-	-	-
Listeria monocytogenes ATCC7644	-	-	7	22	nt	-	-	-
Yersinia enterocolitica NCTC11174	8	7	9	20	nt	-	-	-
Candida albicans RSKK02029	11	10	9	nt	7	-	-	-

EE: Ethanol extract; ME: Methanol extract; AE: Aqueous extract; C: Chloramphenicol; N: Nystatin; nt: not tested; (-): no inhibition

The other antibacterial activity test was MIC. The lowest MIC value is 3250 µg/ml. This value was determined for *Salmonella typhimurium*, *Enterococcus faecalis* and *Escherichia coli*. The lowest MIC value was obtained using the aqueous extract of *Citrus limon* flowers (Table 2).

Table 2. Minimum inhibitory concentrations of *Citrus limon* extracts (µg/ml)

Microorganisms	EE	ME	AE
Bacillus subtilis RSKK245	nt	nt	6500
Staphylococcus aureus RSKK2392	nt	nt	nt
Salmonella typhimurium RSKK19	13000	6500	3250
Enterococcus faecalis ATCC8093	6500	nt	3250
Escherichia coli ATCC11229	6500	6500	3250
Listeria monocytogenes ATCC7644	nt	nt	6500
Yersinia enterocolitica NCTC11174	13000	13000	13000
Candida albicans RSKK02029	6500	13000	>13000

EE: Ethanol extract; ME: Methanol extract; AE: Aqueous extract; nt: not tested

The nonenzymatic antioxidant activity studies were performed by ABTS and DPPH radical scavenging. In the ABTS method, the strongest activity was determined using flower ethanol extract (53%). In addition, DPPH scavenging activity was 95.4 percent (Table 3).

Table 3. Antioxidant activities of *Citrus limon* extracts (300 mg/ml)

Activity / Extracts	ABTS·		DPPH·	
	Scavenging activity (%)	TE	Scavenging activity (%)	TE
EE	58.3	1.95	75.2	2.31
ME	52.15	1.85	95.4	2.57
AE	52.34	1.85	87.4	2.46

TE: mM Trolox equivalent/ g DW; EE: Ethanol extract; ME: Methanol extract; AE: Aqueous extract; DW: Dry weight

Citrus limon extracts showed an effective anti-biofilm action against the strains tested. However, none of the extracts could completely inhibit the formation of bio-film. At the MIC value in this study, *Citrus limon* flower methanol extract had the highest inhibition rate (20.4%) against *Escherichia coli* biofilm formation. Extracts of *Citrus limon* have been found to inhibit the formation of biofilm of *Enterococcus faecalis, Escherichia coli, Salmonella typhimurium, Candida albicans* and *Bacillus subtilis*. The best reduction in biofilm is observed at higher concentrations of extracts (*Citrus limon* AE 56.9%). The aqueous extract of *Citrus limon* at a concentration of 13000µg/ml showed the highest antibiofilm activity (56.9%) against the formation of *Bacillus subtilis* biofilm. The ethanolic extract of *Citrus limon* flowers at a concentration of 13000 µg/ml showed the highest inhibition rate (32.9%) against the formation of *Listeria monocytogenes* biofilms while the methanolic extract of *Citrus limon* exhibited a 52.7% inhibition of the formation of *Salmonella typhimurium* biofilms at a concentration of 13000 µg/ml (Table 4).

The presence of various phytoconstituents was confirmed by thin-layer chromatography. The number of points observed and their corresponding Rf values are given in Figures 1 and 2. Three spots with Rf values of 0.53, 0.60 and 0.64 were observed for ethanol extract from *Citrus limon*, whereas three spots with Rf values of 0.52, 0.69 and 0.77 were observed for methanol extract, respectively. However, two spots were identified from the aqueous extraction (Figure 1 and 2).

