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Phytochemical Analysis of *Euphorbia greenwayi* Aerial Parts: Antioxidant and Anti-Inflammatory Potential

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Abstract

Euphorbia greenwayi P.R.O. Bally & S. Carter, is a member of the Euphorbiaceae family, which includes numerous species used in folk medicine to cure a variety of diseases. However, there have been few reports regarding this species. *E. greenwayi* aerial parts' analysis was done using UPLC-MS/MS technique for methanol extract (ME). The negative ion mode resulted in the identification of three phenolic acids; chlorogenic, ferloyl malic acids, and brevifolin carboxylic acid hexoside. Four flavonoids; rutin, astragalin, kaempferol-*O*-acetyl-hexoside, and isorhamnetin-*O*-rutinoside. Additionally, eight diterpenes were identified as ingenol mebutate, deoxy-oxoingenol butanoate, and ingenol dibenzoate with its isomer, besides, phorbol acetate, phorbol diacetate and its isomer, and phorbol. Also, five triterpenes; taraxasterol, lupeol, β-Sitosterol, hydroxytaraxasterol, and hydroxyoleanolic acid were detected by the positive ion mode. ME was fractionated using chloroform (CF) and ethyl acetate (EF) to evaluate phenolic and flavonoid contents, antioxidant, and anti-inflammatory potential. EF had the greatest flavonoid and phenolic content 141±9 µg RE/mg & 108.76±3 µg GAE/mg, respectively. EF exhibited the highest antioxidant activity in DPPH, metal chelation, and ORAC assays: 30.14±1 µg/mL, 7.36±0.4 µmole EDTA eq/mg, and 7118.83±4 µmole Trolox eq/mg respectively. ME revealed the highest anti-inflammatory activity against the expression of TNF-α and IL6 cytokines 0.241±0.01 and 0.321±0.02 fold change respectively.

Keywords: anti-inflammatory; antioxidant; *Euphorbia greenwayi*; Euphorbiaceae; flavonoids; phenolic acids; terpenes

1. Introduction

Oxidative stress is a condition that is connected to several illnesses like diabetes, aging, and disorders of the degenerative neurological system. It is characterized by reactive oxygen species (ROS), free radicals, and an imbalance in antioxidant levels [1-4]. Chronic and severe oxidative stress in cells increases both apoptosis, which triggers a persistent inflammatory response, and gene expression in some cells, which leads to degenerative diseases [5, 6]. Following an invasion, inflammation serves as a healing mechanism that regenerates the affected area. This is achieved through activation of the immune system, which sends out the inflammatory cells and cytokines to attack the cause of this invasion, such as bacterial attack or to heal damaged tissue [7]. However, persistent (chronic) inflammation can lead to various inflammatory ailments as the body continues sending inflammatory cells even when there is no external threat. This could be observed in different inflammation disorders like rheumatoid arthritis, gastritis, and asthma [8]. Complex inflammatory mediators allow leukocytes to accumulate and localize which causes inflammation. Among leukocytes, macrophages are significant participants in inflammatory reactions and substantial providers of pro-inflammatory cytokines and enzymes such as COX (cyclooxygenase), TNF (tumour necrosis factor), iNOS (inducible nitric oxide synthase), ILs (interleukins), these pro-inflammatory

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mediator genes are highly activated during inflammation and are in charge of its beginning and continuation [9].

Euphorbia greenwayi P.R.O. Bally & S. Carter is a decorative medicinal plant species belonging to Euphorbiaceae Juss (Spurge) family . *E. greenwayi* is native to Tanzania and East Africa. It's a basally branching succulent perennial shrub. It grows up to 1.2 meters tall with sharply 4-edged bluish-green spiny branches with darker spots along the edges. Spines are thin and reddish black in colour. Flowers (cyathia) are pinkish in colour and appear in spring [10]. *Euphorbia* Linn is a major genus among flowering plants [11]. Several *Euphorbia* species were used in folk medicine since the Sumerians and Akkadians and in conventional Australian medicine to treat a range of dermatological, digestive, and respiratory ailments, inflammation, injuries, and migraine [12, 13]. Diverse *Euphorbia* species yielded many diterpenes with a wide range of biological functions. For example, diterpenes from *E. fischeriana* showed antiproliferative effects [14]. Euphopanes diterpenoids isolated from *E. pekinensis* revealed cytotoxic activity [15]. Ingol diterpenoid from *E. marginata* [16]. Tigliane and ingenane diterpenoids from *E. tirucalli* [17]. *E. neorubella* has tigliane diterpenoids with cytotoxic activity [18]. Abietane and para-lianone type diterpenoid from *E. peplus* [19]. Furthermore, triterpenoids, diterpenoids, flavonoids, caffeoyl-quinic acids, and rare disaccharides were identified in *E. peplus* [20]. Also, steroids, fatty acids, and triterpenoids were identified in *E. dendroides* aerial parts [21]. *Euphorbia* genus is also distinguished by its milky latex which contributed to some species' economic importance owing to its richness in biologically active phytoconstituents [22].

