Egypt.J.Chem. **Vol. 62**, No. 10. pp. 1869 - 1877 (2019)

Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Isolation and Structure Elucidation of Compounds from *Coccinia grandis* Leaves Extract

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IN THIS STUDY, preliminary phytochemical screening of dried leaves of *Coccinia grandis* showed presence of triterpenoids, phenolics, flavonoids, saponins and sterols. As well as, five phenolic compounds namely; ferulic acid **(1)**, methyl caffeate **(2)**, ligstroside **(3)**, *trans-p*-coumaric acid **(4)** and kaempferol-3-*O*-*β*-*D*-glucoside **(5)** were isolated from selected fraction of the methanol extract of *Coccinia grandis* leaves. The structures of the isolated compounds were identified using different NMR spectroscopic techniques (^{1}H, ^{13}CNMR, COSY, HSQC, HMBC) and mass spectrometry. Compounds **(1), (2), (4)** and **(5)** were isolated for the first time from *Coccinia grandis* species while compound **(3)** was isolated for the first time from Cucurbitaceae family. Moreover, the anti-microbial activity of the selected fraction of the methanol extract of *Coccinia grandis* leaves was tested against a panel of Gram-positive, Gram-negative bacteria and yeast. A considered activity was recorded against *Bacillus cereus* (inhibition zone of 20 mm with MIC 1mg/ml).

Keywords: *Coccinia grandis,* Phenolics, Flavonoids, NMR, MS, Anti-microbial activity.

Introduction

Nature has always been the primary source of novel pharmacological active compounds. Therefore, isolation and structure elucidation of compounds isolated from plants' extracts is a vital step in determination of their activity as well as establishing suitable methods to synthesize them chemically in large scale. Medicines derived from plants have played a vital role in human health throughout history and it is expected to continue playing its role in health system all around the world [1]. In 1900s, 80% of the drug was derived from nature specially plants. Years later, the trend moved toward development of synthetic drugs. This caused an obvious decline in dependence on medicines of plant origin. However, the significant increase in synthetic drugs' resistance created a demand for exploration for novel classes of drugs with potent biological activity. Low cost, availability and familial experience of herbs made them, again, the best source of new bioactive compounds [2,3]. Nowadays, about 80% of people in the world relay on herbal medicine, which makes it essential to assess their safety and efficacy [1]. Among other phytochemicals, phenolics are considered one of the most important classes of secondary metabolites that offer a fascinating potential of pharmacological applications as they serve well in treatment of various diseases and in both *in-vitro* and *in-vivo* studies [4,5].

Cucurbitaceae is a family of flowering plants [6] that comprises about 130 genera and 800 species [7]. It is characterized by the presence of bitter-tasting triterpenoids (cucurbitacins) which present in high amounts in root tissue as a defense mechanism against mammals. In contrast, fruits of these plants are edible and considered an essential source of nutrients [8].

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Received 20/3/2019; Accepted 11/4/2019.
DOI: 10.21608/ejchem.2019.10925.1700

Cucurbits are vegetable crops cultivated mainly in subtropical and tropical regions [7]. Plants belong to this family are trailers herbaceous annual vines, tendril bearing monoecious, dioecious climbers or woody perennial climbing plant [9].

Coccinia is one of the largest genera of the Cucurbitaceae family that contains 29 species [10]. The name "Coccinia" is derived from "Coccineus", a Latin word means scarlet, indicating color of the fruit [11]. Few species, belong to this genus, are edible*; C. rhemannii* (Malawi), *C. trilobata* (Keyna)*, C. adonesis* (Sudan, Ethiopia, Keyna & Malwai), *C. sessilifolia* (South Africa), *C. grandis* (Ethiopia, India) and *C. abyssinica* (Ethiopia) [12].

Coccinia grandis is a wild plant belongs to *Coccinia* genus and distributed widely in tropical and subtropical regions [13]. It is used traditionally as hypoglycemic agent, digestive agent, carminative, laxative, analgesic and for treatment of abscesses, skin eruptions, leprosy, psoriasis, asthma, ulcer, urinary tract infections, stomach pain, vertigo, filarial swelling, snake poison, high blood pressure and gonorrhea. However, the reported biological activities of the plant are; analgesic, anti-inflammatory, antipyretic, antidiabetic, antiulcer, hepatoprotective, antimalarial, anti-dyslipidemia, antitussive and anticancer activities [10].

