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Bioactive compounds of *Citrullus colocynthis* aerial parts: characterization and biological properties

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ABSTRACT

In this study, the bioactive components of *Citrullus colocynthis* extracts were analyzed in view of their potential use as antioxidant/antibacterial agent. The bioactive compounds were extracted in different organic solvents (hexane, ethanol and ethyl acetate) and analyzed by column chromatography, thin-layer chromatography, high-performance liquid chromatography and gas chromatography-mass spectrophotometry (GC-MS). Three distinct fractions were collected and subsequently examined, one from ethyl acetate extraction (I) and two from ethanolic extraction (II and III). *p*-Coumaric acid was the major phenolic acid from the ethyl acetate extracts (0.11 ± 0.023 mg/kg) while resorcinol was the major phenolic compound from the ethanol extracts (25.00 ± 3.21 mg/kg). Twenty-four bioactive compounds were identified by GC-MS analysis where 1-docosanol pentafluoropropionate was the major bioactive compound in ethanol extracts. Among the ethanol extracts, the highest antioxidant activity was found in fraction III. The highest anti-bacterial activity values, measured in terms of the size of the inhibition zone (mm) against *Bacillus cereus* and *Staphylococcus aureus*, i.e. 24 ± 1.62 and 22 ± 2.19 mm, respectively, were attained at 24 µg/mL for fractions III and I. From the results achieved in this work, ethanol extracts of *C. colocynthis* aerial parts could be considered promising as antioxidant and anti-infectious agents.

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Citrullus colocynthis aerial parts; bioactive compounds; hPLC; GC-ms; antioxidant; antibacterial

Introduction

Members of the gourd family (Cucurbitaceae) are resistant to drought yet vulnerable to frost. About 965 plant species belong to this family and some of the most well-known members of this family are cucumbers, pumpkins, squashes and watermelons.

Within this family, '*Citrullus colocynthis* (L.) Schrad', which is known as handhal (in Arabic) or bitter apple (in English) can be listed (Degola et al. 2019). The Asian desert nations of Kuwait, Saudi Arabia, Iraq, Jordan, Lebanon, Syria and Yemen are home to a large population of *C. colocynthis*. The plant is presently found throughout North Africa in countries including Algeria, Egypt, Libya, Morocco and Tunisia (Al-Snafi 2016a). The World Health Organization (WHO) reports that more than 80% of people in Third World countries still use traditional medicine to treat the majority of common ailments (WHO 2022).

The chemical composition of *C. colocynthis* includes a variety of bioactive compounds as well as carbohydrates, proteins, free amino acids, tannins, saponins, phenolics, flavanoids, flavone glucosides, terpenoids, alkaloids, anthranol, steroids, cucurbitacins, saponarin, trace elements and many others. It has a broad spectrum of pharmacological properties, such as analgesic, gastrointestinal, reproductive, protecting, anti-diabetic, antibacterial, antitumor and anti-inflammatory (Al-Snafi 2015, 2016b). The biological activity and chemical composition of the plant can be impacted by many environmental and developmental factors. The phenolic content and antioxidant activity of various *C. colocynthis* fruit sections have not yet been studied in relation to seasonal change. The weather patterns present at different periods of the year may have an impact on the antioxidant activity, which is typically linked to active phytomolecules like total phenolics (Aires et al. 2011; Ribeiro et al. 2020).

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Polyphenols are naturally occurring chemicals that neutralize free radicals including hydroxyl and peroxyl radicals and act as antioxidants. Phytochemicals such as polyphenols give handhal extracts their antioxidant and free radical-scavenging properties (Sebbagh et al. 2009). All portions of handheld fruits have been studied for their polyphenol content and antioxidant activity (Hussain et al. 2013; Rizvi et al. 2018). Polyphenols, which include phenolic acids, flavonoids and tannins, are also widely known for their antimicrobial properties. Besides, alkaloids, essential oils, glycosides, steroids and terpenoids make up the largest classes of bioactive chemicals (Ferreira et al. 2022).

To properly evaluate the biological properties, it is imperative to employ adequate analytical techniques to determine all biological compounds, both volatile and nonvolatile. Moreover, to guarantee the identification of both polar and nonpolar substances, each method should also include a variety of extraction solvents (Ferreira et al. 2022).

Tests on the inhibitory and bactericidal effects of crude extracts from the aerial parts of *C. colocynthis* and ripe deseeded fruits were conducted on the drug-sensitive standard strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294), 16 drug-resistant strains of *Mycobacterium tuberculosis* and 2 *Mycobacterium* strains other than tuberculosis (MOTT) (Mehta et al. 2013). The *C. colocynthis* fruit extracts in acetone, ethanol, methanol and distilled water showed the strongest antibacterial effects against *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and other bacteria. Petroleum ether extract, however, performed worse against test microorganisms (Rodge and Biradar 2012).

The aim of this study was to investigate the bioactive components in the aerial parts of *C. colocynthis* ethanol extracts along with its antioxidant and antimicrobial activities under specific conditions, such as different extraction methods or variations in growth media, which have been only little explored to date. Results suggest that this extract is a good candidate for practical applications in the pharmaceutical and food industries.

Materials and methods

Materials

Fresh aerial parts of *C. colocynthis* were harvested without any physical damage and microbial exposure in the flowering season (June and July 2022) from northern Iraq with varied climatic temperatures ranging from 38 °C in the daytime and 20 °C during the night. The soil on which *C. colocynthis* was growing consisted of silt and sand. Plant harvesting location was suggested by experts and material was harvested under sterile conditions by wearing hand gloves and preserved in a sterile plastic bag until further use. Authentication of plant material was done by the Ministry of Agriculture, Baghdad, Iraq. The plants were washed with sterile distilled water and then air-dried at room temperature (ca. 25 °C) in darkness as phenolics are prone to photodegradation (Wiktor et al. 2019). Subsequently, the plant materials were kept in a hot air oven (HAO) for 48 h at 36 °C. The dried parts of the plant were vigorously ground into a very fine powder with a mortar and pestle. After shredding, the plant powder was filtered through a sterile chiffon cloth and then stored at −20 °C for further investigation. All chemicals utilized (ethyl acetate, hexane, methanol, ethanol, chloroform, acetonitrile, vanillin-sulphuric acid, formic acid, phosphoric acid, p-coumaric acid, quercetin, resorcinol and cinnamic acid) were of analytical standard and obtained from Sigma Aldrich (Bagdad, Iraq).