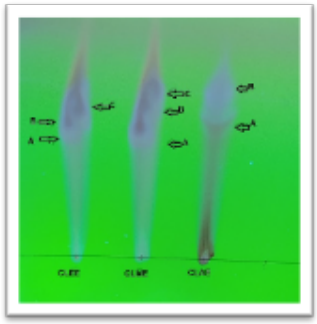

Figure 1: a) The chromatogram of compounds in UV of *Citrus limon* flower extracts

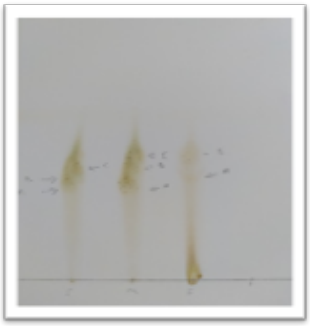

Figure 1: b) TLC image of *C. limon* extracts

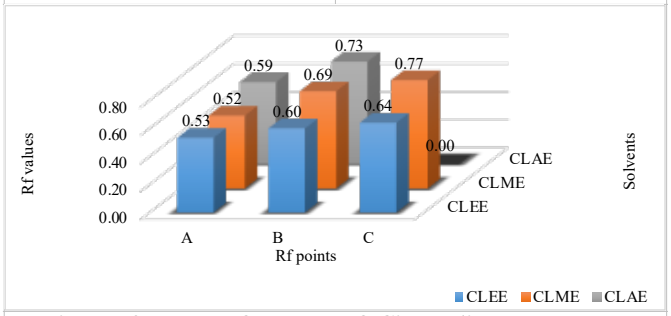

Figure 2: The Rf values of *Citrus limon* extracts
CLEE: *Citrus limon* ethanol extract; CLME: *Citrus limon* methanol extract; CLAE: *Citrus limon* aqueous extract

Table 4. Antibiofilm activities of *Citrus limon* extracts

Organisms	Extracts								
	EE			ME			AE		
	Concentrations of extracts (µg/ml)								
	13000	6500	3250	13000	6500	3250	13000	6500	3250
	Inhibition rates (%)								
B. subtilis RSKK245	27.7	7.8	3.0	46.4	35.5	8.7	56.9	27.3	8.9
S. aureus RSKK2392	17.1	3.4	1.9	39.0	24.2	19.3	36.5	18.3	5.0
S. typhimurium RSKK19	11.7	3.6	1.4	52.7	22.6	13.3	28.9	18.2	10.8
E. faecalis ATCC8093	13.9	9.6	1.9	31.1	25.1	9.5	46.2	26.9	13.8
E. coli ATCC11229	16.6	11.7	10.3	46.0	33.4	20.4	36.8	25.7	9.3
L. monocytogenes ATCC7644	32.9	10.1	5.3	25.1	17.9	13.7	21.1	7.0	3.6
Y. enterocolitica NCTC11174	18.6	16.7	11.9	35.4	17.1	0.7	37.9	22.9	6.2
C. albicans RSKK02029	32.0	19.9	5.4	21.0	11.7	3.7	44.3	29.5	10.0

EE: Ethanol extract; ME: Methanol extract; AE: Aqueous extract

In this study, three metabolites were found in ethanol and methanol extracts, while two metabolites were found in the aqueous extract (Figure 2).

Discussion

Medicinal plants play a major role in the prevention of various diseases and have attracted the attention of numerous researchers in recent years. Herbs and essential oils have served as preservatives and sweetening agents in foods for years. The use of synthetic additives and antimicrobials, which have increased with developing technology, has identified problems such as many side effects on the health and resistance of microorganisms to synthetic antimicrobials, and there is a need to look at additional resources.

In this study, the highest anti-bacterial activity was determined against *E. coli* and this area was 12 mm. However, 8 mm inhibition zones were found for *S. typhimurium*, *E. faecalis* and *Yersinia enterocolitica*, 11 mm for *Candida albicans* (Table 1). Okmen et al. (46) reported that cyclamen ethanolic extract was inhibited by 10 mm against *E. coli*. During the study, Otang and Afolayan (47) detected a 20 mm inhibition zone for *S. typhimurium* and a 15 mm inhibition zone for *E. coli*. Hindi and Chabuck (48) studied the peel, juice and dried form of *Citrus limon* in their study and received different results. Although the peeled parts are effective for *S. aureus* (30 mm) and *E. faecalis* (30 mm), their juice is effective for *S. aureus* (26 mm), *E. faecalis* (28 mm), *Candida albicans* (30 mm), *E. coli* (10 mm) and *Salmonella* Typhi (30 mm). While the literature results are consistent with our studies, there are also differences. This may be because of different phytochemical composition in different parts of the plant, different extraction processes, and environmental factors, as well as differences in the genotypes of the plant used. Tajkarimi et al. (49) reported that plant-based substances can have antimicrobial effects through a variety of mechanisms, including by attacking the phospholipid bilayer in the cell membrane of the bacterium, by interfering with enzymatic systems or by altering genetic material.