The aim of this study is to investigate the phytochemical constituents of the methanol extract of *Coccinia grandis* leaves, isolation and structure elucidation of the isolated compounds using different spectroscopic techniques as well as evaluating its anti-microbial activity against *Bacillus subtilis, Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* microorganisms.

Experimental

Plant material

Fresh leaves of *Coccinia grandis* (Synonym: *Coccinia cardifolia*) were collected from Taiz Governate, Yemen, on February (2017) and were kindly authenticated by Dr. Abdul Wali Ahmed, associate professor in flora and vegetation at the Agricultural Research and Extension Authority ''Southern Upland Station'' Taiz and Ibb, Yemen. Voucher specimen No. PHG-P-CG-299 of the authenticated plant were deposited at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Preliminary phytochemical screening

Sample of *C. grandis* leaves was air-dried and grinded to powder. The powdered sample was screened for the presence of the main classes of secondary metabolites such as flavonoids, sterols, triterpenoids, cardiac glycosides, saponins, tannins, alkaloids and anthraquinones according to the approved methods of preliminary phytochemical tests.

Presence of flavonoids was tested using Shinoda's test [14], sodium hydroxide and ammonia tests [15]. Sterols and / or triterpenoids presence was tested using Liebermann- Burchard's test [16] and Salkowiski's test [17]. Saponins were tested by froth and hemolytic test [19]. Tannins were examined by ferric chloride reagent [15] while, alkaloids were tested by Mayer's test [19]. Presence of anthraquinones were examined using Borntrager's and modified Borntrager's tests [20] and cardiac glycosides were tested by Keller-Killani test [15].

Extraction

Fresh plant material (1300 g) were cleaned, air-dried in shade at room temperature for five days, crushed and then macerated at room temperature in aqueous methanol for 3 days with stirring. The extract was filtered while the marc was re- macerated using fresh aqueous methanol for another 3 days. This process was repeated many times till clear filtrate was obtain. The methanol extract was defatted using petroleum ether and the defatted methanol extract was evaporated using rotary evaporator at 45 ºC till dryness and yielded 177.64 g (13.7% w/w)**.**

Isolation and identification of pure compounds

Defatted methanol extract (used only 100 g) was fractionated using Diaion HP-20 column (100 cm × 8 cm using dry loading method). Elution was performed using mixture of methanol: distilled water (0:100, 25: 75, 50:50, 75:25,100:0, respectively). Fractions were collected from the column, evaporated by rotary evaporator and monitored by TLC plats using DCM: MeOH (8:2) mobile phase. Chromatograms were visualized under UV lamp and similar fractions were combined. The finally combined fractions were re- examined by TLC to select the most promising fraction, rich of quenching spots and has reasonable weight, to be subjected to further purification and fractionation using silica gel column. The selected fraction was water:

methanol (50:50) fraction (yielded 10 g and coded as CG50).

The selected fraction was loaded (using dry method) on normal silica gel 60 F_{254} column packed using wet method. Elution was performed using mixture of DCM: MeOH in increasing polarity (10:0, 9:1, 8:2, 7:3, etc. till 0:10). Fractions were eluted and monitored by TLC plats using DCM: MeOH mobile phase (with the same ratio at which the examined fraction was eluted). Chromatograms were visualized under UV lamp (254 nm and 365 nm) as well as using 10% Sulphuric acid, vanillin sulphuric acid, $FeCl_3$, separately and similar fractions were combined together and weighted. The main fraction (fraction III), which yielded 800 mg and eluted at DCM: MeOH (8:2), was selected (according to the same criteria used before) for further purification and fractionation by smaller silica gel column (35 cm × 2.5 cm) with the same method of packing, loading and ratio of mobile phase used in the previously used silica gel column. Three different sub-fractions; fraction 3 (110 mg), fraction 4 (110 mg), fraction 5 (220 mg) were eluted at 8:2 DCM: MeOH and were further purified using HPLC using (acetonitrile: water) as mobile phase.