Interestingly, due to the scarcity of reports, this research aims to explore the main phytochemical constituents of *E. greenwayi* using UPLC-ESI-MS/MS and estimate the total flavonoid and phenolic constituents, as well as the antioxidant and anti-inflammatory effects.

2. Materials and methods

2.1 Plant material

In March 2019, aerial parts of *E. greenwayi* were obtained from Helal Cactus farm located 17 m above sea level at Al Mansoureyah, Giza Governorate, Egypt (30.10812667354337, 31.105346915336153). The collection and handling of the plant material were in accordance with all the relevant guidelines. Ethical approval was obtained from the research ethics committee, Faculty of Pharmacy, Cairo University; serial number MP (2448). According to a reliable reference, the plant was gratefully authenticated and identified by Professor Dr Reem Samir Hamdy, Professor of Botany, Faculty of Science, Cairo University **[10]**. Voucher specimens were placed in the Pharmacognosy and Medicinal plants Department, Faculty of Pharmacy, Future University in Egypt; labeled EG-111 (FUE), and Herbarium of Pharmacognosy Department Faculty of Pharmacy Cairo University; labeled 2.4.2023.

2.2 Preparation of plant extract

Fresh plant material (12 Kg) was cut into small pieces and exhaustively extracted by maceration in methanol. The methanol extract was concentrated into a 40 g residue. The methanol extract (ME) was then made to a suspension using 150 mL of distilled water followed by successive fractionation (solvent-solvent extraction) using chloroform (CF) (250 mL x 4 times) and ethyl acetate (EF) (250 mL x 4 times). All obtained fractions were evaporated using a rotary evaporator at 40°C and stored in an airtight container at 4°C until required.

2.3 Phytochemical evaluation

2.3.1 Metabolites identification via UPLC-ESI-MS/MS

The UPLC-ESI-MS/MS negative and positive ion modes were executed on a Waters Corporation, Milford, MA01757, USA, XEVO TQD triple quadrupole mass spectrometer. The method was applied as described previously [23-25]. Briefly, the methanol extract (100 µg/mL) was dissolved in methanol (HPLC grade). Reverse phase (ACQUITY UPLC – BEH C18, 1.7 µm particle size, 2.1 × 50 mm) column and gradient elution using two eluents; water acidified with 0.1% formic acid and 0.1% formic acid in methanol was applied with 440°C desolvation temperature, and voltage of capillary and cone are 3kV and 25 eV respectively. The software Maslynx 4.1 was used to detect mass in the *m/z* 100–1000 ESI range. The tentative identification of the main constituents in ME was done based on the retention time peaks, the mass spectrum, and fragmentation profile, together with comparing these data with the previous literature reports in *Euphorbia.*

2.3.2 Estimation of total flavonoid and (TF) phenolic (TP) contents

Estimation of total flavonoid content in ME, CF, and EF was done using the aluminium chloride method [26] employing rutin (Sigma-Aldrich, St. Louis, MO, USA) as a standard. Briefly, in a 96-well microplate 0.015 mL (15 µL) of sample/standard and 0.175 mL (175 µL) of methanol were made into a mixture, 0.03 mL (30 µL) of $AlCl_3$ (1.25%) was then added. Lastly, 0.03 mL (30 µL) of $C_2H_3NaO_2$ (0.125 M) was added and kept for 5 minutes. The resultant yellow colour was measured at 420 nm. Ten serial dilutions of

concentrations to range 6.25-1000 µg /mL were made from 1 mg/mL rutin solution in methanol.

Estimation of the total phenolic constituents in ME, CF, and EF was applied using the Folin-Ciocalteu technique [27]. The results are given in µg Gallic acid equivalent/mg dry extract. In a 96-well microplate, 0.01 mL (10 µL) of sample/standard and 0.1 mL (100 µL) of Folin-Ciocalteu diluted reagent (1:10 *v/v*) were made into a mixture. Then, 0.08 mL (80 µL) of 1M Na_2CO_3 was added and kept away from light for 20 minutes at 25°C. The resultant blue complex colour was measured at 630 nm. Means ±SD were used to represent data. Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) standard series concentrations of 25-1000 µg/mL were prepared. FluoStar Omega (microplate reader) was used to record the results of total flavonoids and phenolics.

2.4 Biological evaluation

2.4.1 In vitro antioxidant activity

Estimated for ME, CF, and EF using various methods including:

2.4.1.1 DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical-scavenging assay

The method was done as specified by [28]. Methanol served as the blank, while Trolox was the standard. From a 1000 µM Trolox stock solution, five concentrations (6.25, 12.5, 25, 50, and 100 µM) were formed. ME, CF, and EF were diluted to provide 5 concentrations. One hundred µL of 0.1% newly prepared DPPH reagent was added to a 96-well plate. After that, the reaction is kept in the dark at 25°C for 30 minutes. The intensity of the DPPH colour at 540 nm was measured. The data is represented by substitution in the equation:

%Inhibition = [(average blank absorbance - average test absorbance)/ (Average blank absorbance)] ×100.

