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Phytochemical Analysis and Antimicrobial Activity of *Euphorbia milii*

Amal Z. Hassan[a], Heba-tollah M. Sweelam[a], Kamel H. Shaker[a,*], Moustafa M. Zohair [b,c], Warda E. Ashour[b], Howaida I. Abd-Alla[a]

[a] *Chemistry of Natural Compounds Department, Pharmaceutical and Drug Industries Institute, National Research Centre, El -Behouth St, Dokki-Giza 12622, Egypt*

[b] *Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industries Institute, National Research Centre, El-Behouth St., Dokki-Giza 12622, Egypt*

[c] *Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 819 -0395, Japan*

Abstract

Antimicrobial activities of *Euphorbia milii* (Euphorbiaceae) extract and its sub-fractions against Gram-negative, Gram-positive bacteria, unicellular yeast and two filamentous fungi were evaluated and the minimum inhibitory concentrations were determined. The ethyl acetate fraction (EAf) exhibited the most significant antimicrobial activity against five tested pathogenic microbes. For the first time, liquid chromatography-electrospray ionization-time-of-flight mass spectrometry analysis (LC-ESI-TOF-MS) of EAf was used to identify its chemical composition. LC-MS/MS analysis allowed the tentative identification of 22 compounds. Quercitrin, luteolin, quercetin, kaempferol-3-glucuronide and naringenin were the major constituents. The chemical composition of un-saponified and saponified from *n*-hexane fraction was analysed by gas chromatography/mass spectrometry (GC-MS). The oxygenated compounds represent the major constituent in both fractions (56.48 and 61.97%, respectively). Terpenes represented by 30.94% in un-saponified fraction while fatty acids and esters constituted by 18.91% in the saponified fraction. Quantification of the total phenolics and flavonoids of the methanolic extract and its successive fractions were determined. The highest percent of the total phenolic was observed for methanolic extract while total flavonoids were recorded for *n*-butanol and ethyl acetate.

Keywords: LC-MS/MS, GC-MS, Euphorbiaceae; *Euphorbia milii*, antimicrobial activity, flavonoids, phenolics

1. Introduction

Antimicrobial resistance possesses a significant challenge for the treatment of bacterial and fungal infections [1]. Several last resort antibiotics are becoming increasingly ineffective for therapy due to antimicrobial resistance which led to the upsurge of novel antimicrobial treatment options [2]. Medicinal plants are fertile source of bioactive compounds which are used to protect against predation by microorganisms, insects, and herbivores [3]. These plants become public because of their low toxicity to humans and their surroundings [4]. *Euphorbia* plants (Euphorbiaceae) form the third largest flowering plants genus with nearly two thousand species in the world. They are distributed all over the world except in very cold areas [5]. They have been used since ancient times as traditional medicinal herbs for treatment of different ailments as asthma, cough, tumours, and warts [6]. The determination of the chemical constituents of herbal samples is an essential issue since plant materials have a variety of compounds with different chemical structures and complex matrices. Nowadays, liquid chromatography–mass spectrometry (LC-MS) is the most widely used technique to characterize the secondary metabolites of plants [7, 8]. *Euphorbia* genus contains a large number of active biological compounds [5, 9]. It was found that *Euphorbia* comprises mainly, triterpenoids, diterpenoids, flavonoids, tannins and polyphenols [5, 9].

Plants are synthetizing flavonoids in response to microbial infection [10]. It was reported that the genus *Euphorbia* is rich in flavonoids, which have been identified in different parts of about thirty species of *Euphorbia*. The alcoholic extracts of some *Euphorbia* species such as *E. falcate*, *E. hirta*, *E.*

*Corresponding author e-mail: kamelshaker11@yahoo.com;(Kamel H. Shaker)

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denticulate, E. macroclada E. cheiradenia E. virgate E. petiolata exhibited antimicrobial activities [5, 11]. Previous phytochemical studies of *E. milii* revealed the presence of *β*-sitosterol, 1-octacosanol, *β*-amyrin acetate, lupeol, cycloartenol, euphol, phenolic compounds, flavonoids, alkaloids, carbohydrates, proteins, anthocyanins, *β*-cyanin, amino acids, cardiac glycosides, steroids, anthraquinones, tannins, phlobatannins, saponins, reducing sugars, coumarins and triterpenes [12,13]. Kaempferol 3-(6'-acetylglucoside)-7-glucoside, kaempferide 5-glucoside-7-glucuronide and herbacetin 8- acetate were detected in alcoholic extract of *E. milii* [12]. The antibacterial activities of flavonoids from other genera in the Euphorbiaceae have been studied against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* [13, 14].

The current study is undertaken to evaluate the antimicrobial activities of various fractions from methanol extract of the aerial parts of *Euphorbia milii*. Identification of the chemical composition of the most active fraction through liquid chromatography associated with mass spectrometry (LC-MS/MS) was carried out for the first time. It is the first systematic study of antimicrobial evaluations for the fractions of the total alcoholic extract using LC-MS/MS technique to determine their chemical composition.

Materials and Methods

2.1 Plant material collection

The aerial parts of *Euphorbia milii* (650 g) were collected from El-Orman Garden, Giza, Egypt, in March, 2021. The parts were air dried to yield 300.4 g. Identification of species was carried out by Dr. Mohamed El-Gebali, Taxonomy Specialist, El-Orman Botanical Garden at Giza. A specimen (EM-AH032021) was deposited and authentic reference material is available at the Department of Chemistry of Natural Compounds, National Research Centre. The plant sample was kept in bags of polyethylene, transported to the laboratory, and dried at room temperature.

2.2 Chemicals and reagents

Methanol, hexane, chloroform, ethyl acetate and *n*-butanol were purchased from Sigma-Aldrich (Chemie Gmbh, Steinhein, Germany). All the other chemicals and solvents were of analytical class and were purchased from Merck Chemical Supplies (Darmstadt, Germany).