High Performance Liquid Chromatography (HPLC)

Knauer HPLC Series consists of two pumps (K-501 and K-1001), semi-preparative column Kromasil 100-5 C_{18} and UV detector (K-2501) (connected to the end side of the column) was used to conduct the HPLC analysis in this study. Samples were prepared by dissolving compounds obtained from separation by silica column chromatography in minimum quantity of HPLC grade methanol. Samples were diluted when necessary before injection into the column and monitored at λ_{max} = 254 nm. Gradient elution was adopted according to the polarity of each fraction using acetonitrile: water solvent system and flow rate of 4 ml/ min. The process resulted in isolation of compound (**1**) & (**2**) from fraction 3, compound (**3**) & **(4)** from fraction 4 and compound (**5**) from fraction 5 as illustrated in **Fig 1.**

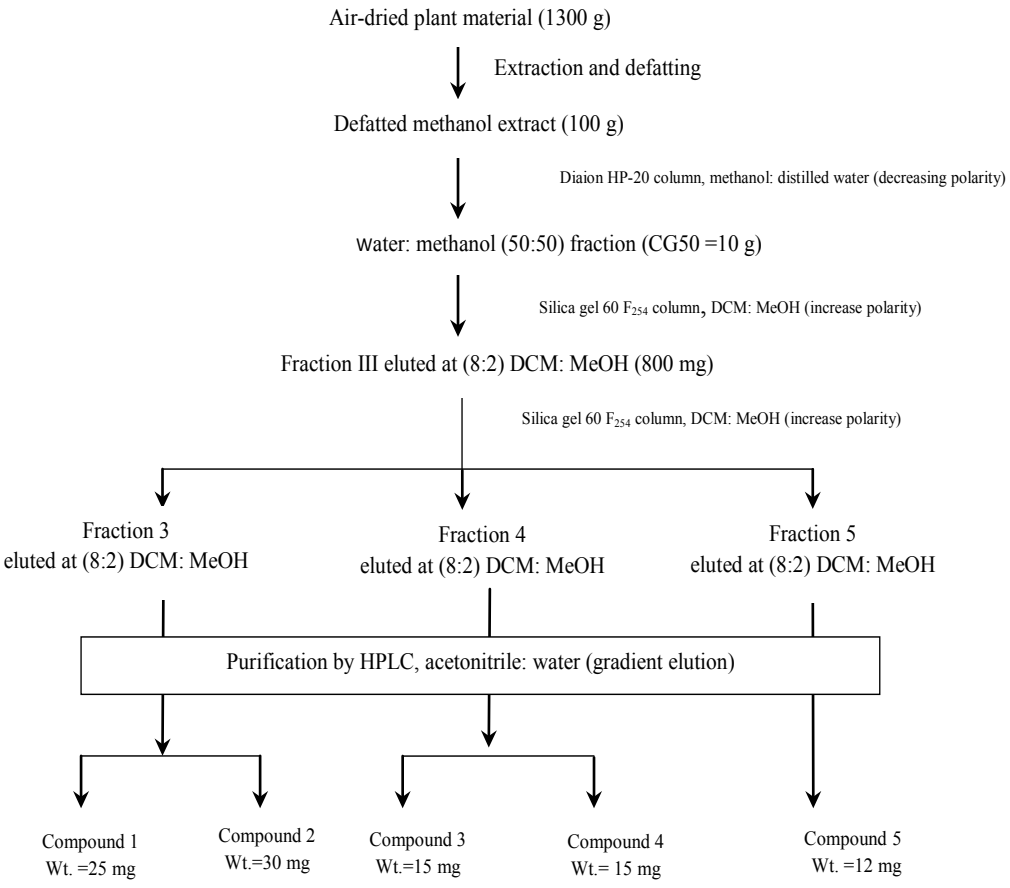

Fig. 1. Schematic illustration of fractionation and separation of compounds from methanol extract of *C. grandis* leaves

Spectroscopic analysis

NMR spectra were acquired in DMSO-d_6 on a Burker Ascend 400 MHz NMR spectrometer (BurkerAvance III, Fallanden Switzerland), at 400 MHz. Standard pulse sequence and parameters were used to obtain 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC and HMBC spectra, respectively. ^{1}H chemical shifts (δ) were measured in ppm, relative to tetramethyl silane (TMS) and ^{13}C NMR chemical shifts to DMSO, and were converted to TMS scale by adding 39.5 ppm. ESI-Mass spectra were measured using a Waters mass spectrometer (Milford MA, US) equipped with a Nanomate ESI interface (Advion).

Anti- microbial assay

Determination of mean of zone of inhibition

Antibacterial susceptibility test was done at the Anti-microbial Unit, National Research Centre (NRC), Giza, Egypt. It was performed according to NCCLS recommendations (National committee for clinical Laboratory Standards, 1993) using agar well diffusion method [21].