2.4.1.2 Metal Chelation (Ferrozine iron) assay

The method was applied using a technique reported by [29]. A 100 µM EDTA (Ethylene di-amine tetra-acetic acid) (Sigma-Aldrich, St. Louis, MO, USA) stock solution in methanol was formed, from which seven serial dilutions were produced (2.5, 5, 10, 15, 20, 40, and 50 µM) and the calibration curve was plotted providing the linear regression equation Y = 1.6068 × X + 3.4588 (R^2= 0.9974). Samples concentration was made to 1 mg/mL in methanol. In 96 well plates, 0.05 mL (50 µL) of sample and 0.02 mL (20 µL) of freshly made 0.3 mM ferrous sulfate were made into a mixture. Each well was then filled with 0.03 mL (30 µL) of 0.8 mM ferrozine. The mixture was reserved at 25°C for 10 minutes. The decrease in generated colour intensity was detected at 562 nm. As a control, methanol was used. The %inhibition is calculated using the DPPH assay equation. Results are presented as µmole EDTA equivalent/mg sample.

2.4.1.3 ORAC (Oxygen radical absorbance capacity)

The method applied as previously reported [30]. In brief, 7 serial dilutions (50, 100, 200, 400, 500, 800, and 1000 µM) were formed from Trolox (Sigma-Aldrich, St. Louis, MO, USA) stock solution (1 mM in methanol) so that a calibration curve could be achieved. Samples were dissolved in methanol 100 µg/mL. 0.01 mL (10 µL) of the prepared samples were incubated with 0.03 mL (30 µL) fluoresceine (100 nM) at 37°C for 10 minutes. Three fluorescence measurement cycles were performed (wavelengths of excitation and emission are 485 and 520 nm respectively) (cycle time, 90 sec.). Then, 0.07 mL (70 µL) of newly produced 300 mM AAPH (2,2'-Azobis (2-amidinopropane) dihydrochloride) was added. Measurement of the fluorescence took 60 minutes (40 cycles, each 90 sec). Results of the extract and fractions were recorded as µmole Trolox equivalents by substituting in the linear regression equation extracted from the calibration curve; Y = 335.2 × X + 20079 (R^2 = 0.9937).

2.4.2 Anti-inflammatory activity

2.4.2.1 Cell culture

The macrophage cells RAW 264.7 (ATCC, Rockville, MD) were cultivated as a monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, General Electric Healthcare Life Sciences, Mississauga, Canada) supplemented with 10 % heat inactivated FBS (fetal bovine serum) (HyClone), and 1% P/S (100 IU/ mL penicillin and 100 µg/mL streptomycin (Solarbio life sciences, Beijing, P. R. China).

2.4.2.2 Cell viability assay

Cytotoxic activity of *E. greenwayi* extracts was evaluated on RAW 264.7 cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. RAW 264.7 cells were spread in 96-well plates (4×10^3 cells per well) for 24 h. ME, CF, and EF with different concentrations (4, 16, 63, 250, and 1000 µg/mL) were added and left for 24 h after the addition of LPS to be incubated. Then to each well, a solution of 5 mg/mL MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was then added and left in an incubator (37°C, 5% CO_2). After 4 h the cell supernatants were discarded, and then the insoluble formazan product was solubilized by adding DMSO (Dimethyl sulfoxide). One hour later, the absorbance was measured with ROBONIK P2000 Elisa Reader at 490 nm. Cell viability percentage was estimated and IC_{50} for each extract was determined.

2.4.2.3 Anti-inflammatory potential of E. greenwayi extracts against pro-inflammatory cytokines

The levels of mRNA of TNF-α and IL-6 were marked using reverse transcription polymerase chain reaction (RT-PCR) that utilized the cellular total RNA extracted from RAW 264.7 cells that were stimulated by LPS (Lipopolysaccharide) and pre-treated with 0.1 mg/mL of each tested fraction.

Into 24-well plates RAW 264.7 macrophages (3×10^5 cells per well) were seeded with 0.1 mg/mL of ME, CF, and EF for 24 h. The cells were washed using Phosphate-buffered saline (PBS) (1 mL/well), followed by exposure of cell monolayers exposed to a Cell-Lysis (CL) Buffer, thus preparing cell lysates. The lysates were assembled cautiously so as not to mix up the remnants of cell monolayers and stored frozen. Analysing the levels of mRNA of IL-6 and TNF-α (pro-inflammatory cytokines) was done through reverse transcription polymerase chain reaction with β-actin as a housekeeping gene/internal control using specific primers (Table 1). iScript RT-qPCR kit (Bio-Rad) was used to perform the RT-qPCR analysis as instructed by the manufacturer. Celecoxib was used as a reference drug (positive control) (Sigma-Aldrich, St. Louis, MO, USA).

Table 1 Real-time PCR primer sequences.

Gene		Primer sequence
TNF-α	Forward	5'-CTCTTCTGCCTGCTGCACTTTG-3'
	Reverse	5'-ATGGGCTACAGGCTTGTCACTC-3'
IL-6	Forward	5'-AGACAGCCACTCACCTCTTCAG-3'
	Reverse	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
β-Actin	Forward	5'-GCACCACACCTTCTACAATG-3'
	Reverse	5'-TGCTTGCTGATCCACATCTG-3'

2.5 Statistical analysis

All experiments were performed in triplicate. Data are expressed as means ± standard deviation (SD). P-values of 0.05 or less were statistically significant.