2.3 Strains and standards source

Gram positive bacteria (*Staphylococcus aureus* ATCC29213 and *Bacillus subtilis* ATCC 6633), Gram negative bacteria (*Escherichia coli* ATCC 25922), unicellular yeast fungi (*Candida albicans* ATCC 10321 and *Candida tropicalis* ATCC 750 and filamentous fungi (*Fusarium* NRC15 and *Fusarium oxysporum* NRC2017) were used. Bacteria and yeast strains are American Type Culture Collection, and fungal isolates were obtained from the culture collection of the Department of Chemistry of Natural and Microbial Products, National Research Centre, Cairo, Egypt. Thiophenicol (Thiamphenicol, Sanofiaventis, France) and Treflucan (Fluconazole, Egyptian Pharmaceutical Industries Company EIPICO) were used as antibacterial and antifungal positive controls, respectively, in a concentration of 100 µg/disk and DMSO was used as a negative control. The disks (1 mg/5 µl) were applied on inoculated nutrient agar and PDA media plates and incubated for 24 h at 37 °C for bacteria and 72 h at 28 °C for fungal strains, respectively. The zone of inhibition was recorded and compared with the positive control treatments

2.4 Extraction and fractionation

The air-dried powdered aerial parts of *E. milii* (300.4 g) was exhaustively extracted with 80% methanol (3 × 500 ml) at room temperature. The methanolic extract was distilled in vacuum at 40℃ to yield 8.4 g of the total methanolic extract Fr1 (representing 2.8% of the air-dried aerial parts). The total Fr1 was suspended in water and successively partitioned with various solvent systems to obtain various fractions including *n*-hexane (Fr2, 0.43 g), ethyl acetate (Fr3, 2.44 g) and *n*-butanol (Fr4, 1.60 g).

2.5 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed according to previously reported methods [14].

2.6 Lipid extraction

The plant powder (100 g) was extracted with *n*-hexane (2 x500 ml) over 24 h each time, at room temperature. The *n*-hexane extract was dried over anhydrous sodium sulphate and evaporated under pressure at 40℃ to dryness to get 0.52 g lipid content. The lipid material was saponified by refluxing with 50 ml of methanolic KOH (10%, w/v) for 3h [15]. The saponifiable fraction (SAP) was extracted by acidification with 2N HCl of the alkylated aqueous solution. Identification of components of both fractions UNS and SAP were achieved by comparing their relative retention time and mass spectra with those of the NIST, WILLY mass spectral library data of the GC-MS system.

2.7 Total phenolics and flavonoids

The The phenolic amounts in the total extract Fr1 of *E. milii* and its successive fractions Fr2 to Fr4 were

determined according to Attard method [16]. A stock solution of standard- gallic acid (GA) 1 mg/ml in methanol was prepared followed by preparation of seven serial dilutions in the concentrations of 1000, 800, 600, 400, 200, 100, and 50 µg/ml. Each of the 7 gallic acid (GA) standard concentrations and 1 sample under estimation were pipetted in the plate wells in six replicates. Measurement was performed at 630 nm through reader microplate Fluostar Omega (BMG Labtech, Chicago, IL, USA). To estimate flavonoid contents in fraction samples Fr2 to Fr4, a standard rutin was prepared as stock solution of 1 mg/ml in methanol then preparation of 7 serial dilutions in the concentrations of 1000, 600, 400, 200, 100, 50 and 10µg/ml. Each of the seven rutin standard concentrations and 1 sample were pipetted in the plate wells in six replicates. Measurement was performed at 420 nm by the reader Fluostar Omega. The total phenolics and flavonoids in the tested samples were calculated and expressed as gallic acid and rutin equivalent in mg/g of sample, respectively.

2.8 Antibacterial and antifungal activities

The sequential fractions Fr1 to 4 were screened *in vitro* by an agar diffusion method [14] against Gram positive bacteria (*Staphylococcus aureus* ATCC29213 and *Bacillus subtilis* ATCC 6633), Gram negative bacteria (*Escherichia coli* ATCC 25922), unicellular yeast fungi (*Candida albicans* ATCC 10321 and *Candida tropicalis*), and filamentous fungi (*Fusarium solani* NRC15 and *Fusarium oxysporum* NRC2017).

2.8.1 The minimum inhibitory concentration (MIC)

MICs of the total methanolic extract Fr1 and the ethyl acetate fraction Fr3 as well as, the unsaponified UNS and saponified SAP fractions were determined *in vitro* against Gram positive bacteria (*Staphylococcus aureus* ATCC29213), Gram negative bacteria (*Escherichia coli* ATCC 25922), unicellular yeast fungi (*Candida albicans* ATCC 10321), and filamentous fungi (*Aspergillus niger* NRC53) by microtitre broth dilution method. MIC was performed using serial dilution, as previously described according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [17, 18]. The serial dilutions from the stock solution were made (500, 250, 125 and 62. 5 ug) using nutrient broth and potato dextrose broth for bacteria and fungi respectively in 96-well microplates. The bacterial suspension containing approximately 5×10^5 colony-forming units/ml was prepared from a 24 h culture plate. From this suspension, 100 µl was inoculated into each well. A sterility control well and a growth control well were also studied for each strain. Positive controls for antibacterial and antifungal activity were thiophenicol and treflucan at concentrations of 500, 250, 125, and 62.5 g, respectively. The microtiter plates were incubated at 37 °C, for 24 h for bacteria and 48 h for fungi. After incubation; the MIC values are visually determined. The lowest concentration of each extract/fraction displaying no visible growth was recorded as MIC.