Extraction of C. colocynthis by Soxhlet

Five grams of harvested and powdered plant material was subjected to Soxhlet apparatus using diverse organic solvents (100 mL) including hexane, ethanol and ethyl acetate. The extraction process was carried out at a temperature of 60 °C in a water bath and the complete process was replicated three times. The extracted solvents were evaporated using a rotary evaporator. Further, the extracts were filtered with a polytetrafluoroethylene (PTFE) 0.22-µm filter. The extracted materials were weighed and preserved in sterilized dark airtight vessels for further assays (Wiktor et al. 2019).

Isolation and fractions

Column chromatography (CC) was utilized to isolate and fractionate the *C. colocynthis* extracts. The column was packed with 300 g silica gel (Sigma Aldrich, Baghdad, Iraq) (60–120 Adsorbent) spending wet packaging method. The extracts were mixed homogeneously with a minimal amount of silica gel on the column. Different solvent mixtures were used as a mobile phase for column elution systems including ethyl acetate, hexane and ethanol. Each fraction was then purified by paper chromatography. Out of the collected fractions, only the ethanol one was subsequently subjected to thin-layer chromatography (TLC) prior to further analysis (Mohammed et al. 2021).

Thin-layer chromatography (TLC)

Thin-layer chromatography was executed based on the previously adopted methodology (Bourgou et al. 2016). An aluminum-backed TLC device was employed with 2 µL of crude plant extracts. The TLC plates (20 cm x 20 cm) were coated with 0.2 mm thickness silica gel 60 F254 (Millipore, E. Merck, Billerica, MA, USA). Different ratios of solvents including chloroform, ethyl acetate and formic acid (10:8:2 *v/v/v*) were utilized. Using a UV-visible spectrophotometer (Sunny UV.7804C, Tokyo, Japan), the developed plate was observed at the wavelengths of 254 nm and 365 nm. The TLC plate was treated with vanillin-sulphuric acid prior to heating. The Relative Factor (R_f) value for each spotted compound was calculated and compared with each corresponding standard. Fractions with the same $R_f$zone were distinguished and individual bands were concentrated through the rotary evaporator. Fractions were further examined by high-performance liquid chromatography (HPLC) for the determination of the phenolic compounds.

Analysis of phenols by HPLC

HPLC was utilized to analyze the phenolic components extracted from the crude extracts. The analytical system, equipped with silica-based C18 column (Agilent Technologies, Santa Clara, CA, USA), consisted of a system controller (SCL-10A VP), an UV-VIS detector (SPD-10A), an LC pump (LC-10AT) and an auto-sampler (SIL-10AF). The analytical column was a Chiralcel® OD-RH with the following dimensions: 150 *L* × 4.6 mm I.D.; 5 µm particle size (Chiral Technologies Inc. Exton, PA, USA). Acetonitrile, water and phosphoric acid (30:70:0.08, *v/v/v*) was used as a mobile phase under isocratic conditions at a temperature of 25 ± 1 °C, run at a flow rate of 0.4 mL/min. Individual run time was fixed at 8 min, followed by a clean-up of 15 min. Isolated phytocompounds were detected at a wavelength of 288 nm (Skendi et al. 2017). Compound identification was carried out by comparing retention times of the analytes with the corresponding reference standards.

Analysis of volatiles by GC-MS

Esterification of fatty acids was carried out using 0.5 mL of extracts in a test tube. Two mL of ethanol was added, and the mixture was homogenized for 1 min, then 0.5 mL of concentrated sulfuric acid was added in drops and left to cool at room temperature. Two mL of n-hexane and two mL of distilled water were added to the tube and shaken well several times until the layers separated. The upper layer represents approximately 20% of the methyl esters produced from fatty acids and thus is ready for direct injection in gas chromatography connected to the mass spectrometer. A gas chromatography-mass spectrometry (GC-MS) apparatus (Shimadzu-QP2010 Ultra, Japan) in the Consumer Protection laboratory at the College of Agriculture, University of Basrah, equipped with an Inert Cap WAX column (GI Sciences, Tokyo, Japan) (30 *M* × 0.32 mm) was used. The sample was injected at an amount of 5 µL, the injection temperature was set to 280 °C in the split-less injection mode. GC-MS parameters were as follows: EI source temperature: 230 °C; Interface temperature: 150 °C; initial oven temperature in GC: 40 °C for 3 min, raised to 400 °C at the rate of 10 °C per minute; pure helium gas (99.9%); linear velocity: 36.1 cm/sec; acquisition of MS spectra was carried out in full scan mode, in the mass range of 100 to 700 m/z, with an event time of 0.2 s. Peak identification was finalized by matching with the National Institute of Standards and Technology (NIST08) database. Percent areas were determined without the use of response factors.

DPPH radical scavenging activity assay

Free radical scavenging analysis was performed using DPPH (2,2-diphenyl 1-picryl hydroxyl), as adapted from the previous protocol described by Amiri et al. (2020) with minor changes. Different concentrations (10, 20, 40, 80, 160-µg/mL) of fractions were prepared and, afterward, DPPH (20 mg) diluted in ethanol (100 mL) was added. The whole mixture was mixed well and kept undisturbed under ambient temperature for 30 min under dark conditions. Ascorbic acid and DPPH were used as reference standard and control respectively. A UV-Visible spectrophotometer was utilized to measure the optical density (OD) at 517 nm.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity was investigated through ferric reducing antioxidant power (FRAP) assay according to a previously described protocol (Oyaızu 1986) with minor changes. The fractions with different dosages (10, 20, 40, 80 and 160 µg/mL) and ascorbic acid used as standard were mixed with 2.5 mL $K_3Fe(CN)_6$ (1% w/v), 2.5 mL 0.2 M phosphate buffer (pH 6.6) and 1 mL of distilled water. After 20 min of incubation at 50 °C, 22.5 mL of trichloro-acetic acid (10% *w/v*) were added and the mixture was centrifuged at 3000 rpm for 10 min. For each sample, 2.5 mL of supernatant were mixed with distilled water (2.5 mL) and 0.5 mL $FeCl_3$ (0.1%, w/v). Finally, the OD was measured at 700 nm using a spectrophotometer (Sunny UV.7804C, Tokyo, Japan).