3. Results

3.1 Phytochemical evaluation

3.1.1 Plant extract preparation

Maceration of 12 Kg of fresh *E. greenwayi* aerial parts in methanol yielded 40 g of methanol extract residue (ME). Fractionation of methanol extract with chloroform (CF) and ethyl acetate (EF) yielded 1.3 g and 2.6 g residues, respectively.

3.1.2 Identification of E. greenwayi ME metabolites via UPLC-ESI-MS/MS

Tentative characterization of the main constituents in *E. greenwayi* ME results in the identification of twenty different phytoconstituents.

Results (Tables 2 & 3, Figures 1, 2, and 3) allowed the identification of seven phytochemical components using negative ionization mode. Three phenolic acids and four flavonoids were recognized. Compound (1) showed pseudo molecular ion $[M-H]^-$ at *m/z* 353 related to the deprotonated chlorogenic acid with product ions at *m/z* 191 representing the caffeoyl part loss combined with *m/z* 179 showing the loss of quinic acid part. Compound (6) is tentatively identified as feruloyl-malic acid as it revealed a parent ion peak at ($[M-H]^-$ ion at *m/z* 309), and loss of malic acid was recognised from the product ion peak at *m/z* 193. Compound (2) brevifolin carboxylic acid hexoside appeared at ($[M-H]^-$ ion at *m/z* 453), the loss of the hexose $[M-H\text{-hexoside}]^-$ was related to the product ions at *m/z* 291, while the loss of the hexose and CO_2 was observed at *m/z* 247 $[M-H\text{-hexoside-}CO_2]^-$.

The four tentatively identified flavonoids were mainly having flavanol aglycone. Compound (3) was identified as rutin ($[M-H]^-$ ion at *m/z* 609), the loss of the sugar part $[M-H\text{-rutinose}]^-$ was recognised from the fragment ion peak at *m/z* 301 presenting the quercetin aglycone, additionally, the loss of carbonyl group from the quercetin aglycone $[M-H\text{-rutinose-CO}]^-$ was assigned to the fragment ion peaks at *m/z* 273. Moreover, two kaempferol glycosides were found; astragalin (kaempferol-*O*-glucoside) *m/z* 447 and kaempferol-*O*-acetyl-hexoside *m/z* 489, both showed the kaempferol aglycone at *m/z* 285 which indicates the loss of hexoses moiety in astragalin $[M-H\text{-hexose}]^-$, while $[M-H\text{-hexose-acetyl}]^-$ in kaempferol-*O*-acetyl-hexoside. In both compounds, the fragment ion peak at *m/z* 255 was found to present the loss of $(H_2CO)^-$ from the kaempferol aglycone part [31, 32]. Compound (7) was recognized as isorhamnetin-*O*-rutinoside $[M-H]^-$ ion at *m/z* 623 with a fragmentation pattern showed the loss of sugar part where the isorhamnetin aglycone appeared at *m/z* 315 $[M-H\text{-rutinose}]^-$, the fragment ion peak at *m/z* 300 denotes loss of a methyl group from the isorhamnetin aglycone. Fragmentation patterns are illustrated in Table 2.

Positive ionisation, on the other hand, results in the tentative identification of thirteen phyto-constituents. Eight of them are diterpenoids, ingenane and tigliane (phorbol), and five are triterpenes.

Four ingenane diterpenes were identified. The ingenol esters; ingenol mebutate (ingenol angelate) showed $[M+Na]^+$ ion at *m/z* 453 with a fragment ion peak at *m/z* 353 indicating $[M+Na\text{-angelic acid}]^+$. Additionally, deoxy-oxoingenol-butenoate showed the parent ion peak at *m/z* 437 $[M+Na]^+$, the loss of butenoic acid part appeared as a fragment ion peak at *m/z* 353, the ingenol moiety appeared at fragment ion *m/z* 295, the loss of the carbonyl group was also observed at *m/z* 267. Ingenol dibenzoate and its isomer displayed a parent ion molecular peak $[M+Na]^+$ at *m/z* 579 with fragmentation at *m/z* 457 due to the loss of one benzoic acid and at *m/z* 317

resulting from the loss of the second benzoic acid moiety.[33]

Besides, four tigliane diterpenes were found. Phorbol acetate is recognized from the main molecular ion peak $[M+Na]^+$ (*m/z* 429), it demonstrated MS^2 fragment ions at *m/z* 387 $[M+Na\text{-acetic}]^+$. phorbol-diacetate (*m/z* 448) and its isomer presented a molecular ion peak $[M+Na]^+$ (*m/z* 471), the loss of the two acetate moieties was detected from the fragment ion peak of phorbol-diacetate *m/z* 411 $[M+Na\text{- acetic acid}]^+$, 333 $[M+Na\text{-2(acetic acid)-}H_2O]^+$. Furthermore, phorbol $[M+Na]^+$ (*m/z* 387) showed fragmentations at *m/z* 369 after losing one water molecule and *m/z* 333 after losing three portions of H_2O [33].