2.9 LC-ESI-TOF-MS Analysis

2.9.1. Sample preparation

Sample preparation was done as previously reported [14]. A stock solution of the extract was prepared from 50 mg of the lyophilized ethyl acetate extract dissolved in 1000 µl of the solvent mixture composed of water: methanol: acetonitrile (H_2O:MeOH: ACN) in a ratio of 2:1:1. Complete solubility of stock solution was obtained by sample vortexed and ultra-sonication at 30 KHz for 10 min. An aliquot, 20 µl of the stock solution was again diluted with 1000 µl of the H_2O: MeOH: ACN (2:1:1) and centrifuged at 10,000 rpm for 5 min, and 10 µl (1 µg/ml) was used for injection. The LC-MS analysis was also performed for blank and quality control samples/internal standard (IS) for confidence in the experiment. The sample was injected in negative mode.

2.9.2 Instruments and acquisition method

Small molecules were separated on an ExionLC system (AB Sciex, Framingham, MA, USA) connected with an autosampler system, an in-line filter disks pre-column (0.5 µm×3.0 mm, Phenomenex, Torrance, CA, USA), and an Xbridge C18 (3.5 µm, 2.1×50 mm) column (Waters Corporation, Milford, MA, USA) was maintained at 40 °C, and at a flow rate of 300 µL/min was utilized. The mobile phase consisted of solution (A): 5 mM ammonium formate in 1% methanol, pH adjusted to 8.0 by using sodium hydroxide, and solution (B): consisting of acetonitrile (100%). The gradient elution was performed with the following program: 0–20 min, 10% B; 21–25 min, 90% B; 25.01–28 min, 10% B; and then 90% B for equilibration of the column. The mass spectrometry (MS) was performed on a Triple TOF 5600+system equipped with a Duo-Spray source operating in the ESI mode (AB SCIEX, Concord, ON, Canada). The sprayer capillary and declustering potential voltages were − 4500 and − 80 V. The source temperature was set at 600 °C, the curtain gas was 25 *psi*, and gas 1 and gas 2 were 40 psi. The collision energy − 35 V (negative mode) with CE spreading 20 V and the ion tolerance for 10 ppm were used. The TripleTOF5600+was operated using an information dependent acquisition (IDA) protocol. Batches for MS and MS/MS data collection were created using Analyst-TF 1.7.1. The IDA method was used to collect full-scan MS and MS/MS information simultaneously. The method consisted of high-

resolution survey spectra from 50 to 1100 *m/z* and the mass spectrometer was operated in a pattern where a 50-ms survey scan was detected. Subsequently, the top 15 intense ions were selected for acquiring MS/MS fragmentation spectra after each scan [19].

2.9.3 LC–MS data processing

MS-DIAL 3.70 open-source software [20] was used for non-targeting, small molecule comprehensive analysis of the sample. According to the acquisition mode, ReSpect negative (1573 records) databases were used as reference databases. The search parameters were set as MS1 and MS2 mass tolerance: 0.01 Da and 0.05 Da for data collection, for peak detection; minimum peak height: 100 amplitude, mass slice width: 0.05 Da, smoothing level: 2 scans, minimum peak width: 6 scans, for identification MS1 and MS2 tolerance: 0.2 Da/each, for alignment; retention time tolerance: 0.05 min and MS1 tolerance: 0.25 Da. The MS-DIAL output was used to run again on PeakView 2.2 with the MasterView 1.1 package (AB SCIEX) for feature (peaks) confirmation from Total Ion Chromatogram (TIC) based on the criteria; aligned features having signal to-noise ratio greater than 5 and intensities of the sample: blank greater than 5.

2. Results and Discussion

2.1. GC-MS analysis of *n*-hexane extract

The components of both the unsaponified (UNS) and saponified (SAP) fractions from *n*-hexane extract were achieved by GC-MS analysis. Analysis of UNS fraction revealed the presence of 29 compounds accounting for 96.52% of the total fraction (Table 1). The detected compounds were categorized as oxygenated compounds (56.48%), terpenes (30.94%), sterols (4.91%) and hydrocarbons (4.19%). The acyclic diterpene alcohol, phytol was the main detected compound (19.67%) followed by the heterocyclic compound oxirane (dodecyloxy) methyl (8.57%), the fatty alcohol-1-hexadecanol (8.16%) and the carbotricyclic compound veridiflorol (5.99%). GC-MS analysis of the saponifiable fraction (SAP) allowed identification of 34 different compounds with 97.24% (Table 2). The oxygenated compounds represent the major constituent (61.97%) followed by fatty acids and esters (18.91%) and hydrocarbon compounds (16.36 %). 1,5-Hexdiene-3,4-diol,2,5-dimethyl was the major compound (22.67%) followed by 2,4,6-tripropyl-1,3,5-trioxane (9.24%) and 10-undecenoic acid, methyl ester (5.05%). GC-MS showed the presence of unsaturated fatty acids (15.41%) more than saturated one (3.5%) (Table 2). It could be noted that this is the first GC-MS identification of the lipid portion of *E. milii*.

3.2 Total phenolics and flavonoids determination

The amount of the total phenolics (TP), expressed as mg of gallic acid (GA) per gram sample, is shown in Fig. 1. The number of phenolic compounds (mg GA/g) was arranged as follows: Fr1 (43.25 ± 3.05) > Fr4 (24.89 ± 1.18) > Fr3 (19.84 ± 1.45) > Fr2 (7.79 ± 0.55). The higher quantities of phenolic compounds were observed in the total methanolic extract in agreement with previously reported literature [21]. The total flavonoids (TF) of *E. milii* extract /fractions, expressed as mg of rutin/g (Fig.1). They were very analogous in Fr3 and Fr4 (6.65± 0.75 and 6.54 ± 1.34 mg rutin/g, respectively while, they were 1.21± 0.05 and 0.42± 0.02 for fractions Fr1 and Fr2, respectively. Flavonoids are thought to play an important role in many aspects of plant life, including interactions with the environment, where they protect plants from various biotic and abiotic stresses [13, 22, 23].

