

Total phenolics and flavonoids content, antioxidant activity and GC/MS analyses of *Euphorbia grandialata*

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ABSTRACT

Objective: This study has been designed to study phenolic and flavonoid contents quantitatively; screen antioxidant activity and investigate unsaponifiable and saponifiable matters of *Euphorbia grandialata* R. aerial parts.

Methods: The phenolics and flavonoids content were assayed by Folin-Ciocalteu reagent and aluminium chloride reagent respectively, while the anti-oxidant activity was done by 2, 2-diphenyl-1-picrylhydrazyl (DPPH). GC/MS analysis was used to analyze unsaponifiable and saponifiable matters.

Results: Total phenolics result was calculated as $(17.61 \pm 1.2 \mu\text{g gallic acid/g})$, total flavonoids as $(4.495 \pm 0.39 \mu\text{g rutin/g})$ and anti-oxidants activity as $(140.6 \% \mu\text{g ascorbic acid/g})$. The identified compounds in unsaponifiable matter were hydrocarbons (51.1 %), steroids and triterpenes (35.92%). The saponifiable matter showed fourteen components from which six were identified as methyl esters of saturated fatty acids (46.26%) and eight as methyl esters of unsaturated fatty acids (53.74 %).

Conclusion: *Euphorbia grandialata* could be considered as a valuable source of natural antioxidants. This is the first report for phenolics and flavonoids content, antioxidant activity and GC/MS analysis of this plant.

INTRODUCTION

Euphorbiaceae, called the spurge family, is considered as a large family of flowering plants comprising around 300 genera with 7,500 species (Rahman, 2013). The medicinal uses of *Euphorbia* is due to their carminative (Bondarenko *et al.*, 1972), diuretic (Liu *et al.*, 2002), anti-leukemic (Kupchan and Uchida, 1976), anti-inflammatory, analgesic (Singh *et al.*, 1984; Heimann, 1994), anti-asthmatic (Shelke *et al.*, 2014), antioxidant (Sharma and Dey, 2007) and antimicrobial (Hussain *et al.*, 2014) activities. The genus *Euphorbia* is rich in phenolic compounds such as scopoletin, scoparone, isoscopoletin, gallic acid (Wu *et al.*, 2012), esculetin and phenylpropanoid-2-hydroxydihydrocinnamic acid (Nazemiyeh *et al.*, 2010) and flavonoids such as quercetin, quercitrin, quercitol, kaempferol, luteolin (Wu *et al.*, 2012), kaempferol-3-glucoside, kaempferol

-3-galactoside (Awaad *et al.*, 2013), quercetin-3-glucoside, quercetin-3-glucuronide, quercetin-3-rhamnoside (Saleh, 1985), rutin and 5'-methoxy-8-methyl-6-prenyl-5,7-dihydroxy-3',4'-methylenedioxy-flavone (Kumar and Intekhab, 2013). *Euphorbia grandialata* R. commonly known as "Candelabra tree", "Spurge", "Milkweed" or "Penge Euphorbia", is widely distributed in South Africa, rarely cultivated in Egypt.

It prefers well-drained soil, during the active growing season from March to September and the flowering stage during Summer (Dyer, 1937). Free radicals, which are produced by the chemical reaction of organic compounds, could damage the body's tissues and cells, leading to human aging and causing a variety of diseases. Therefore, it is important to find the antioxidants for scavenging these free radicals. Generally, two methods have been developed for the assessment of antioxidative activities including *in vitro* and *in vivo* screening. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method is one of the universal *in vitro* tools in which DPPH radical, a very stable nitrogen-centered radical, can be used to determine the free radical scavenging ability, which is

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related to their antioxidative activities. This method depends on spectrophotometric measurement of changes in concentration of DPPH[•] which results from the reaction of the free radicals with an antioxidant (Meng *et al.*, 2016).

All the reported studies about Euphorbias were related to polar constituents and nothing concerned their fixed oil, although fatty acids were known to have anticancer, antimutagenic and antimicrobial activities (Al Ashaal *et al.*, 2010). Therefore, it was interesting to quantitatively study phenolic and flavonoid contents; screen the antioxidant activity and find its correlation with the phenolics and flavonoids content; in addition to the investigation of unsaponifiable and saponifiable matters of *E. grandialata* aerial parts growing in Egypt.

MATERIALS AND METHODS

General

UV-visible spectrophotometer (Shimadzu UV-1601 PC) was used. Analysis using GC/MS was carried out using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS and an electron ionization system with energy of 70 eV for the ionization process. For GC of unsaponifiable matter, capillary column (30 m x 0.25 mm) packed with TG-5MS fused silica was used. The temperature of the oven was programmed at an initial temperature 50 °C (hold 2 min.) to 150 °C at an increasing rate of 7 °C /min. then to 270 °C at an increasing rate 5 °C /min. (hold 2 min.) then to 310 °C as a final temperature at an increasing rate of 3.5°C /min. (hold 10 min.). The carrier gas was helium gas and used at a constant flow rate of 1mL/min. For GC of saponifiable matter, capillary column (30 m x 0.25 mm) packed with 70% cyanopropyl–polysilphenylenesiloxane was used. The temperature of the oven was programmed at an initial temperature 80 °C increased to 230 °C by the rate of 3 °C/min, then kept isothermal for 20 min. The carrier gas was helium gas and used at a constant flow rate of 1.5 mL/min. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagents, gallic acid, rutin and ascorbic acid were purchased from E-Merck. Other chemicals used were of analytical grade.