CG50 fraction of *Coccinia grandis* leaves extract was tested against a panel of Gram-positive and Gram-negative bacterial pathogens; *Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC6633, *Bacillus cereus* ATCC14759, and *Staphylococcus aureus* ATCC2921, *Candida Albicans* ATCC10231 by using agar well diffusion method [22]; using 100 µl of suspension as inoculum containing 1 x10^6 CFU/ml of the pathogenic bacteria. The tested micro-organism inoculums were spread on a sterile Petri dishes containing Müller-Hinton agar. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 µl of CG50 fraction prepared by dissolving 10 mg of the fraction in one ml of dimethyl sulfoxide (DMSO). The inculcated plates were then incubated for 24 h at 37 °C. After incubation time, antibacterial activity was evaluated by measuring the zones of inhibition against the tested organisms. The observed zones of inhibition were recorded and expressed as inhibition diameter zone in millimeters (mm) compared to 5 mg/ml of the standard antibiotic. The experiment was carried out in triplicate and the average zone of inhibition was calculated [23],[24].

Statistical analysis

Results of zone of inhibition were expressed as mean and standard deviation (mean ± S.D.). Statistical analysis was performed with student's t-test and differences were considered as significant at *p*-values below 0.05.

Determination of The Minimum Inhibitory Concentration (MIC)

The MIC activity of CG50 fraction of the plant extract was evaluated using the two-fold serial dilution [25]. Two-fold serial dilutions of CG50 were prepared using the proper nutrient broth. The final concentrations of the solutions prepared from the fraction were 2000, 1000, 500 and 250 µg/ml. The tubes were then inoculated with the test organism, grown in their suitable broth at 37 °C for 24 hours for tested microorganisms, each 5 ml received 0.1 ml of the above inoculum and incubated at 37 °C for 24 hours. The lowest concentration showing no growth of the microbe was taken as the minimum inhibitory concentration (MIC).

Results and Discussion

Preliminary phytochemical screening

Preliminary phytochemical screening of *Coccinia grandis* leaves was carried out to explore the phytochemical composition of the methanolic extract. Results showed that *C. grandis* contains phenolic compounds, flavonoids and tannins, as well as saponins. While cardiac glycosides, alkaloids and anthraquinones were absent.

Isolation and identification of natural compounds

Five compounds were isolated for the first time from *C. grandis* species. The isolated compounds were investigated using various spectroscopic techniques and identified by comparing the resulted data with data published in literature. They were identified as; ferulic acid **(1)**[26], methyl caffeate **(2)**[27], ligstroside **(3)**[28], *trans-p*-coumaric acid **(4)**[29], kaempferol-3-*O*-*β*-*D*-glucoside **(5)**[30]

Ferulic acid (1) was isolated as a light-yellow amorphous powder. ESI-MS showed a molecular ion peak at m/z 194 (M+, base peak).^{1}H NMR spectral analysis (DMSO-d_6, 400 MHz) gave the following signals (δ ppm): 7.22 (1H, *d, J* = 1.8 Hz, H-2), 7.04 (1H, *d*, *J* = 8.1 Hz, H-5), 6.78 (1H, *dd*, *J* = 8.1;1.8 Hz, H-6), 7.44 (1H, *d*, *J* =16, H-7), 6.34 (1H, *d, J* = 16 Hz, H-8), 3.82 (3H, *s*, OCH_3).^{13}C NMR spectral analysis (DMSO-d_6,100 MHz) gave the following signals (δ ppm): 126.13 (C-1), 111.10 (C-2), 147.96 (C-3), 148.89 (C-4), 115.63 (C-5), 122.54 (C-6), 144.22 (C-7), 116.42 (C-8), 55.71(OCH_3).

TABLE 1. Preliminary phytochemical screening of *Coccinia grandis* leaves

Phytoconstituents	Results
Flavonoids	+
Sterols and / or triterpenoids	+
Saponins	+
Cardiac glycosides	-
Tannins	+
Alkaloids	-
Anthraquinones	-

(-): Absent, (+) Present

Methyl caffeate (2) was isolated as a yellow amorphous powder. ESI-MS showed a molecular ion peak at m/z 194 (M+, base peak).^{1}H NMR spectral analysis(DMSO-d_6, 400 MHz) gave the following signals (δ ppm): 7.04 (1H, *d, J* = 2.0 Hz, H-2), 6.73 (1H, *d*, *J* = 8.0 Hz, H-5), 6.97 (1H, *dd, J* = 8.0;2.0 Hz, H-6), 7.48 (1H, *d, J* = 15.9 Hz, H-7), 6.25 (1H, *d, J* = 15.9 Hz, H-8), 3.68 (3H, *s*, OCH_3). ^{13}C NMR spectral analysis (DMSO-d_6, 100 MHz) gave the following signals (δ ppm): 126.42 (C-1), 115.17 (C-2), 146.12 (C-3), 149.09 (C-4), 114.66 (C-5), 122.72 (C-6), 146.22 (C-7), 116.72 (C-8), 168.58 (C-9), 52.36 (OCH_3).