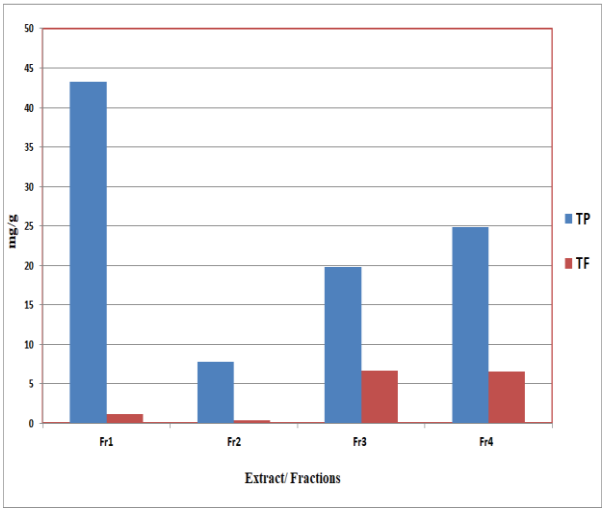

Figure 1: Total phenolics (TP) and total flavonoids (TF) in extract/fractions of the aerial parts of *E. milii*, Fr1: Total methanol extract, Fr2: *n*-Hexane fraction, Fr3: Ethyl acetate fraction, Fr4: Butanol fraction

3.3 Antibacterial and antifungal activities

Antibacterial and antifungal activities of the sequential fractions Fr1 to 4 were screened *in vitro* against Gram positive bacteria (*Bacillus subtilis* ATCC 6633), Gram negative bacteria (*Escherichia coli* ATCC 25922), unicellular yeast fungi (*C. tropicalis* ATCC 750 and filamentous fungi (*F. solani* NRC15 and *F. oxysporum* NRC2017) and the results were presented in Table 3.

Table 1
GC-MS analysis of UNS fraction of *E. milii* aerial parts

No	RT	Compound	Area %	Molecular Formula	Molecular Weight
1	5.16	*cis*-4-Methylcyclohex-3-ene-1,2-diol	2.97	$C_7H_{12}O_2$	128
2	24. 41	*cis*-Sesquisabinene hydrate	1.09	$C_{15}H_{26}O$	222
3	28. 24	1-Tetradecene	0.95	$C_{14}H_{28}$	196
4	29. 34	Oxirane, [(dodecyloxy)methyl]	8.57	$C_{15}H_{30}O$	242
5	30. 08	1-Tridecanol	4.22	$C_{13}H_{28}O$	200
6	30. 70	*cis*-Phytol	6.27	$C_{20}H_{40}O$	296
7	30. 96	Heneicosanoic acid, methyl ester	3.06	$C_{22}H_{44}O_2$	340
8	31. 77	1,2-Benzenedicarboxylic acid, dibutyl ester	1.37	$C_{16}H_{22}O_4$	278
9	32. 14	(*cis*)-2-nonadecene	1.76	$C_{19}H_{38}$	266
10	32. 24	Octacosanoic acid, 2,4,6,8-tetramethyl-, methyl ester	1.68	$C_{33}H_{66}O_2$	494
11	33. 21	Octadecane, 1-(ethenyloxy)	2.19	$C_{20}H_{40}O$	296
12	33. 54	11-Hexadecen-1-ol, (Z)	2.57	$C_{16}H_{32}O$	240
13	33. 77	Patchoulane	1.22	$C_{15}H_{26}$	206
14	33. 88	1-Hexadecanol	8.16	$C_{16}H_{34}O$	242
15	34. 09	Linoleic acid, methyl ester;	3.67	$C_{19}H_{34}O_2$	294
16	34. 19	Oleic acid, methyl ester	3.31	$C_{19}H_{36}O_2$	296
17	34. 42	Phytol	19.67	$C_{20}H_{40}O$	296
18	34. 91	4-Hexenoic acid, 3-methyl-2,6-dioxo-	1.09	$C_7H_8O_4$	156
19	35. 42	(-)-Caryophyllene oxide	1.13	$C_{15}H_{24}O$	220
20	35. 78	Geranyl linalool isomer B	1.84	$C_{20}H_{34}O$	290
21	37. 07	E-7-Tetradecenol	1.92	$C_{14}H_{28}O$	212
22	37. 47	Tridecane	1.48	$C_{13}H_{28}$	184
23	38. 07	*cis*-Oleic Acid	0.80	$C_{18}H_{34}O_2$	282
24	38. 47	4,8,12,16-Tetramethylhep tadecan-4-olide	1.86	$C_{21}H_{40}O_2$	324
25	39. 04	Gitoxigenin	1.95	$C_{23}H_{34}O_5$	390
26	40. 37	Z-(13,14-Epoxy)tetradec11-en-1-ol acetate	2.77	$C_{16}H_{28}O_3$	268
27	51.99	7,8-Epoxylanostan-11-ol,3-acetoxy	0.91	$C_{32}H_{54}O_4$	502
28	52. 58	9,19-Cyclolanostan-3-ol, acetate, (3á)-	2.05	$C_{32}H_{54}O_2$	470
29	52. 85	Veridiflorol	5.99	$C_{15}H_{26}O$	222