In the current study, the MIC for *Salmonella typhimurium*, *E. faecalis* and *E. coli* was 3250 µg/ml (Table 2). Rahman et al. (50) reported that *Citrus limon*'s MIC value for *Salmonella* Typhi was 3125 ppm. Ewansiha et al. (51) found the MIC for *C. limon* peel essential oils for *S.* Typhi to be 50 mg/ml. These literatures-based results are parallel to our studies, but there are a few differences. These differences may be due to differences in sample cultures, substance levels and extraction methods for essential oils (52). The mechanism of the antimicrobial activity of essential oils is associated with the attack of phospholipids present in the cell membrane, thereby increasing the permeability and leaching of the cytoplasm or their interaction with enzymes located in the cell wall. This means that phenolic compounds in essential oils inhibit the growth of microorganisms by sensitizing the cell membrane phospholipid bilayer, thus increasing its permeability and leading to leakage of vital intracellular components or disruption of bacterial enzyme systems (53, 54, 55, 56, 57). Danila et al. (58) reported that *C. limon* contains numerous monoterpenes, oxygenated monoterpenes and sesquiterpenes.

Recently, interest in natural antioxidants has grown steadily because synthetic antioxidants have side effects such as carcinogenesis. In the current study, the highest antioxidant activity of *Citrus limon* was determined from the ethanol extract by ABTS method, with a scavenging rate 58.3%. However, the highest antioxidant potency of methanol extract was found to be 95.4% in the DPPH method (Table 3 and 4).

Xi et al. (59) reported that the greatest DPPH recovery activity from the peel was 7.45 µM TE/g DW, while the greatest ABTS recovery activity was 8.65 µM TE/g DW from the peel of the plant. Yang et al. (60) found that lemon has greater scavenging activity than mint in their study. Oekeh et al. (61) examined the antioxidant activity of various fruit juices and reported DPPH scavenging activity in lemon juice to be 5%. Makni et al. (62) indicated that the antioxidant activity (70%) of tasty lemon at 1000 µg/ml was higher than that of succulent lemon (20%). Rowshan and Najafian (63) reported that the scavenging activity for DPPH from *Citrus limetta* skin was 87.7%. Differences in radical scavenging activities may depend on geographical origins, weather and seasonal conditions, collection time, stage of development, extraction method and the presence of new chemotypes (64). It can be linked to changes in the chemical content of plants, soil composition, daily or seasonal changes in plant material during collection, physiological development of plants, with the type of bacteria (65, 66, 67, 68).

Thin-layer chromatography is usually carried out to better identify bioactive compounds. This study demonstrated that TLC profiling of all plant extracts had diverse metabolites such as alkaloids, flavonoids, phenols and tannins. Among the three solvents (ethanol, methanol and water), ethanol and methanol were observed to be efficient at extracting the maximum number of secondary metabolites (Figure 1 and 2). Kumar et al. (69) have also reported various phytochemical compounds in lemon peel ethanolic extract. Various Rf values of the compounds give an idea of their polarity which can also help to choose a particular solvent system for the additional isolation of any compound of plant extracts using chromatographic and spectroscopic techniques (70). The sites from which plant samples were collected are of varying altitudes, soil pH, and soil types, which likely contributed to differences in the phytochemical composition of plant samples. This is consistent with earlier findings that plants growing in areas of different altitudes and soil types have different phytochemical compositions (71, 72).

Conclusion

In this study, different extracts of *Citrus limon* have different effects against microorganisms. One of the most important outcomes of this study is radical scavenging activities. The presence of strong antioxidants in all extracts has been observed by DPPH

research. *Citrus limon* extracts can be beneficial as a good anti-oxidant protective system for the human body against oxidative damage. Based on the current findings, it can be suggested that herbal extracts can be used as a source of a natural antibacterial compound with strong antioxidant potential. The study reveals that *C. limon* flowers are a rich source of active compounds with diverse medicinal and pharmacological properties that make it an attractive product, alternative, and cheap source of functional ingredients for functional food and nutraceutical formulation. However, further studies are required to focus on in vivo studies using biologically active compounds. As well, further studies are required for fractions, characterization, and study of active ingredients.

Conflict of interest

The authors state that there is no conflict of interest concerning the publication of this document.

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