Origin and selection of microbial strains

The crude extracts were tested against four human pathogenic bacteria for *in-vitro* antimicrobial activities. All four microbial strains were acquired from laboratory stock cultures composed of the most common food-borne pathogens, namely *Bacillus cereus* (ATCC 14579) and *Staphylococcus aureus* (ATCC 6538) as Gram-positive strains and *Escherichia coli* (ATCC 8739) and *Proteus vulgaris* (ATCC 7829) as Gram-negative strains. The microbial cultures were preserved using bead vials under extremely low temperatures (−18 °C).

Preparation of the inoculums

The selected bacterial strains were cultured on nutrient agar (NA) containing Mueller-Hinton broth under 37 °C for 24 h and subcultured for further assays. The inoculum was prepared by suspension of culture in a 0.85% NaCl sterile saline solution. The suspension OD was kept from 0.4 to 0.6 with 405 nm, which indicates a cell density close to that of 0.5 McFarland, identical to an inoculum assessed at 10^6 to 10^8 colony forming units per mL (CFU/mL) (Andrews 2009).

Disk diffusion method on agar

The antibiotic assay was conducted using the conventional disk diffusion method as adapted from Zazharskyi et al. (2019) with slight changes. The plates of Mueller-Hinton agar (MHA) were streaked with previously prepared inoculums using a sterile swab. Afterwards, 5 µL of each fraction including ethyl acetate (I-II) and ethanol (III-V) ones were filtered through a Whatman filter paper and dissolved in deionized water and 10% *v/v* dimethyl sulfoxide. The same procedure was followed for positive and negative controls added with Amikacin and Gentamycin (5 µg/mL) antibiotics along with

the same solvents employed for the crude extracts respectively. The plates were preserved at room temperature and incubated at 37 °C for 24 h. Finally, antibacterial activity was estimated by determining the diameter of inhibition zones (along with the disc's diameter) measured in millimeters with three triplications.

Statistical analysis

All analyses were carried out in triplicate ($n = 3$), and all datasets obtained were statistically analyzed using Statistical Package for the Social Sciences (SPSS) software and the efficacy of plant extract fractions was analyzed using analysis of variance (ANOVA). Significant differences and mean comparisons were determined using Tukey's test with STATISTICA 13 (alpha = 0.05). Finally, the graph plots and results were analyzed using Microsoft Excel (2007) and BioStat software.

Results and discussion

Phenolic compound estimation

Four phenolic compounds, namely p-coumaric acid, quercetin, resorcinol and cinnamic acid were identified by using HPLC from different extracts of fresh aerial parts of *C. colocynthis*. The ethyl acetate extracts were associated with fraction I (one peak), the ethanol extracts were associated with fraction II (one peak), whereas fraction III exhibited two peaks. Phenolic compounds were determined by comparing retention times and peak areas with reference standards as shown in Table 1 and Figure 1. *p*-Coumaric acid was the major phenolic acid from the ethyl acetate extracts reaching 0.112 ± 0.02 mg/kg while resorcinol was the major phenolic compound from the ethanol extracts (25.00 ± 3.21 mg/kg), followed by quercetin and cinnamic acid (12.96 ± 2.20, 4.33 ± 1.04 mg/kg, respectively. In this regard, Yaser (2013) reported the alcohol extracts of the callus from *C. colocynthis* with concentrations of rutin and quercetin of 40.61% and 39.59%, respectively. According to the work of Bilal and Khorsheed (2022), the phytochemical composition of *C. colocynthis* seed extracts was measured using a continuous Soxhlet apparatus and sequence solvent systems based on polarity. The extracts were analyzed in hexane (CI1), chloroform (CI2), ethyl acetate (CI3) and ethanol (CI4). Additionally, acid hydrolysis was performed on the extracts (CI3, CI4 and CI5) to obtain the free phenolic components, which were then identified by HPLC. Extracts from the seeds of *C. colocynthis* contained phenolic compounds (rutin, caffeic acid, ellagic acid, gallic acid, quercetin, myricetin, luteolin). The extracts (CI3, CI4 and CI5) contained rutin and ellagic acid; additionally, CI3 and CI4 contained both caffeic acid and gallic acid. Determination of phenolic compounds in fractions of *C. colocynthis* was also reported by Ibrahim et al. (2010) and chlorogenic acid was the most abundant phenolic compound in the methanol extract (16.3 mg per 100 g dry sample). Sarah et al. (2022) detected no coumaric acid and cinnamic acid by using HPLC in the water extracts of *C. colocynthis* although quercetin was detected at a concentration of 0.21 µg/mL. Finally, Hussain et al. (2013) analyzed the extracts of *C. colocynthis* from roots, leaves and fruits indicating the presence of quercetin in both ethanol and hexane extracts with different concentrations, while p-coumaric acid was detected only in the ethanol extracts.

Table 1. Phenolic compounds detected in *Citrullus colocynthis* fractions by HPLC analysis.

Fractions	Number of peaks	Retention time (min)	Concentration (mg/kg)c	Identified compounds
I[a]	1	3.61	0.112 ± 0.02	p-coumaric acid
IIb	1	3.27	12.96 ± 2.20	quercetin
IIIb	1	2.68	25.00 ± 3.21	resorcinol
	2	3.52	4.33 ± 1.04	cinnamic acid

[a]Fractions identified from ethyl acetate extraction;
[b]Fractions identified from ethanol extraction;
[c]Values represent mean and standard deviation ($n = 3$).