Table 2
GC/MS analysis of SAP fraction of *E. milii* aerial parts

No.	RT	Compound	Area %	Molecular Formula	Molecular Weight
1	3.58	*cis,cis*-Cyclopentene oxide ether	2.99	$C_{10}H_{14}O_3$	182
2	4.34	1,5-Hexdiene-3,4-diol,2,5-dimethyl	22.67	$C_8H_{14}O_2$	142
3	4.56	5-Isopropenyl-2-methylcyclohexanol	2.29	$C_{10}H_{18}O$	157
4	4.70	Cyclopentanol,1-cyclopentyl	2.27	$C_{10}H_{18}O$	154
5	5.19	7-Methylbicyclo[2.2.1]-hept-2-en-7-ol	1.37	$C_8H_{12}O$	128
6	7.47	2,4,6-Tripropyl-1,3,5-trioxane	9.24	$C_{12}H_{24}O_3$	216
7	10.88	1,2,3,4,5-Cyclopentanepentol	3.60	$C_5H_{10}O_5$	150
8	11.05	5,9-Dodecadien-2-one,6,10-dimethyl-, (E,E))	2.62	$C_{14}H_{24}O$	208
9	12.19	3-(Prop-2-enoyloxy)dodecane	1.96	$C_{15}H_{28}O_2$	240
10	12.36	3,6,9,12-Tetraoxadocosan-1-ol	1.73	$C_{18}H_{38}O_5$	334
11	12.54	3-(1,3-Dihydroxyisopropyl)-1,5,8,11, 14,17-hexaoxacyclononadecane	2.50	$C_{16}H_{32}O_8$	352
12	15.40	Octaethylene glycol monododecyl ether	2.70	$C_{28}H_{58}O_9$	538
13	15.57	l-Gala-l-ido-octose	1.65	$C_8H_{16}O_8$	240
14	16.06	3-Nonenoic-3,4-D_2 acid, methyl ester	2.49	$C_{10}H_{16}D_2O_2$	172
15	16.94	4-Cyclopentene-1,3-diol,trans	1.41	$C_5H_8O_2$	102
16	17.12	*trans*-3-Cyclopentene-3,5-diol	1.59	$C_5H_8O_2$	101
17	17.22	1-Dodecene	1.48	$C_{12}H_{24}$	168
18	18.15	3-(Prop-2-enoyloxy)dodecane	2.95	$C_{15}H_{28}O_2$	240
19	18.57	*trans*-2-Undecenoic acid	1.42	$C_{11}H_{20}O_2$	184
20	19.04	1,2-Epoxynonane	1.84	$C_9H_{18}O$	142
21	19.32	2-Octen-1-ol, (E)-	1.48	$C_8H_{16}O$	128
22	19.51	10-Undecenoic acid,methyl ester	5.05	$C_{12}H_{22}O_2$	198
23	20.05	Benzene,[(1-methylethylidene) cycleopropyl]	1.09	$C_{12}H_{14}$	158
24	20.14	1,2-Benenedicrboxylic acid, *bis* (2- methylpropyl)ester	1.81	$C_{16}H_{22}O_4$	278
25	20.58	Cyclododecanol, 1-ethenyl	1.84	$C_{14}H_{26}O$	210
26	20.67	Ethanol,2-[(2-methyl-2-propenyl)oxy]	1.99	$C_6H_{12}O_2$	116
27	21.13	1,12-Tridecadiene	1.17	$C_{13}H_{24}$	180
28	21.23	Methyl 12,13-tetradecadienoate	1.94	$C_{15}H_{26}O_2$	238
29	21.23	3-Hepten-1-ol	1.94	$C_7H_{14}O$	114
30	21.43	Methyl 12,13-tetradecadienoate	1.43	$C_{15}H_{26}O_2$	238
31	21.83	13,16-Octadecadiynoic acid, methyl ester	1.93	$C_{19}H_{30}O_2$	290
32	22.27	E,E-1,9,17-Docasatriene	1.15	$C_{22}H_{40}$	304
33	22.99	2-Methymalonic acid	1.69	$C_4H_6O_4$	118
34	28.54	3-(Prop-2-enoyloxy)dodecane	1.96	$C_{15}H_{28}O_2$	240

The ethyl acetate fraction Fr3 revealed a promising activity against Gram negative bacteria *E. coli* and filamentous fungi *F. solani* with inhibition zone 1.4 mm and showed a moderate activity against tested yeast *C. tropicalis* ATCC 750 with an inhibition zone of 1.3 mm as well as a considerable effect against tested Gram-positive bacteria *B. subtilis* with inhibition zone of 0.9 mm. The total methanol extract Fr1 has a good antimicrobial activity against Gram negative bacteria *E. coli* and tested yeast *C. tropicalis* ATCC 750 with inhibition zone 1.2 mm. The *n*-hexane fraction Fr2 revealed very strong activity against filamentous fungi *F. solani* with an inhibition zone of 1.5 mm and showed a moderate activity against *E. coli* and tested yeast *C. tropicalis* ATCC 750 with an inhibition zone of 1.0 mm (Table 3). It was mentioned that *E. milii* extract was proactive against *Bacillus subtilis* [4] and also, exhibit antifungal, immune boosting and molluscicidal properties [9, 24]. Based on these obtained results, the ethyl acetate fraction Fr3 showed the most potent microbial activity against five tested microbes comparing with other tested fractions. So, it could be proposed that ethyl acetate fraction contains wide spectrum antimicrobial drugs. Also, it was been detected active at low amounts, which is very important for antimicrobial drugs. The activity of ethyl acetate fraction has been attributed to the presence of flavonoids [25, 26]. Some Egyptian medicinal plants (included *Euphorbia hirta*) have used in the treatment of some diseases in folk medicine of Egypt and as disinfectants, contain abundance of flavonoids, including quercetin, kaempferol, apigenin, luteolin, and their respective glycosides [27]. So, it was selected for phytochemical investigation using LC-MS/MS analysis to determine its components that may be accountable for its activity.