The Plant material

Euphorbia grandialata R. was collected in October 2014 (from Shebin El-Qanater, Qalubiya, Egypt). It was identified by Dr. Abdelhalim Mohamed (Plant Taxonomy Department, Agricultural Research Institute, Egypt). A voucher specimen was placed at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Beni-suef University under registration number BUPD-44. The fresh aerial parts were cut into small pieces and used in the subsequent experiments.

Extraction

The extraction of the fresh cut aerial parts of *E. grandialata* (1g) was carried out at room temperature using 50 mL methanol (80%) for 2h on an orbital shaker adjusted at 200 rpm. The supernatant produced from mixture centrifugation for 20 min.

was transferred to a 100 mL volumetric flask. The procedure was repeated and respective supernatant was pooled. The final volume was adjusted to 100 mL.

Total phenolics content

Using Folin–Ciocalteu reagent for determination of total phenolics content (Abu Bakar *et al.*, 2009). The methanol extract (300 µl) was added to 2.25 mL of Folin–Ciocalteu reagent and wait for 5 min at room temperature then add to the mixture 2.25 mL of sodium carbonate (60 g/L) solution and stand at room temperature for 90 min then measure the absorbance at 725 nm. The standard curve was prepared using gallic acid for quantification purpose and the results were calculated as µg of gallic acid equivalent per g fresh weight.

Total flavonoids content

Using aluminium chloride method for determination of total flavonoids content (Abu Bakar *et al.*, 2009). In a test tube, methanol extract (0.5 ml) was added to 2.25 mL of distilled water then 0.15 mL of 5% NaNO₂ solution was added to the mixture. Wait for 6 min. then add to the mixture 0.3 mL of 10% AlCl₃.6H₂O solution. Stand for another 5 minutes then add 1.0 mL of 1 M NaOH. Mix the mixture well using vortex. Immediately, the absorbance was measured spectrophotometrically at 510 nm. The standard curve was prepared using rutin for quantification purpose and the results were calculated as µg of rutin equivalent per g fresh weight.

Antioxidant activity

Using DPPH free radical model to determine the antioxidant activity (Abu Bakar *et al.*, 2009). An aliquot of 300 µl of samples were added to 3.0 mL of 500 µM DPPH in absolute ethanol. Shake the mixture vigorously and wait for 30 min in the dark at room temperature. The absorbance was measured spectrophotometrically at 517 nm. Ascorbic acid was used as a positive control. Using the following equation to calculate the free radical scavenging activity

Scavenging effect (%) = [1 – (absorbance of sample/absorbance of control)] x 100

Statistical analysis

All experiments were carried out three times and the result was represented as mean ± standard errors. Microsoft Excel Windows 2013 was used to calculate the linear regression analysis and Pearson's correlation coefficient

Preparation of the unsaponifiable matter (USM), the saponifiable (SM) and the fatty acid methyl esters (FAME) derivatives of SM

The fresh cut aerial parts of *E. grandialata* (7 kg) were exhaustively extracted by cold maceration (2000 ml, x4, each 48 h) with aqueous ethanol (70%). The extract was evaporated under reduced pressure using a rotary evaporator. Distilled water (1L) was used to suspend the obtained residue (200g) and fractionated

successively with *n*-hexane (500 mL x 4) followed by evaporation of hexane under reduced pressure. The *n*-hexane extract (10 g) was saponified by subjecting it to heat under reflux with 100 mL 10% alcoholic potassium hydroxide together with 40 mL benzene for 24 h to make sure that hydrolysis had been completed. The saponified solution was concentrated under reduced pressure and suspended in 100 mL distilled water followed by extraction with diethyl ether (10 x 50 mL) till exhaustion. Distilled water was used to wash the combined ether extract several times to remove alkalinity. Anhydrous sodium sulphate was used to dry ether extract, followed by evaporation till dryness. The solvent-free residue obtained representing the unsaponifiable matters (USM), was orange-yellow in color and amounted to 8 g calculated as 80% of the total lipoidal composition of the corresponding *n*-hexane extract. The dried residue was kept in a desiccator for GC/MS analysis (Vogel, 1975). Liberation of the free fatty acids (FA) from the aqueous alkaline solution left after separation of the unsaponifiable matter was done by acidification with dilute HCl (10%). The FA was extracted with diethyl ether (5 x 50 mL). Distilled water was used to wash the combined ether extract to remove acidity. Anhydrous sodium sulphate was used to dry ether extract, followed by evaporation till dryness. The oily residue obtained representing the saponifiable matters (SM), amounted to 2 g calculated as 20% of the total lipoidal composition of the corresponding *n*-hexane extract (Vogel, 1975). SM was subjected to methylation to afford fatty acid methyl esters (FAME) derivatives of SM (Vogel, 1975). Chemical investigation using GC/MS technique was conducted to identify components of USM and FAME. A tentative identification of both USM and FAME components was done by comparison between their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system. The quantification of all identified components was based on the integration of the area under the peak.