Moreover, the five triterpenes identified in *E. greenwayi* methanol extract are lupeol $[M]^+$ at *m/z* 426, β-Sitosterol $[M]^+$ at *m/z* 414, taraxasterol $[M]^+$ at *m/z* 426, hydroxytaraxasterol $[M]^+$ at *m/z* 442, and hydroxyoleanolic acid $[M+H]^+$ at *m/z* 473. Taraxasterol fragmentation ion peaks appeared at *m/z* 411 $[M\text{-}CH_3]^+$ and *m/z* 408 $[M\text{-}H_2O]^+$, while hydroxytaraxasterol appeared at *m/z* 424 $[M\text{-}H_2O]^+$. Lupeol demonstrated MS^2 peaks at *m/z* 411 due to the loss of a methyl group, followed by the loss of $[C_2H_4]^+$ moiety at *m/z* 383, and *m/z* 189 due to the loss of $[C_{13}H_{22}]^+$ and 1 molecule of water [34]. β-Sitosterol showed fragments at *m/z* 396 due to the loss of water, with a characteristic fragment ion peak *m/z* 273 indicating the loss of the β-Sitosterol side chain and two hydrogen atoms [35]. Hydroxyoleanolic acid fragmentation ion peaks appeared at *m/z* 411 $[M+H\text{-}H_2O\text{-}CO_2]^+$, 248 $[C_{16}H_{24}O_2]^+$, and 203 $[C_{15}H_{23}]^+$ due to losing COOH from *m/z* 248. Table 3 shows all fragmentation patterns and compares them to the previously published data.

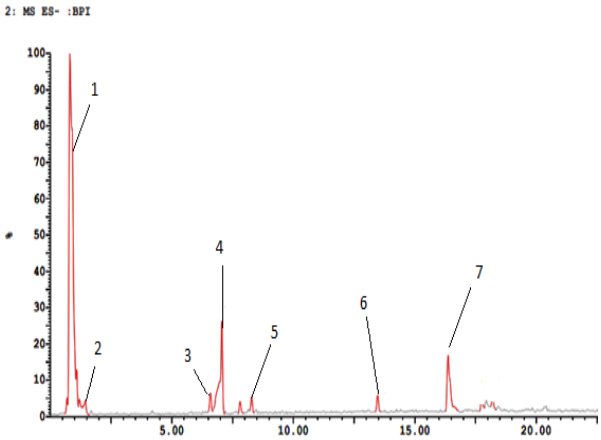


Fig. 1 HPLC-ESI-MS chromatogram (negative ion mode) of *E. greenwayi* ME.

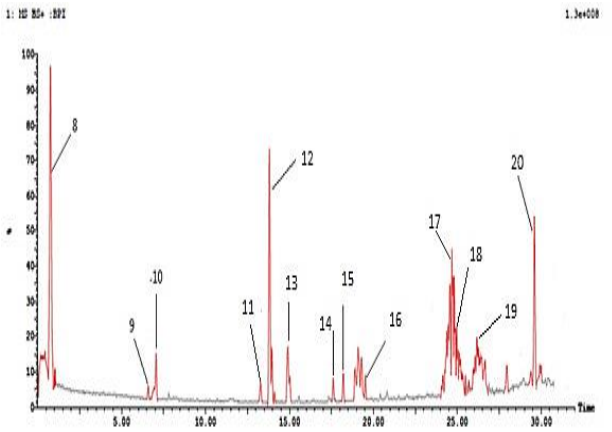


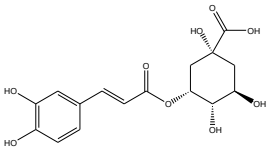
Fig. 2 HPLC-ESI-MS chromatogram (positive ion mode) of *E. greenwayi* ME.

Table 2 Tentatively identified compounds assigned in *E. greenwayi* ME using the negative ion mode of UPLC-ESI-MS/MS

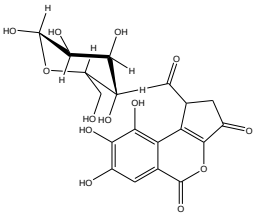
	Rt min.	Tentative assignment	$[M\text{-}H]^-$ *(m/z)*	MS^2 ion fragments *(m/z)*	Ref.
1	0.8	Caffeoylquinic acid (Chlorogenic acid)	353	191, 179	[31]
2	2	Brevifolin carboxylic acid hexoside	453	291, 247	[32]
3	6.6	Quercetin-*O*-rutinoside (Rutin)	609	301, 273, 179, 151	[32]
4	7.07	Kaempferol-*O*-glucoside (Astragalin)	447	285, 255	[31]
5	8.28	Kaempferol-*O*-acetyl-hexoside	489	327, 285, 255	[32]
6	13.47	Feruloyl-malic acid	309	193	[32]
7	16.37	Isorhamnetin-*O*-rutinoside	623	315, 300, 151	[32]

Table 3 Tentatively identified compounds assigned in *E. greenwayi* ME using the positive ion mode of UPLC-ESI-MS/MS