3.3.1 Minimum inhibitory concentration (MIC)

The MICs of the total extract Fr1, ethyl acetate fraction Fr3, unsaponified UNS and saponified SAP fractions derived from the *n*-hexane extract were investigated by microtitre broth dilution method and the results were presented in Table 4. Fr1 showed MIC against tested pathogens ranged from 62.5 ug/ml against *E. coli* ATCC25922 and *A. niger* NRC53 to 125 µg/ml against *S. aureus* ATCC29213 and *C. albicans* ATCC 10321. The most significant activity was recorded for Fr3 against the tested pathogens with MIC 62.5 µg/ml and moderate activity against Gram positive (*S. aureus* ATCC29213) with MIC of 259 ug/ml. The UNS fraction had good inhibition activity with MIC 125 ug/ml against all tested pathogens. The UNS activity can be attributed to the presence of aliphatic alcohols, hydrocarbons, terpenoids and sterols [28, 29]. Phytol, which is an acyclic diterpene alcohol, was reported to have high antimicrobial activity through stimulating oxidative exertion reaction and also, has low toxicity [30]. Previously, some terpenes and their derivatives were examined to be active as antimicrobial drug resistant to pathogens which include fungi and bacteria [31]. Sterols also, have an inhibitory effect on pathogen growth [32]. Fatty acids such as oleic and linoleic acids have been established to have antibacterial activity [33]. The SAP fraction had higher activity against all tested pathogens except Gram negative (*E. coli* ATCC25922). These results may be partly attributed to the presence of fatty acids and their esters. It was reported that medium-chain fatty acids have more antibacterial activity than long-chain fatty acids [34]. Whereas, the monounsaturated and polyunsaturated fatty acids have higher antimicrobial activity, according to the place of double bonds in the long chain fatty acids, than saturated fatty acids with lower chain lengths. Esters have also been shown to have antibacterial properties [35]. It was reported that antibacterial feature of fatty acids is applied by considerable creatures in order to protect against bacteria causing disease but their antibacterial mechanism is still unknown [36].

3.4 LC-MS/MS of ethyl acetate fraction

A combination of liquid chromatography (LC) and mass spectrometry (MS) has become a powerful tool for analysing the quality, quantity and chemical diversity of plant metabolites [37]. Determination of the phytocomponents of ethyl acetate fraction Fr3, the most active antimicrobial fraction of *E. milii* was analyzed via LC-MS/MS. The analysis was performed in negative ionization mode (Fig. 2) and the comparison with the public reference database (HMBD, FOODB) as well as previously reported data of the precursor ion and fragment daughter ions was determined. Twenty-two characterized peaks of different classes of compounds with total ratio of 95.68 % were tentatively identified (Table 5). Flavonoids comprised the main components (91.08 %) in addition to acids (0.42%), terpenes (2.21%) and coumarin (1.97%) were tentatively identified (Table 5). LC-MS chromatogram exhibited flavonoids at retention time from 5.6-10.9 min and the common characteristic MS^2 fragments of flavonoids were observed at 151 and 125 amu. with neutral loss of CO_2, CO and H_2O. The first major flavanol (Fig. 3) was detected at *m/z* 447.0920 $[M\text{-}H]^-$ ($C_{21}H_{19}O_{11}$) followed by neutral loss of deoxyhexose unit ($C_6H_{10}O_4$) at *m/z* 301.0351($C_{15}H_9O_7$) then *m/z* at

Table 3

Antimicrobial activity of *E. milii* extract /fractions

Pathogenic / Fraction No.	Inhibition Zone diameter (mm)				
	Gram positive bacteria	Gram negative bacteria	Yeast	Fungi	
	B. subtilis ATCC 6633	*E. coli* ATCC25922	*C. tropicalis*	*F. solani NRC15*	*F. oxsporium* NRC2017
Fr1	0.8	1.2	1.2	0.8	NA
Fr2	0.8	1.0	1.0	1.5	NA
Fr3	0.9	1.4	1.3	1.4	NA
Fr4	0.7	0.7	NA	0.9	NA
Positive control	1.2	2.0	0.7	1.4	1.5

Notes: The inhibition zone diameter expressed in mm. Thiophenicol and Treflucan were used as positive controls at concentration of 100 µg/disc, DMSO was used as negative control. NA: not appeared, Fr1: total methanol extract, Fr2: *n*-hexane fraction, Fr3: ethyl acetate fraction, Fr4*: n*-butanol fraction

Table 4
Minimum inhibitory concentrations (MICs) of *E. milii* fractions against four different micro-organisms

	Minimum Inhibitory Concentration (MIC) (µg/ml)			
	Gram positive bacteria	Gram negative bacteria	Yeast and Fungi	
	S. aureus ATCC29213	*E. coli* ATCC25922	*C. albicans* ATCC 10321	*A.niger* NRC53
Fr1	125	62.5	125	62.5
Fr3	259	62.5	62.5	62.5
UNS	125	125	125	125
SAP	62.5	250	62.5	62.5
PC*	125	125	125	125

PC*: Positive control, Fr1: total methanolic fraction, Fr3: ethyl acetate fraction, UNS: unsaponified fraction, SAP: saponified fraction