Ligstroside (3) was isolated as a yellow powder. ESI-MS showed $[M-H]^-$ion peak at m/z 523.^{1}H NMR spectral analysis (DMSO-d_6, 400 MHz) gave the following signals (δ ppm): 5.86 (1H, *s*,H-1), 7.51 (1H, *s*, H-3), 3.83 (1H, *m*, H-5), 2.40 (2H, *m*, H-6), 5.97 (1H, *q*, H-8), 1.60 (3H, *d*, *J* = 6.0 Hz, H-10), 4.09 (2H, *m*, H-1'), 2.74 (2H, *t*, H-2'), 7.02 (2H, *d*, *J* = 8.4, H-4' and H-8'), 6.67 (2H, *d*, *J*=8.4, H-5' and H-7'), 3.64 (3H, *s*, OCH_3), 4.64 (1H, *d*, *J*=8, H-1''). ^{13}C NMR spectral analysis (DMSO-d_6, 100 MHz) gave the following signals (δ ppm): 92.98 (C-1), 153.52 (C-3), 107.73 (C-4), 30.21 (C-5), 170.76 (C-7), 123.13 (C-8), 127.89 (C-9), 13.02 (C-10), 166.60 (C-11), 65.15 (C-1'), 33.53 (C-2'), 129.15 (C-3'), 129.86 (C-4' & C-8'), 115.22 (C-5' & C-7'), 155.90 (C-6'), 99.04 (C-1''), 70.02 (C-2''), 76.57 (C-3''), 73.33 (C-4''), 77.44 (C- 5''), 61.18 (C-6''), 51.33 (OCH_3).

***Trans-p*-coumaric acid (4)** was isolated as a colorless powder. ESI-MS showed a molecular ion peak at m/z 164 (M+, base peak).^{1}H NMR spectral analysis (DMSO-d_6, 400 MHz) gave the following signals (δ ppm): 7.44 (2H, *d, J* = 8.4, H-2 and H-6), 6.78 (2H, *d*, *J* = 8.4, H-3 and H-5), 7.40 (1H, *d*, *J* =16, H-7), 6.27 (1H, *d*, *J* =16, H-8). ^{13}C NMR spectral analysis (DMSO-d_6, 100 MHz) gave the following signals (δ ppm): ^{13}C NMR spectral analysis (DMSO-d_6, 100 MHz) gave the following signals (δ ppm): 126.24 (C-1), 130.56 (C-2 and C-6), 116.45 (C-3 and C-5), 159.67 (C-4), 144.02 (C-7), 117.38 (C-8), 169.97 (CO).

Kaempferol-3-*O*-*β*-*D*-glucoside (5) was isolated as a yellow powder. ESI-MS showed a molecular ion peak at m/z 448 (M+, base peak).^{1}H NMR spectral analysis (DMSO-d_6, 400 MHz) gave the following signals (δ ppm): 6.11 (1H, *d*, *J* = 1.6 Hz, H-6), 6.32 (1H, *d*, *J* = 1.6 Hz, H-8), 8.03 (2H, *d, J*=8.8, H-2' and H-6'), 6.88 (2H, *d, J*=8.8, H-3' and H-5'), 5.44 (1H, *d, J* = 7.6, H-1''). ^{13}C NMR spectral analysis (DMSO-d_6, 100 MHz) gave the following signals (δ ppm): 156.86 (C-2), 133.41 (C-3), 177.49 (C-4), 161.40 (C-5), 99.51(C-6),168.25 (C-7), 94.29 (C-8), 156.38 (C-9),121.27 (C-1'), 131.17 (C-2' and C-6'), 115.46 (C-3' and C-5'), 160.18 (C-4'), 101.32 (C-1''). 74.51 (C-2''), 76.68 (C-3''), 70.15 (C-4''), 77.66 (C-5''), 61.12 (C-6'').