GC-MS analysis

Table 2 shows the bioactive compounds identified in ethanol extracts of *C. colocynthis* by using GC-MS analysis associated with their reported biological activities. Compounds were identified by performing a similarity search on each peak using the NIST08 library database and PubChem database following the National Center of Biotechnology Information (NCBI) (PubChem, n.d2013.). Twenty-four components were identified. Through the use of PubChem and ChEBI databases in conjunction with a literature search, the compounds identified by GC-MS analysis were selected to choose just the 28 substances with known antioxidant properties previously reported for *C. colocynthis* extracts (Al-Nablsi et al. 2022). Based on the peak area (%), the prevalent bioactive compounds were 1-docosanol, pentafluoropropionate (28.86%); phytol (12.16%); 2-methylbicyclo [3.2.1] octane (10.28%); palmitic acid (7.64%); stigmast-5-en-3.beta.-ol, (24S)- (4.18%); octacosanol (4.12%); n-tetratetracontane (4.4%); diisooctyl phthalate (3.41%); diethylene glycol monostearate (2.81%); delta-tocopherol (2.41%); squalene (2.16%), n-hentriacontane (2.16%); beta-stigmasterol (1.93%); linolenin, 1-mono- (1.88%); (2E)-3,7,11,15-tetramethyl-2-hexadecene (1.63%); and ethyl palmitate (1.16%). In addition, many compounds had a peak area less than 1%, such as (3E)-5-isopropyl-6-methyl-3,5-heptadien-2-ol; 4-allyl-2-ethoxyphenol; 7, 8-dimethyltocol; 1-octacosanol, heptafluorobutyrate; linoleic acid chloride; farnesyl acetone; and arachidic acid ethyl ester. In this regard, Gupta et al. (2018) reported that, based on GC-MS analysis, 30 chemically different compounds were identified in hexane extracts of *C. colocynthis* including hydrocarbons, carboxylic acids of alkyl, fatty acids, alcoholic fatty acids, phenols, terpenes, vitamins and sterols. The two main compounds of hexane extracts in seeds were reported to be linoleic and palmitic acid. The peak area % of palmitic acid reached values as high as 11.43 ± 0.92 and 27.55 ± 1.79% for leaves and seeds, respectively. The mean of linoleic acid content in fruit pulp was 13.86 ± 0.97%, with values of 4.63 ± 0.28% in leaves and 33.92 ± 1.65% in seeds. Bilal and Khorsheed (2022) demonstrated that, after the saponification process for *C. colocynthis* seed extracts, the compounds present in the hexane (CI1), chloroform (CI2), ethyl acetate (CI3), ethanol (CI4)

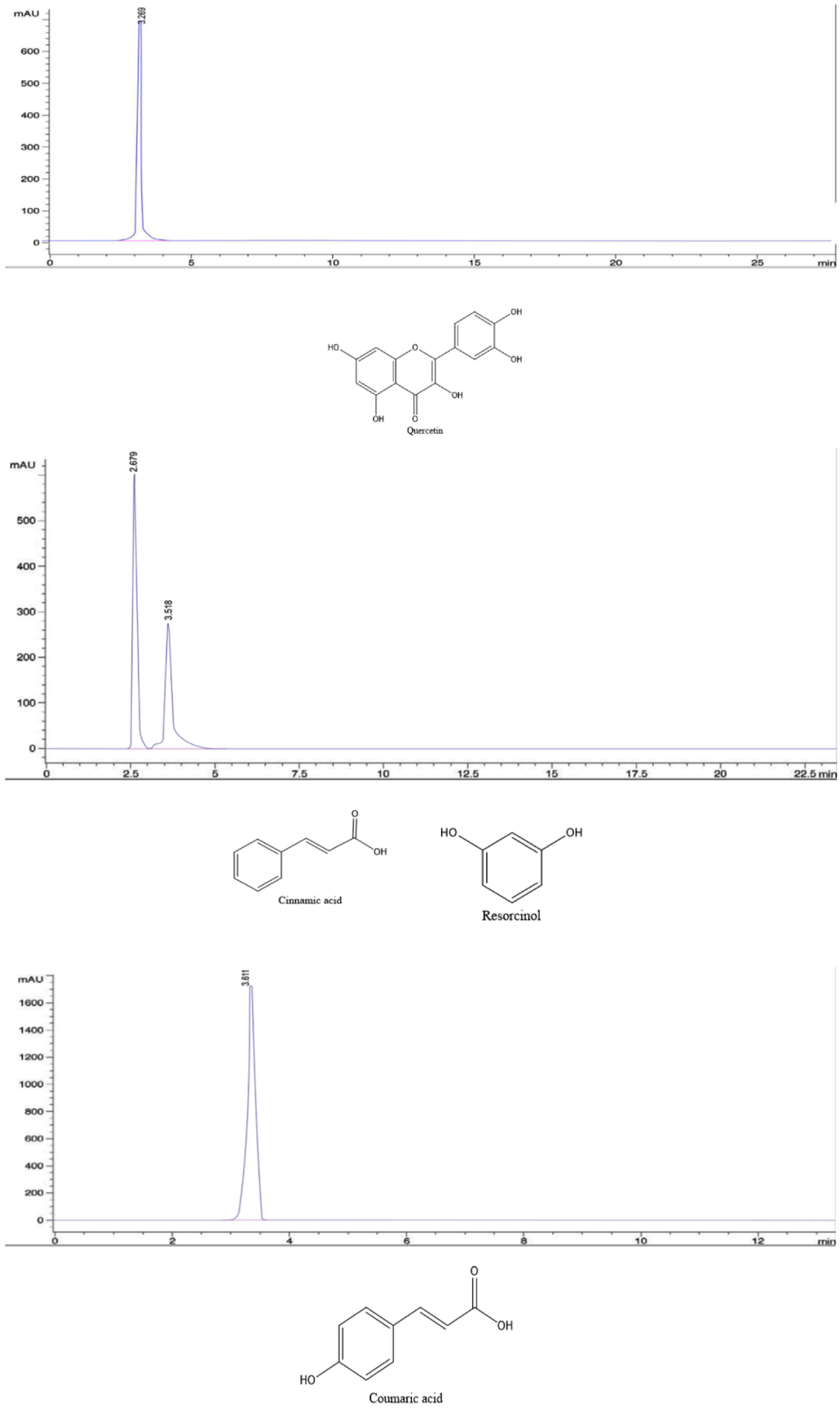

Figure 1. HPLC Chromatograms of phenolic compounds detected in *Citrullus colocynthis* fractions.

and hot aqueous (CI5) extracts were five fatty acids, namely palmitic, stearic, oleic, linoleic and elaidinic acids. Overall, 43 phytoconstituents were found in *C. colocynthis* seed extracts, 21 were found in the hexane extract, 12 in the chloroform extract, and 16 in the methanolic extract. In the hexane and chloroform extracts, monoterpenes predominated with values

Table 2. The bioactive molecules identified in *Citrullus colocynthis* extracts by GC-MS.