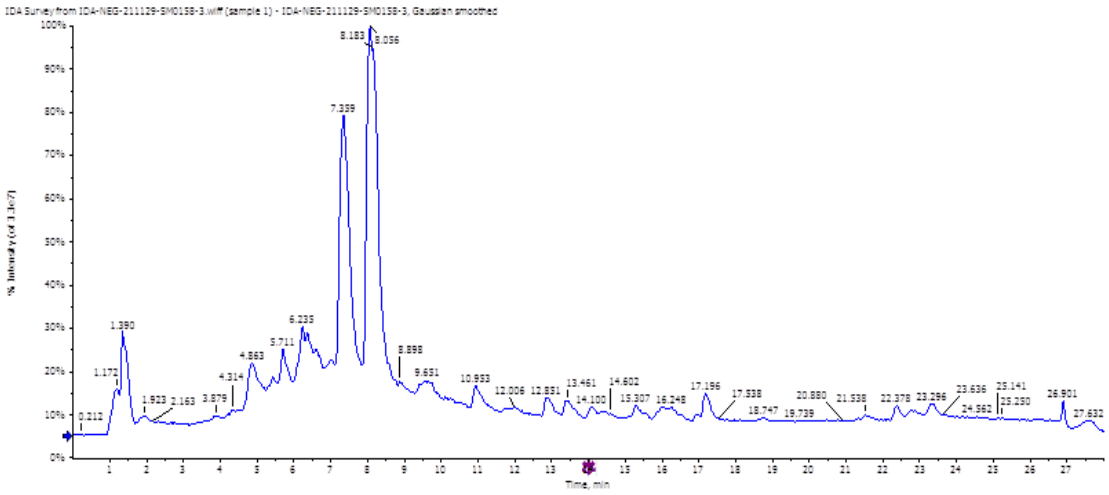

Figure 2: LC-MS chromatogram of ethyl acetate fraction of *E. milii* in negative ionization mode

Table 5
LC-MS/MS of ethyl acetate fraction of *E. milii*

Number	Retention time	Area %	Precursor ion *m/z*	Molecular formula (Error ppm)	MS/MS	Proposed compound	Reference
1	1.04	0.21	133.0874	$C_4H_6O_5$ (1.2)	133,115, 114, 89	D- (+)-Malic acid	[50]
2	1.01	0.04	173.1944	$C_8H_{14}O_4$ (3)	173,111	Suberic acid	[51]
3	1.18	0.01	129.0987	$C_5H_6O_4$ (-0.4)	129,68.84	Citraconic acid	[52]
4	1.38	0.71	353.3087	$C_{16}H_{18}O_9$ (-3.3)	353,191,190,179,173,161, 155,135	Chlorogenic acid	[53]
5	4.61	0.16	359.0759	$C_{18}H_{16}O_8$ (-4.2)	359,197, 161	Rosmarinic acid	[54]
6	5.10	1.97	177.1430	$C_9H_6O_4$ (7)	177, 17	Aesculetin	[55]
7	5.44	0.42	477.3598	$C_{21}H_{18}O_{13}$ (2.3)	478,477,301,178,151	Quercetin-3-glucuronide	[56]
8	5.65	9.90	461.0691	$C_{21}H_{18}O_{12}$ (-0.5)	461,285,229,175,85	Kaempferol-3-glucuronide	[40]
9	5.94	0.06	609.5606	$C_{28}H_{34}O_{15}$ (-0.6)	609, 284,	Hesperetin-7-*O*-neohesperidoside	[57]
10	6.14	0.06	301.2788	$C_{16}H_{14}O_6$ (4.7)	301,120, 164	Hesperetin	[58]
11	6.61	3.30	463.0874	$C_{21}H_{20}O_{12}$ (-1)	463, 300, 255	Isoquercitrin	[59]
12	6.68	0.08	433.3503	$C_{20}H_{18}O_{11}$ (-2.9)	433, 387	Quercetin-3-D-xyloside	[60]
13	6.71	0.13	331.3047	$C_{17}H_{16}O_7$ (-3.7)	303,242,177,153,125, 107	(+)-Taxifolin	[56]
14	7.35	10.07	301.0344	$C_{15}H_{10}O_7$ (0.5)	301,273,178	Quercetin	[40]
15	7.39	0.65	477.4029	$C_{22}H_{22}O_{12}$ (5.5)	477,59	Isorhamnetin-3-*O*-glucoside	[61]
16	7.89	0.37	463.3763	$C_{21}H_{20}O_{12}$ (4.3)	463, 301	Quercetin-4'-glucoside	[62]
17	7.91	0.28	435.4093	$C_{21}H_{24}O_{10}$ (0.40)	435,273	Phlorizin	[63]
18	8.05	45.53	447.0919	$C_{21}H_{20}O_{11}$ (-0.5)	447, 301,271	Quercitrin	[38]
19	8.18	14.60	285.0405	$C_{15}H_{10}O_6$ (-0.5)	285,266,257,151	Luteolin	[39]
20	10.32	6.19	271.2528	$C_{15}H_{12}O_5$ (0.9)	271,177,151, 119,107	Naringenin	[42]
21	10.65	0.09	269.0458	$C_{15}H_{10}O_5$ (-1.1)	269, 197,107	Apigenin	[64]
22	13.37	0.74	345.3700	$C_{19}H_{22}O_6$ (-2.5)	345,227,301	Gibberellin A3	[65]