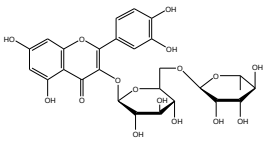
	Rt min.	Tentative assignment	$[M]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*)	MS^2 ion fragments (*m/z*)	Ref.
8.	0.75	Ingenol mebutate		453	353 295	[33]
9.	6.59	Phorbol acetate		429	387	[33]
10.	7.06	Phorbol diacetate	448		411 351 333 329 315 311 293	[33]
11.	13.29	Taraxasterol	426		411 408 189	[36, 37]
12.	13.81	Phorbol diacetate (isomer)		471	411 333 293	[33]
13.	14.90	Deoxy-oxoingenol butenoate		437	353 295	[33]
14.	17.76	Ingenol dibenzoate		579	457 317 295	[33]
15.	19.09	Ingenol dibenzoate isomer		579	457 335 317 313 307 295 285	[33]
16.	19.52	Phorbol		387	369 333 293	[33]
17.	24.97	Lupeol	426		411 383 189	[34]
18.	25.43	β-Sitosterol	414		396 273 255	[35]
19.	26.38	Hydroxytaraxasterol	442		424 189 207	[38]
20.	30.9	Hydroxyoleanolic acid	473		411 409 248 203 154	[39]



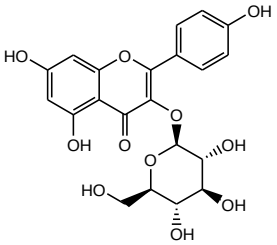
Chlorogenic acid (1)



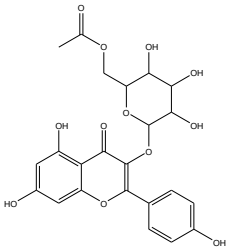
Brevifolin carboxylic acid hexoside (2)



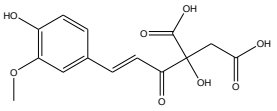
Rutin (3)



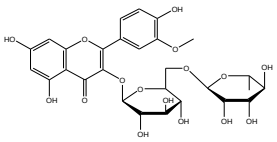
Astragalin (4)



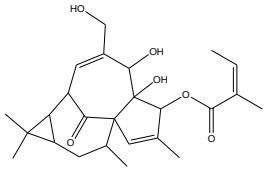
Kaempferol-*O*-acetyl-hexoside (5)



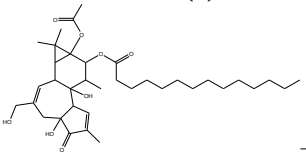
Feruloyl-malic acid (6)



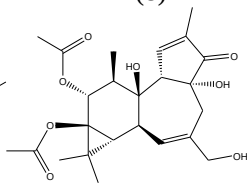
Isorhamnetin-*O*-rutinoside (7)



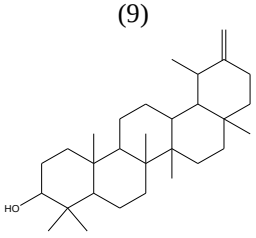
Ingenol mebutate (8)



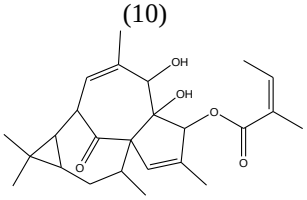
Phorbol acetate (9)



Phorbol diacetate (10)



Taraxasterol (11)



Deoxy-oxoingenol butanoate (13)

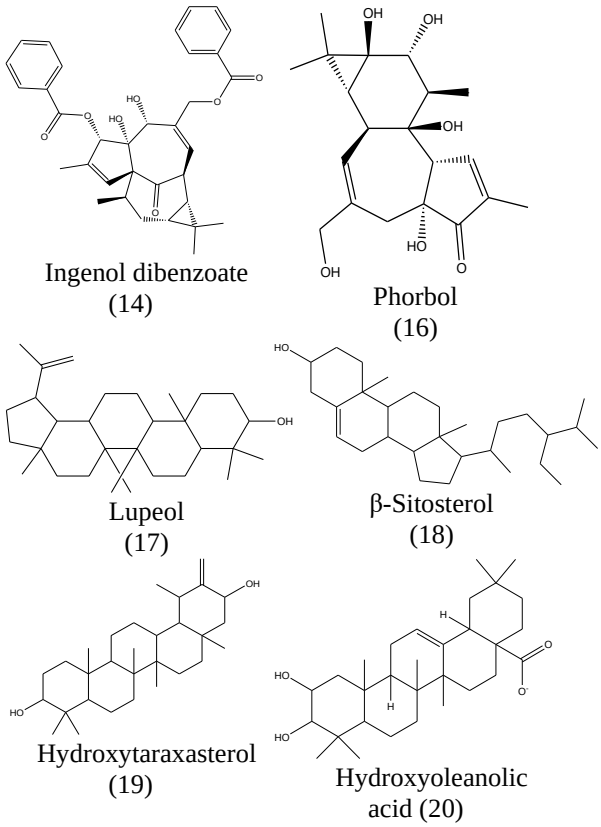


Fig.3 Identified compounds in *E. greenwayi* methanol extract.

3.1.3 Estimation of total flavonoid and phenolic contents

Table 4 presented that EF had the greatest contents of both flavonoids 141±9 µg rutin equivalent/mg and phenolics 108.76±3 µg Gallic acid equivalent/mg. ME and CF exhibited relatively lower values in both total flavonoids and phenolics than EF.