NO.	Compound	LRI (exp)	LRI (lib)*	Area %	Synonyms	Bioactivity**
1	n-hexadecanoic acid	1978	1974	7.64	palmitic acid	Antioxidant and microbicide inhibitors
2	5-isopropyl-6-methyl-hepta-3, 5-dien-2-ol	2022	2023	0.44	(3E)-5-isopropyl-6-methyl-3,5-heptadien-2-ol	No activity reported
3	phenol, 2-ethoxy-4-(2-propenyl)-	2052	2049	0.76	4-allyl-2-ethoxyphenol	Anti-inflammatory and antioxidant
f4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	2110	2221	12.16	Phytol	Cytotoxicity, antioxidant, and anti-microbial agents
5	2-hexadecene, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]	2214	2210	1.63	(2E)-3,7,11,15-tetramethyl-2-hexadecene	Anti-microbial activity
6	hexadecanoic acid, ethyl ester	2278	2281	1.19	ethyl palmitate	Anti-inflammatory, and immunological activity
7	2-methylbicyclo [3.2.1]octane	2323	2319	10.28	2-methylbicyclo [3.2.1]octane	Pharmaceutically active and anti-cancer agent
8	7-tetradecenal, (Z)	2358	2350	5.23	Z-7-tetradecenal	Pesticide active substance
9	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester	2447	2450	1.88	linolenin, 1-mono-	Antimicrobial, antioxidant, and cytotoxicity agent
10	octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester	2588	2581	2.81	diethylene glycol monostearate	Surfactant—emulsifying, antioxidant, and anti-cancer agents
11	eicosanoic acid, ethyl ester	2646	2650	0.61	arachidic acid ethyl ester	Cytotoxicity agent
12	5,9,13-pentadecatrien-2-one, 6,10,14-trimethyl-	2762	2761	0.41	farnesyl acetone	Antioxidant and Flavoring Agents
13	9,12-octadecadienoyl chloride, (Z,Z)	2801	2799	0.53	linoleic acid chloride	No activity reported
14	1,2-benzenedicarboxylic acid, diisooctyl ester	2915	2915	3.41	diisooctyl phthalate	Antimicrobial activity
15	stigmasterol	3002	3014	1.93	–	Therapeutic and antiviral agents
16	1-octacosanol	3178	3150	4.12	octacosanol	Antidiarrheal, antioxidant and anti-arthritic, anti-microbial activities
17	2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl	3258	3241	2.16	squalene	Antibacterial, anticancer, antioxidant, antidetox agent
18	γ-sitosterol	3349	3325	4.18	stigmast-5-en-3.beta.-ol, (24S)-	Hypolipidemic, antiviral and anti-cancer agent
19	tetratetracontane	3455	3450	4.40	n-tetratetracontane	Antioxidant, antibacterial and anti-fungal agent
20	docosyl pentafluoropropionate	3499	3512	28.86	1-docosanol, pentafluoropropionate	No activity reported
21	2H-1-benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2R-[2R	3556	3547	2.41	δ-tocopherol	Preservative and antioxidant agent
22	octacosyl heptafluorobutyrate	3660	3650	0.36	1-octacosanol, heptafluorobutyrate	No activity reported.
23	γ-tocopherol	3691	3685	0.48	7,8-dimethyltocol	Preservatives, antioxidant, anti-cancer agent
24	hentriacontane	3425	3430	2.16	n-hentriacontane	Anti-inflammatory, anti-bacterial agent

*NIST08 database.
**National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/12410#section=Thieme-References (accessed Feb. 04, 2023).

of 40.4 and 41.1%, respectively, whereas in the methanolic extract, oxygenated aliphatic hydrocarbons predominated with a ratio of 77.2% (Khan et al. 2023). On the other hand, Aldughaylibi et al. (2022) detected four chemical compounds by GC-MS in the chloroform fraction of *C. colocynthis* including 1,2-benzenedicarboxylic acid, 9,12-octadecadienoic acid, 11-octadecadienoic acid, phytol and hexadeanoic acid. In another study, Bourhia et al. (2021) revealed the existence of many unique chemicals, especially 2,4-nonadienal, tetradecane, hexadecane and pentadecane.

Estimation of the antioxidant activity by the DPPH method

The two *C. colocynthis* extracts were shown to have significantly different DPPH radical scavenging capabilities of each fraction I, II and III in comparison to the quercetin-based standard as shown in Table 3. The antioxidant impact of fraction III was comparable to the standard, reaching values of 46.23 ± 3.22, 57.34 ± 3.80, 61.66 ± 3.29, 72.43 ± 3.42, 83.95 ± 4.06 and 97.22 ± 4.52 (%) at concentrations of 5, 10, 15, 30, 60 and 120 (µg/mL), as compared to the standard, which reached 47.43 ± 3.11, 59.09 ± 3.09, 65.75 ± 4.34, 77.85 ± 3.75, 88.36 ± 4.51 and 97.42 ± 4.94 (%) at the same concentrations. Comparatively, low antioxidant activity was observed for fractions I and II (63.31 ± 4.16 and 81.41 ± 4.06%, respectively), at 120 µg/mL compared to the

Table 3. DPPH activity of isolated fractions.