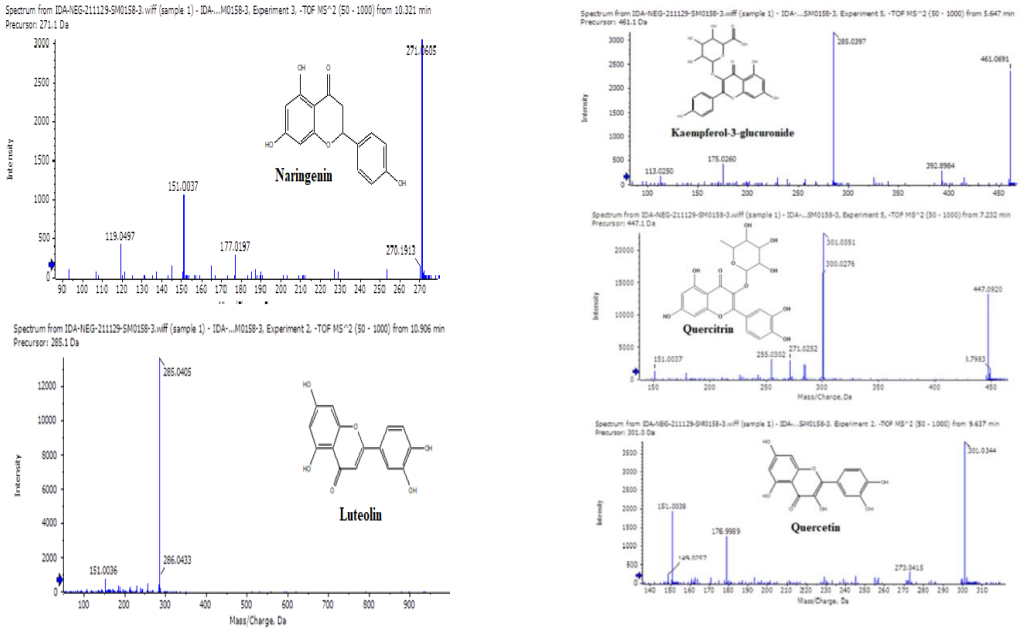

Figure 3: Major identified metabolites *via* LC-MS/MS

300.0276 ($C_{15}H_8O_7$), 271.0252 ($C_{14}H_7O_6$) and the comparison with the reference database, the flavanol identified as quercitrin [38]. The second flavone was determined at *m/z* 285.0405 $[M-H]^-$ ($C_{15}H_9O_6$) followed by neutral loss of H_2O and CO at *m/z* 267.0288 ($C_{15}H_7O_5$), 257.0467 ($C_{14}H_9O_5$) respectively, which identified as luteolin [39]. The other flavonoids were detected at *m/z* 301.0344 ($C_{15}H_9O_7$), 461.0691 ($C_{21}H_{17}O_{12}$) and 271.2528 ($C_{15}H_{11}O_5$), $[M-H]^-$ and were identified as quercetin, kaempferol-3-glucuronide and naringenin, respectively [40-42]. The flavonoids, quercetin-3-glucuronide, kaempferol-3-glucuronide, quercitrin and naringenin were previously reported from different *Euphorbia* species [14, 43].

3.4.1 Antimicrobial activity of identified flavonoids

The flavanone naringenin has been reported to have antibacterial activity against methicillin-resistant *Staphylococcus aureus* [10, 26]. The antibacterial effect of this flavonoid was noticed to be caused by reducing the fluidity in the hydrophobic and hydrophilic regions of the both outer and inner cellular membrane. Zhang [44] reported that apigenin and quercetin were shown to inhibit 3-hydroxyacyl-ACP dehydrase from *Helicobacter pylori*. The flavonol quercetin has been assigned to upregulate the expression of several iron siderophore proteins. This resulted in limiting the amount of Fe^{3+}/Fe^{3+} is required for the biofilm formation of *Pseudomonas aeruginosa*. A study by Barboza [45] recorded that the same compound inhibits enteroaggregative *E. coli* EAEC 042 biofilm. It has proposed that autoinducer-2-mediated cell–cell signalling is a significant regulatory factor for the biofilm production in *Salmonella typhimurium* and *Escherichia coli*. Multiple research teams reported that some *Citrus* flavonoids, such as kaempferol, apigenin, naringenin and quercetin are effective antagonists of cell–cell signalling [10, 46]. Quercetin and its derivative quercetin-3-*O*-rutinoside have been reported to decrease the bilayer thickness, quercitrin (quercetin-3-*O*-rhamnoside), isoquercetin (quercetin-3-glucoside) are known to prevent ATP hydrolysis [47]. The antimicrobial activity displayed by Fr3 could be due to the overall activity of their phenolic compounds as quercitrin and quercetin. Reports mentioned that quercetin is capable of inhibiting the growth of fungi, different types of Gram-negative and Gram-positive bacteria as well as different drug-resistant microorganisms, through damaging the cell membrane [48]. The antibacterial activity of polyphenols (as flavonoids), was attributed to their propensity to delay the outgrowth of a large scale of infectious microorganisms, including bacteria and fungi [49].

The ethyl acetate fraction was the most potent against tested pathogens followed by the saponified fraction of *n*-hexane. The highest percent for the total phenolic composition was observed for the methanolic extract whereas total flavonoids were recorded for *n*-butanol and ethyl acetate. LC-MS/MS analysis of the ethyl acetate fraction showed that quercitrin was the major metabolite while GC-MS exhibited the presence of 1,5-hexdiene-3,4-diol,2,5-dimethyl. Ethyl acetate and saponified fraction may serve as a promising potential antimicrobial agent after evaluating the mode of action in *in vivo* studies. Additionally, *E. milii* could be a beneficial source for quercitrin and quercetin metabolites. It is the first systematic study of antimicrobial evaluations for the fractions of the total alcoholic extract using LC-MS/MS technique to determine the chemical composition of most active fraction. It could be noted that this is the first GC-MS identification of the lipid portion of *E. milii.* Evaluations for the fractions of the total alcoholic extract using LC-MS/MS technique and GC-MS identification of the lipid portion of *E. milii* resulted in the identification of promising chemical composition for the first time.

Conclusion

The ethyl acetate fraction was the most potent against tested pathogens followed by the saponified fraction of *n*-hexane. The highest percent for the total phenolic composition was observed for the methanolic extract whereas total flavonoids were recorded for *n*-butanol and ethyl acetate. LC-MS/MS analysis of the ethyl acetate fraction showed that quercitrin was the major metabolite while GC-MS exhibited the presence of 1,5-hexdiene-3,4-diol,2,5-dimethyl. Ethyl acetate and saponified fraction may serve as a promising potential antimicrobial agent after evaluating the mode of action in *in vivo* studies. Additionally, *E. milii* could be a beneficial source for quercitrin and quercetin metabolites.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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