Biological activity

CG50 fraction of *C. grandis* leaves was tested *in vitro* for antibacterial activity against different strains of Gram +ve, Gram −ve and yeast. Results in Table 2 indicate that CG50 has no activity against *Bacillus subtilis* but it has good activity against *Staphylococcus aureus* (19 ± 0.534 mm). On the other hand, significant inhibition was recorded against *Bacillus cereus* (20 ± 0.711 mm). Therefore, *B. cereus* was employed as a test bacterium to determine the minimum inhibitory concentration of the extract. MIC of CG50 fraction against *B. cereus* was1 mg/ml.

Bacillus cereus is considered as an important pathogen responsible for a wide array of gastrointestinal and non- gastrointestinal human infectious [31]. It was also reported to be associated with postoperative and post- traumatic

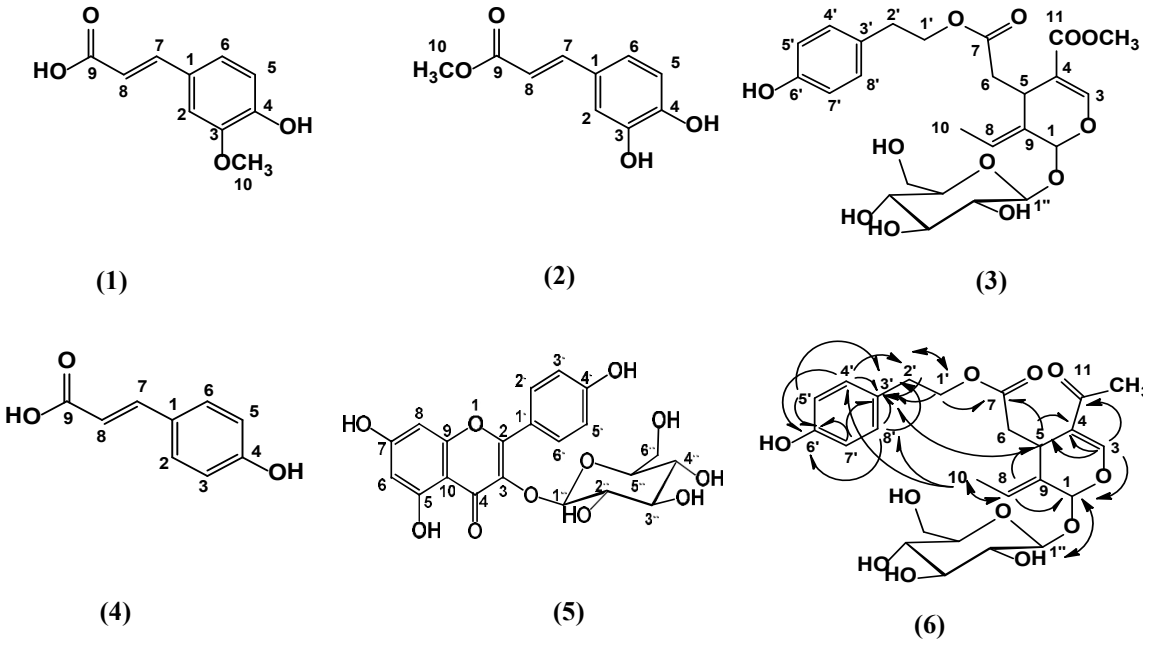

Fig. 2. Compounds isolated from CG50 fraction of methanol extract of *Coccinia grandis* leaves; (1) ferulic acid, (2) methyl caffeate, (3) ligstroside, (4) *trans-p-* coumaric acid, (5) kaempferol-3-*O-β- D*-glucoside, (6) HMBC correlation of ligstroside.

TABLE 2. Zones of inhibitions of CG50 fraction against tested pathogenic microbes

Tested pathogenic microbe Zones of inhibition	Gram +ve		Gram-ve		Yeast
(mm)	*B. subtilis*	*B. cereus*	*S. aureus*	*E. coli*	*C. albicans*
(CG50)	NA	20 ± 0.711	19 ± 0.534	15 ± 0.323	19 ± 0.080
Ciprofloxacin	30 ± 0.78	29 ± 1.52	28 ± 1.51	30 ± 1.73	-
Ketoconazole	-	-	-	-	28 ± 0.52

Data are measured in triplicates (n = 3) and presented as means ± SD, well diameter: 10 mm (100 µL were tested) Concentration of the tested sample =10 mg/mL, Concentration of standard antibiotics = 5 mg/mL, NA: No Activity

wounds infections [32]. The anti-microbial activity of CG50 fraction shown in this study against *B. cereus* may explain the traditional use of the plant in treatment of cuts and wounds [33].