Table 4 Total flavonoid and phenolic contents in ME, CF, and EF of *E. greenwayi*.

Extract (5 mg/mL)	Total flavonoid TF (µg rutin equivalent/mg)	Total phenolic content TPC (µg Gallic acid equivalent/mg)
ME	6.54±0.5^{c}	26.24±1^{c}
CF	35.57±3^{b}	44.16±2^{b}
EF	141±9^{a}	108.76±3^{a}

Values are reported as mean ± S.D (n=3), Different letters indicate significant differences in the extracts (p< 0.05); (a) indicates the highest significance. ME (methanol extract), CF (chloroform fraction), and EF (ethyl acetate fraction).

3.2 Biological evaluation

3.2.1 In vitro antioxidant activity

3.2.1.1 DPPH

ME, CF, and EF revealed moderate to low scavenging activity. Minimum IC_{50} values were exhibited by EF 30.14±1 µg/mL followed by the ME 206.00±1 µg/mL, while CF showed the highest IC_{50} value 221.90±1 µg/mL. All fractions showed higher IC_{50} values than Trolox 4.42±0.2 µg/mL.

3.2.1.2 Metal chelation

All extracts were examined for their ability to chelated metal complexes (iron). All extracts showed an ability to chelate metal ions (Table 5). The results of all tested samples were calculated as µmole EDTA equivalent/mg sample.

ME and CF (1 mg/mL) metal chelation ability is equal to 18.08±1 and 16.82±1 µmole EDTA eq/mg respectively and the inhibition percentage for both was evaluated as 32.51 and 30.48 % respectively. However, EF showed the least inhibition (15.29%) and demonstrated the highest metal chelation activity 7.36±0.4 µmole EDTA eq/mg.

3.2.1.3 ORAC

This assay is based mainly on producing peroxyl radicals initiated through an azo compound AAPH. The potential of ME, CF, and EF of *E. greenwayi* to scavenge peroxyl radicals initiated by the spontaneous decomposition of (AAPH) was evaluated.

Results were consistent with DPPH and metal chelation assays. EF was superior to the other fractions 7118.83±4 µmole Trolox eq/mg. Contrariwise, ME and CF had lower antioxidant capacities 961.14±2 and 3110.43±3 µmole Trolox eq/mg respectively.

Table 5 Antioxidant activity of ME, CF, and EF of *E. greenwayi* aerial parts.

	Antioxidant activity Mean ± SD			
	DPPH	**Metal chelation**		**ORAC**
	(IC_{50}) µg/mL	(µmole EDTA eq/mg extract)	% Inhibition	(µmole Trolox eq/mg extract)
ME	206.00±1^{b}	18.08±1^{b}	32.51%^{b}	961.14±2^{b}
CF	221.90±1^{c}	16.82±1^{c}	30.48%^{c}	3110.43±3^{c}
EF	30.14±1 ^{a}	7.36±0.4^{a}	15.29%^{a}	7118.83±4^{a}
Trolox	4.42 ±0.2	-	-	-

Values are reported as mean ± S.D (n=3), Different letters indicate significant differences in the extracts (p< 0.05). (a) indicates the highest significance. ME (methanol extract), CF (chloroform fraction), and EF (ethyl acetate fraction).

3.2.2 Anti-inflammatory activity

3.2.2.1 Cell viability

The effect of various concentrations (4 µg/mL to 1000 µg/mL) of *E. greenwayi* extracts on RAW 264.7 macrophage cell viability was detected by the MTT colourimetric assay using celecoxib as a reference. Concentration at 0.1 mg/mL of *E. greenwayi* ME, CF, and EF showed no toxic effects on RAW 264.7 macrophage cells (Table 6).

Table 6 Effect of *E. greenwayi* ME, CF, and EF on RAW 264.7 Cells viability.

Extract	IC_{50} against RAW 264.7 cells µg/mL)
ME	319.28 ±16 [b]
CF	224.76 ±11[c]
EF	653.257 ±33 [a]

Values are reported as mean ± S.D (n=3), Different letters indicate significant differences in the extracts ($p< 0.05$). (a) indicates the highest significance. ME (methanol extract), CF (chloroform fraction), and EF (ethyl acetate fraction).

3.2.2.2 Effect of extracts on pro-inflammatory cytokines gene expression by macrophages.

When a cell is exposed to LPS, pro-inflammatory proteins like TNF-α and IL-6 are released, which causes an inflammatory response. The proteins levels can be detected by measuring their levels upon release or by measuring the levels of their mRNA and gene expression using the RT-PCR technique, which was the method of choice to assess the effect of *E. greenwayi* ME, CF, and EF on TNF-α and IL-6 inhibition. Levels of mRNA of TNF-α and IL-6 were elevated in untreated cells (negative control); however, the mRNA levels of corresponding extracts-treated cells were reduced significantly (Figure 4).
ME showed results that were comparable to celecoxib (positive control) 0.241±0.01 and 0.224±0.01 respectively against the expression of the TNF-α gene, and 0.321±0.02 and 0.288±0.02 respectively against the expression of the IL-6 gene (Figure 4).