Concentration (µg/mL)	Standard	Fraction I	Fraction II	Fraction III
5	47.43 ± 3.11^{a}	25.21 ± 2.01^{a}	28.15 ± 2.07^{a}	46.23 ± 3.22^{a}
10	59.09 ± 3.09^{b}	31.16 ± 3.73^{b}	34.17 ± 3.29^{b}	57.34 ± 3.80^{b}
15	65.75 ± 4.34^{c}	42.04 ± 4.09^{c}	48.55 ± 3.11^{c}	61.66 ± 3.29^{c}
30	77.85 ± 3.75^{d}	53.65 ± 3.75^{d}	59.48 ± 2.85^{d}	72.43 ± 3.42^{d}
60	88.36 ± 4.51^{e}	59.41 ± 3.03^{e}	68.22 ± 3.03^{e}	83.95 ± 4.06^{e}
120	97.42 ± 4.94^{f}	63.31 ± 4.16^{f}	81.41 ± 4.06^{f}	97.22 ± 4.52^{f}

$^{a-f}$means with same superscript letters are not significantly different ($p > 0.05$) within the same column for a particular fraction.

standard. The difference in the antioxidant potential in the different fractions might due to the fact that different phenolic compounds have distinct redox characteristics, which make them reducing agents, hydrogen donors and singlet oxygen quenchers. Nevertheless, all fractions demonstrated antioxidant activity. Prior studies have shown that several factors affect how effective phenolic compounds are as anti-radicals and antioxidants. One of the most important factors is the ratio of hydroxyl groups directly linked to aromatic ring (Sroka and Cisowski 2003). The antioxidant capacity measured using the DPPH assay, of various *C. colocynthis* fruit parts (rinds, pulps and seeds) was previously shown to be higher in seeds than in the pulp and rinds (74.1% vs. 32.6% and 38.0%, respectively) (Al-Nablsi et al. 2022). Substantial variations in the DPPH radical scavenging capability of methanolic extracts of *C. colocynthis* leaves, stem, root, fruit pulp and seeds was also reported by Gupta et al. (2018). Fruit pulp extracts were shown to be more effective (196.44 ± 17.78 µg/mL) compared to the leaves (413.33 ± 39.78 µg/mL), stems (374.68 ± 38.22 µg/mL), roots (288.46 ± 29.07 µg/mL) and seeds (314.66 ± 31.66 µg/mL). Isosaponarin, isovitexin and isoorientin 3in, isovitexin13.33be more effective (196.44 the pulp and rinds *C. colocynthis* fruits and demonstrated strong antioxidant activity (Mehta et al. 2013). *C. colocynthis* methanolic fruit extracts were tested for their ability to quench free radicals. At a concentration of 2500 mg/mL, the fruit extracts were shown to have the maximum free radical scavenging capacity (Kumar et al. 2008). Extracts of *C. colocynthis* seeds were tested for their antioxidant effects at a concentration of 2000 g/mL in DPPH assay; the results showed that the ethyl acetate extract was the most effective, with a reducing percentage of 88.8%, followed by 74.5% the hydromethanolic extracts and 66.2% the crude aqueous extracts (Benariba et al. 2013). According to *in vitro* antioxidant tests, *C. colocynthis* fruit methanolic extracts inhibited DPPH radicals by 62% at 800 g/mL (Rajangam and Christina 2013). The polyphenolic content of organic *C. colocynthis* extracts derived from different tissues has been demonstrated to have a significant antioxidant capacity *in vitro* (Benariba et al. 2013; Rizvi et al. 2018). Antioxidant activity varied more widely among stem and leaf extracts than root extracts and this variation was dependent on the solvent used for extraction. Methanol leaf extracts and ethyl acetate root extracts were shown to have the highest antioxidant capacity, followed by chloroform and methanol root extracts (Degola et al. 2019). The capacity of *C. colocynthis* fruit extracts to neutralize DPPH free radicals reached values as high as 42.13, 47.74, 51.67, 69.24 and 90.13% at concentrations of 5, 10, 15, 20 and 25 µg/mL, respectively (Sarah et al. 2022). The level of radical scavenging activity in all *C. colocynthis* ethanol extracts ranged from 56.8% to 67.2%. Root and fruit hexane extracts demonstrated much less radical scavenging activity than ethanol extracts reached (39.8 and 35.7%, respectively). Additionally, fruit hexane extracts demonstrated strong anti-radical action reaching 60.2% which is higher than fruit ethanol extracts which reached 58.1%. One possible explanation for the plant extracts' capacity to scavenge DPPH radicals is that they include flavonoids and phenolic acids (Hussain et al. 2013).

Table 4. Ferric reducing power % of isolated fractions.

Concentration (µg/mL)	Standard	Fraction I	Fraction II	Fraction III
5	40.43 ± 2.03[a]	23.98 ± 2.07[a]	26.61 ± 2.11[a]	41.01 ± 2.19[a]
10	49.09 ± 2.18[b]	29.04 ± 2.31[b]	32.34 ± 3.05[b]	52.22 ± 3.85[b]
15	56.75 ± 3.05[c]	38.12 ± 2.96[c]	47.22 ± 3.26[c]	59.50 ± 3.26[c]
30	66.85 ± 4.15[d]	48.01 ± 3.04[d]	56.91 ± 3.18[d]	68.33 ± 3.02[d]
60	79.36 ± 3.97[e]	57.92 ± 3.22[e]	68.71 ± 4.04[e]	79.72 ± 3.14[e]
120	92.42 ± 3.84[f]	67.69 ± 4.08[f]	80.87 ± 4.23[f]	94.37 ± 3.83[f]

[a–f]means with same superscript letters are not significantly different ($p > 0.05$) within the same column for a particular fraction.

Estimation of the antioxidant activity by ferric reducing power

The ferric-reducing antioxidant power (FRAP) assay was utilized as another evaluation of antioxidant activity in this investigation. Table 4 displays the results of a FRAP assay comparing two *C. colocynthis* extracts to a quercetin-based standard. The results demonstrate that the C. *colocynthis* extracts differ significantly in each of the three fractions tested. Fraction III had the highest FRAP reaching values as high as 41.01 ± 2.19, 52.22 ± 3.85, 59.50 ± 3.26, 68.33 ± 3.02, 79.72 ± 3.14 and 94.37 ± 3.83% at concentrations of 5, 10, 15, 30, 60, 120 µg/mL, respectively as compared to the standard, which reached 40.43 ± 2.03, 49.09 ± 2.18, 56.75 ± 3.05, 66.85 ± 4.15, 79.36 ± 3.97 and 92.42 ± 3.84 (%) at the same concentrations. Fractions I and II had comparatively the lowest FRAP reaching (67.69 ± 4.08 and 80.87 ± 4.23) % at 120 µg/mL compared to the standard. In a previous study, the FRAP assay was reported to be a valuable tool to assess the antioxidant activity of diverse substances since it is repeatable and linearly proportional to the molar concentration of the antioxidants present in the test samples (Hodzic et al. 2009). In addition, the lowest antioxidant activity was found in the aqueous extracts of *C. colocynthis* among six medicinal plants which were estimated using FRAP assay reaching 6.50 ± 0.01% (Asgharpour et al. 2013). Moreover, both the aqueous and butanolic extracts of *C. colocynthis* had a reducing power of 0.36 and 0.55 at 3 mg/mL, respectively, which was lower than that of ascorbic acid (1.89 at 3 mg/mL) (Chekroun et al. 2015). Using the FRAP assay, the reducing potential of *C. colocynthis* root was assessed and compared with ascorbic acid as a standard. At the same concentration and experimental conditions, *C. colocynthis* had reducing powers that were 20% of those of ascorbic acid (Owoade et al. 2018).