Staphylococcus aureus is considered the main pathogen responsible for most infections in humans especially in skin and soft tissues such as abscesses [34]. Our result goes in agreement with the reported data about the activity of *C. grandis* leaves extract against other strains of *S. aureus* [35]. Therefore, the use of *C. grandis* in treatment of abscesses and skin eruption in folk medicine may be attributed to the anti-bacterial activity of extract against *S. aureus.*

Escherichia coli is a commensal bacterium that found in human's intestine. However, some of its strains are pathogenic and may cause diarrhea (enteropathogenic *E. coli*) or other infections such as urinary tract infections (UTI), meningitis and septicemia (extraintestinal *E. coli*) [36]. Findings in this study go in agreement with previously reported studies examined the anti-microbial activity of the *C. grandis* extract against different strains of *E. coli* [35]. These results may interpret the use of *C. grandis* for treatment of UTIs in folk medicine.

Candida albicans is an opportunistic yeast that occurs as a commensal microbe in the

mouth, genitourinary and gastrointestinal tracts. It causes infections in children, elderly persons or immunocompromised patients [37]. Our findings go in agreement with previously published data concerning the activity of the plant against *C. albicans* [38]. These findings may explain the efficacy of using *C. grandis* for treatment of mouth ulcers in folk medicine [33].

Conclusion

Methanol extract of *Coccinia grandis* leaves was fractionated and purified using different techniques of chromatography. Five pure compounds were isolated from a selected fraction of the extract (CG50) and their structure were confirmed using different techniques of spectroscopy. The isolated compounds were reported for the first time from *C. grandis* species and ligstroside was reported for the first time from Cucurbitaceae family.

CG50 fraction was tested against different types of pathogenic bacteria and yeast and showed maximum anti-microbial activity against *Bacillus cereus*. The observed activity could be attributed to the presence of phenolic structures. Further chemical investigation studies are needed to explore the modes of action of the extract.

Acknowledgment

This research was supported and financed by the Faculty of Pharmacy, Sana'a University, Sana'a, Yemen, through its Master linkage program with the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The authors also want to acknowledge the Center of Drug Discovery, Research and Development, Faculty of Pharmacy, Ain Shams University for hosting NMR and mass measurments.

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فصل و تعرف على التركيب الكيميائي لمركبات من خلاصة اوراق نبات "كوكسينيا جراندس"

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في هذه الدراسة تم إجراء فحص كيميائي أولي لاوراق نبات "كوكسينيا جراند" و قد أظهر هذا الفحص ان النبات يحتوي على التيربينويدات والفلافونويدات ومشتقاتهم من الجلايكوسيدات، والصابونينات والستيرول. كما تم فصل خمسة من المركبات النقية من جزء مختار من المستخلص الميثيلي لنبات " كوكسينيا جراندس" أعطي الكود (CG50) وهي : حمض الفيروليك **(١)**، ميثيل كافيات (٢)، ليجستروسيد **(٣)** ، ترانس-بارا-حمض الكيوماريك **(٤)** و كامفيرول ٣--*O*- بيتا- دي-جليكوسايد **(٥)**. و تم التعرف على تركيبها الكيميائي باستخدام العديد من الوسائل الطيفية مثل ^{1}H NMR، ^{13}C NMR، HSQC وHMBC و جهاز قياس طيف الكتلة .كما تبين أن المركبات **(١)** ، **(٢)** ، **(٤)** و **(٥)** تم فصلها لأول مرة من نبات كوكسينيا جراندس في حين تم عزل المركب **(٣)** لأول مرة من العائلة القرعية. علاوة على ذلك فقد تم اختبار النشاط المضاد للميكروبات للجزء المختار من المستخلص الميثيلي لاوراق النبات (CG50) ضد العديد من البكتريا موجبة الجرام و سالبة الجرام و الخميرة ووجد أن له تأثير واضح ضد بكتيريا *Bacillus cereus* حيث بلغ قطر منطقة التثبيط ٢٠ مم و بلغ أقل تركيز للجزء المختار المؤدي الى تثبيط نمو البكتيريا المعنية (MIC(١ملليغرام / مل.