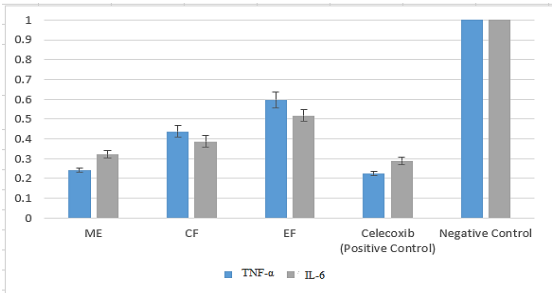


Figure 4 Effects of *E. greenwayi* extracts on the expression of TNF-α and IL6 in RAW 264.7.
Values are reported as mean ± S.D (n=3). ME (methanol extract), CF (chloroform fraction), and EF (ethyl acetate fraction), Results are expressed as fold change.

4. Discussion

The *Euphorbia* genus is distinguished by its richness in biologically active phytoconstituents [22]. The current study is carried out because of little-known information regarding the primary components of *E. greenwayi* and its biological activity.

Negative and positive ionization mode LC-MS/MS analysis results in the identification of twenty phytoconstituents. The negative ion mode identified three phenolic acids and four flavonoids. Furthermore, the positive ion mode detected eight diterpenes and five triterpenes. All the identified compounds were previously reported in different *Euphorbia* species such as *E. hirta*, *E. tirucalli*, *E. lagascae*, and *E. cooperi* [17, 32, 40] but to our knowledge, this is the first report in *E. greenwayi* species.

Owing to the extensive biological activity associated with phenolics and flavonoids, their contents were assessed in ME, CF, and EF. The greatest contents of both flavonoids and phenolics were detected in EF compared to ME and CF. This could be owed to the existence of other non-phenolic constituents in ME, which affects the total concentration of flavonoids and phenolics. Furthermore, this could confirm the selectivity of the ethyl acetate to the polar constituents in the total extract.

Due to the effectiveness of natural antioxidants in treating a wide range of diseases, the value of traditional herbal remedies has increased in the modern era. [41-44]. Several members of the Euphorbiaceae family demonstrated antioxidant potential due to their flavonoid and polyphenolic contents. [13, 45]. The antioxidant potential is usually classified based on the mode of action, which can be electron transfer, transition element chelation, or hydrogen atom shift. These methods have applications such as DPPH, metal chelation, and ORAC. Results demonstrated that EF has the greatest antioxidant activity in all tested techniques; this could be owing to its high flavonoid and phenolic contents. This is because of their capability in acting as radical scavengers, besides restoring the antioxidant enzyme activities [46]. Moreover, many reports relate the antioxidant activity to different phenolic constituents such as kaempferol, quercetin, caffeic acid, ferulic acid, and chlorogenic acid which were identified in ME [47, 48].

Inflammation is considered a body`s defense mechanism against harmful causes. The body induces the production of pro-inflammatory mediators, but when the inflammatory response and the mediators are more than normal this causes pathological inflammation which is associated with different illnesses. The suppression of pro-inflammatory mediators is thought to be significantly aided by flavonoids and phenolic acids [48-50]. As a result, the anti-inflammatory activity was investigated because the results revealed a significant amount in all analyzed fractions. ME inhibited pro-inflammatory cytokines more effectively than celecoxib (the positive control). Although EF had the highest phenolic and flavonoid content values, the synergistic activity of ME constituents and the presence of many terpene types (Table 3) may explain the superior anti-inflammatory activity. This may be aligned with the review research, which described diterpenoids extracted from 45 *Euphorbia* species and their various

pharmacological effects, like anti-inflammatory potential [11].

5. Conclusion

The current study focused on the phytochemical analysis, antioxidant, and anti-inflammatory potential of *E. greenwayi* aerial parts ME (methanol extract), CF (chloroform fraction), and EF (ethyl acetate fraction). EF demonstrated the greatest flavonoid and phenolic contents which could be the source of the observed antioxidant potential. The anti-inflammatory activity was more detectable in ME; this could be related to the synergistic effect of its different constituents identified using UPLC-MS/MS analysis as phenolic acids, flavonoids, and terpenes. However, CF demonstrated the lowest results in all tested assays. Further phytochemical and biological studies are required to investigate its potential to be a source of natural bioactive components.

Declarations

Competing interests: Authors declare that no competing interest exists.

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Authors' contributions: Conceptualization, Ahmed Zaghlol, Zeinab Kandil, Miriam Yousif, Riham Salah EL-Dine and Wafaa Elkady; Data curation, Ahmed Zaghlol; Investigation, Ahmed Zaghlol; Methodology, Riham Salah EL-Dine, and Wafaa Elkady; Supervision, Zeinab Kandil, Miriam Yousif, Riham Salah EL-Dine and Wafaa Elkady; Visualization, Ahmed Zaghlol; Writing – original draft, Wafaa Elkady; Writing – review & editing, Zeinab Kandil, Miriam Yousif, Riham Salah EL-Dine and Wafaa Elkady.

Conflicts of Interest: The authors declare no conflict of interest.

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