Phenolic substances have antioxidant activity based on their chemical structure, particularly the presence of hydroxyl groups. The antioxidant action is linked to the arrangement of the 3ntioxiitionalydroxy on the B cycle and the 4-carbonyl group on the C cycle, as well as the 3-OH and 5-OH groups on the C cycle (Wojdylo et al. 2007; Benariba et al. 2013). The FRAP assay requires a single electron donor species or antioxidants to accomplish the reduction of the Fe(TPTZ)2(III) complex, which is pale yellow, into the Fe(TPTZ)2(II) complex, which is blue. According to the findings of Huang et al. (2005), the standard reduction potential of Fe(III)TPTZ salt is approximately 0.7 V. Therefore, any species having a reduction potential that is lower than 0.7 V is capable of reducing Fe^{3+}–TPTZ to Fe^{2+}–TPTZ and contributing to the FRAP value. At a

Table 5. Antimicrobial activity of fractions I-III.

	Concentration µg/mL	Zone of Inhibition (mm)			
		B. cereus	*S. aureus*	*P. aeruginosa*	*E. coli*
Fraction I	1.5	5 ± 1.04^{a}	4 ± 0.18^{a}	0	0
	3	8 ± 1.07^{b}	9 ± 1.04^{b}	7 ± 0.84^{a}	7 ± 0.88^{a}
	6	10 ± 2.14^{b}	11 ± 1.87^{b}	9 ± 1.06^{a}	10 ± 1.35^{b}
	12	15 ± 2.16^{c}	18 ± 2.53^{c}	15 ± 1.74^{b}	14 ± 1.08^{c}
	24	20 ± 2.34^{d}	22 ± 2.19^{d}	20 ± 2.17^{c}	18 ± 1.22^{d}
Fraction II	1.5	0	0	0	0
	3	5 ± 0.98^{a}	6 ± 1.11^{a}	0	6 ± 0.65^{a}
	6	10 ± 1.86^{b}	9 ± 1.77^{b}	5 ± 0.87^{a}	10 ± 1.09^{b}
	12	13 ± 1.94^{c}	14 ± 1.88^{c}	8 ± 1.98^{b}	14 ± 1.54^{c}
	24	19 ± 2.07^{d}	20 ± 1.33^{d}	15 ± 1.11^{c}	20 ± 1.56^{d}
Fraction III	1.5	7 ± 0.77^{a}	5 ± 0.76^{a}	0	0
	3	9 ± 1.43^{a}	6 ± 0.48^{a}	7 ± 1.32^{a}	5 ± 0.71^{a}
	6	15 ± 1.43^{b}	10 ± 1.15^{b}	11 ± 1.56^{b}	9 ± 0.62^{b}
	12	19 ± 1.47^{c}	15 ± 1.38^{c}	16 ± 1.08^{c}	12 ± 1.01^{c}
	24	24 ± 1.62^{d}	20 ± 1.49^{d}	21 ± 2.02^{d}	15 ± 1.11^{d}
Control	Gentamycin	20	19	20	18

[a–d]means with same superscript letters are not significantly different ($p > 0.05$) within the same column for a particular fraction; *B. cereus: Bacillus cereus; S. aureus: Staphylococcus aureus; P. aeruginosa: Pseudomonas aeruginosa; E. coli: Escherichia coli.*

pH of 3.6, the FRAP assay is used to determine the ability of any species to convert Fe(III)TPTZ to Fe(II)TPTZ.

C. colocynthis fractions were assessed for reducing power and compared to a calibration curve using iron(II) sulphate as the standard reduction agent. *C. colocynthis* FRAP values decreased from methanol to ethyl acetate to n-hexane (Asghar et al. 2011).

According to our HPLC and GC-MS analyses, *C. colocynthis* extracts present secondary metabolites that are relevant for antioxidant activities, which are also supported by DPPH and FRAP tests: quercetin, resorcinol, cinnamic acid, phytol, linolenin, 1-mono-, diethylene glycol monostearate, farnesyl acetone, octacosanol, squalene, n-tetratetracontane, delta-tocopherol, 7,8-dimethyltocol and others. The results of this study are consistent with those of previous studies (Sroka and Cisowski, 2003; Hodzic et al. 2009; Benariba et al. 2013; Gupta et al. 2018; Owoade et al. 2018; Al-Nablsi et al. 2022).

Evaluation of the antibacterial activity

Table 5 shows that the two extracts were effective against four pathogenic bacterial strains [*Bacillus cereus* and *Staphylococcus aureus* (as a gram-positive bacteria), *Pseudomonas aeruginosa* and *Escherichia coli* (as a gram-negative bacteria)]. *B. cereus* and *S. aureus* were inhibited at concentrations of 1.25 µg/mL for Fractions I and III, 3 µg/mL for Fraction II. *P. aeruginosa* and *E. coli* were inhibited at concentrations of 3 µg/mL for Fractions I and III and 3 µg/mL for Fraction II. The results show at Fraction II, that *P. aeruginosa* was inhibited at a concentration of 6 µg/mL while *E. coli* was inhibited at a concentration of 3 µg/mL.

The highest inhibition zone (mm) for *B. cereus* and *S. aureus* were 24 ± 1.62 and 22 ± 2.19 mm, respectively, at 24 µg/mL for Fractions III and I compared to control that reached 20 and 19 mm, respectively. The highest inhibition zone (mm) for *P. aeruginosa* and *E. coli* were 21 ± 2.02 and 20 ± 1.56 mm, respectively, at 24 µg/mL for Fractions III and II compared to control that reached 20 and 18, mm, respectively. On the other hand, the inhibition zones (mm) of *B. cereus, S. aureus, P. aeruginosa* and *E. coli* were not observed at 1.5 g/mL for Fraction II. Evidence suggests that p-coumaric acid, quercetin, resorcinol and cinnamic acid, present in Fractions I, II and III, respectively, all play key roles in preventing the growth of the tested strains.

Using radiometric BACTEC, crude extracts, fractions and compounds of *C. colocynthis* plant aerial parts and ripe deseeded fruits were tested against the drug-sensitive standard strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294), 16 drug-resistant strains and two MOTT strains. The most effective bioactive ingredient against *M. tuberculosis* H37Rv was found in the methanolic extract of ripe, deseeded *C. colocynthis* fruit (MIC 31.2 µg/ml). Sixteen clinical isolates of Mycobacterium TB, including seven non-multidrug resistant, eight multidrug-resistant, one highly drug-resistant and two MOTT bacilli with MICs of 50–125, 31.2–125 and 62.5–125 µg/ml, were still suppressed by three bioactive fractions. The primary indicators effective against Mycobacterium TB H37Rv (MICs 50 and 25 µg/ml) and the 18 clinical isolates were ursolic acid and cucurbitacin E 2-0-d-glucopyranoside (Mehta et al. 2013).

The *C. colocynthis* fruit extracts tested against *E. coli*, *S. aureus*, *S. typhi, S. shigella and C. albicans* showed the strongest antibacterial activity in acetone, ethanol, methanol and distilled water. In contrast, petroleum ether extracts had lower potency against test microorganisms (Rodge and Biradar 2012). *S. aureus* was more susceptible to the inhibitory effects of the ethanolic extracts than the water extracts. A standard strain of *S. aureus* was inhibited by the ethanolic extracts of *C. colocynthis* fruits at a concentration of 5 mg/mL in a manner comparable to that of novobiocin (Najafi et al. 2010).

The MIC and MBC/MFC of plant organs were measured at various stages of development. Gram-negative and Gram-positive bacteria (*E. coli, P. aeruginosa, S. aureus and Enterococcus faecalis*) as well as *Candida* species were examined in aqueous and diluted acetone extracts from the plant's roots, stems, leaves and three stages of its fruit and seeds (*Candida glabrata, Candida albicans, Candida parapsilosis and Candida kreusei*). All extracts were effective against all strains (Rasool and Jahanbakhsh 2011).

The fruit extracts of *C. colocynthis* had the greatest MICs and MBCs/MFCs (0.10 mg/mL against *Candida albicans* and *Candida glabrata*, 0.20 mg/ml against *E. coli* and *P. aeruginosa*), while the root extracts had the lowest antibacterial and anticandidal activity (Rasool and Jahanbakhsh 2011). Using the agar disc diffusion method, five local bacterial isolates (*E. coli, S. aureus, Streptococcus* sp., *Bacillus cereus* and *Klipsella* sp.) were tested for the antibacterial activity of an alkaloid isolated from *C. colocynthis. Streptococcus* sp. was the target of the extracted alkaloid's most potent antibacterial action. The isolated alkaloid's MIC was calculated using the broth dilution technique. MIC values of 600 g/mL and 3000 g/mL, respectively, were recorded for *S. aureus* and *E. coli* isolates (Al-hejjaj et al. 2010). Methanolic and aqueous extracts

of *C. colocynthis* seeds were tested *in vitro* for their capacity to inhibit the growth of fungi and mycotoxins. *Aspergillus ochraceus* and *Aspergillus flavus* were used as test organisms for the antifungal and anti-mycotoxigenic activities of methanolic and aqueous extracts. The findings indicate that the extracts had excellent antifungal efficacy against *A. ochraceus*, but not *A. flavus*. The extracts exhibited effective anti-ochratoxigenic properties in liquid media (Gacem et al. 2013).

These findings suggest that *C. colocynthis* extracts contain compounds that are important for antimicrobial activities, for example, quercetin, resorcinol, cinnamic acid, palmitic acid, phytol, (2E)-3,7,11,15-tetramethyl-2-hexadecene, linolenin, 1-mono-, diisooctyl phthalate, beta-stigmasterol, octacosanol, squalene, stigmast-5-en-3.beta-ol, (24S)- and n-hentriacontane. The findings of this study are in line with the findings of earlier studies (Bourhia et al. 2021; Aldughaylibi et al. 2022; Khan et al. 2023).

Conclusion

Based on the findings of this investigation, ethanol extracts of *C. colocynthis* are a valuable source of bioactive compounds with interesting antibacterial capability and antioxidant activity. Four phenolic compounds were positively identified by HPLC. Resorcinol was the major phenolic compound from the ethanol extracts of *C. colocynthis*. Twenty-four bioactive compounds were identified by GC-MS analysis. 1-Docosanol, pentafluoropropionate was the major bioactive compound in ethanolic extracts followed by phytol; 2-methylbicyclo [3.2.1] octane, palmitic acid and octacosanol. The optimized Soxhlet extraction method used in this study accounted for superior antioxidant and antibacterial activities. *C. colocynthis* aerial parts could be considered promising as antioxidant and anti-infectious agents, although further studies are necessary to propose their role in medicinal and nutritional applications.

Authors' contribution

Ammar B. Altemimi: Conceptualization, Writing—Original draft, Project administration. Muthanna J. Mohammed: Investigation. Zina T. Alkanan: Investigation. Shaymaa H. Abdulrahman, Investigation. Shatha A. Allaitha: Investigation. Tarek Gamal Abedelmaksoud: Writing—Reviewing and editing, Supervision. Qausar Hamed ALKaisy: Investigation. Qausar Hamed ALKaisy: Investigation. Mazin A. A. Najm: Writing—Reviewing and editing. Francesco Cacciola: Conceptualization, Writing—Reviewing and editing, Supervision. P. Vasantha Srinivasan: Writing—Reviewing and editing, Supervision.

Disclosure statement

The authors declare no conflict of interest.

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