RESULTS AND DISCUSSION

Total phenolics, flavonoids content and antioxidant activity

Spectrophotometric determination of total phenolics content in *E. grandialata* alcoholic extract was done according to Folin-Ciocalteu method using gallic acid as the standard. Results showed that 17.61 ± 1.2 μg of gallic acid were equivalent to 1g of fresh weight, and the results of total flavonoids content determination by aluminium chloride method was 4.495 ± 0.39 μg rutin were equivalent to 1g fresh weight. Antioxidant assay of the methanol extract showed scavenging capacity to the free radical DPPH 140.6 % μg ascorbic acid/gin comparison to standard ascorbic acid as positive control.

Plants with high contents of secondary metabolites especially phenolics and flavonoids were known to have antioxidant efficacy because of their redox properties and chemical structures. Phenolic compounds are one of the most abundant phytochemicals in plants. They play important role since they safeguard against ultraviolet rays and ensure security against

microbial attack and predators (Nacz,2006).The abilities of phenolic compounds for scavenging free radical are due to their hydroxyl groups (Soobrattee *et al.*, 2005). It showed antioxidant efficacy by different strategies like donating hydrogen atoms to free radicals or through binding to transition metal ions resulting in more stable forms (Kumar and Sandhir, 2014). Accordingly, polyphenol showed some physiological activities such as the protection against neurodegenerative and cardiovascular diseases and cancer since they have a high antioxidant capacity (Wootton-Beard and Moran,2011). Also, flavonoids showed *in vitro* and *in vivo* antioxidant activities which depends on the free OH groups, particularly 3-OH (Geetha *et al.*, 2003; Shimoi *et al.*, 1996).

DPPH assay has been used worldwide as a method to determine the free radical scavenging capacity of various plant extracts due to its simplicity and relatively short time compared to other methods (Majewska *et al.*, 2011). *E. grandialata* alcoholic extract showed high antioxidant activity. According to previous studies (Pietta 2000; Michalak, 2006) this activity was thought to be due to the high content of phenolics and flavonoids in this plant. The high content of phenolics and flavonoids is the accountable for the antioxidant efficacy of the plant extract. Flavonoids are considered as the highly efficacious scavengers of majority of oxidizing molecules and free radicals involved in many diseases (Bravo, 1998). They have the ability to inhibit the formation of reactive oxygen, bind to trace elements implicated the production of the free radical and clean out reactive species (Agati *et al.*, 2012). Crude extracts of plant materials rich in phenolics were known have antioxidative properties and health benefits, so far it could used in the food industry for (Baba and Malik 2015) This study has been conducted for other *Euphorbia* species as *E. hirta* (Asha *et al.*, 2016), *E. helioscopia* (Maoulainine *et al.*, 2012), *E. echinus* (Lahlou *et al.*, 2014)and *E. heterophylla* (Abbasi *et al.*, 2013), but this is the first report for antioxidant study of *E. grandialata*.

Identification of SM and USM components using GC/MS technique

The results of GC/MS analysis of the saponifiable matter of *E. grandialata* aerial parts (Fig.1 and Table 1) revealed the presence of 14 components from which six were identified as methyl esters of saturated fatty acids (46.26%) and eight were identified as methyl esters unsaturated fatty acids (53.74 %). Methyl isohexadecanoate was the major saturated fatty acid (33.13%) while 8, 11-octadecadienoic acid was the predominant unsaturated fatty acid (25.70%) followed by 9,12,15-Octadecatrienoic acid (14.45%) then 9-Octadecenoic acid (10.35%).On the other hand, 29 components were identified in USM (Fig.2 and Table 2). The identified compounds were hydrocarbons (51.1%) from which 1-nonanolwas the major (7.23%). In addition to sterols and triterpenes (35.92%) from which lanosta-8, 24-dien-3-olwas the major sterol (4.48%),A'-neogammacer-5-en-3-ol (14.35%), and α -amyrin (5.39%) were the major triterpenes. It could be considered the first GC/MS analysis report of one species from genus *Euphorbia*.

Table 1: GC/MS analysis of the saponifiable matters of the *n*-hexane extract of *Euphorbia grandialata* R.aerial parts.

| Peak no | Identified Components | R _t | % Area | Molecular weight | Molecular formula |
|---|--|----------------|---------------|------------------|--|
| 1 | Dodecanoic acid, methyl ester (C ₁₃) | 13.22 | 2.23 | 214 | C ₁₃ H ₂₆ O ₂ |
| 2 | Methyl tetradecanoate (C ₁₅) | 18.59 | 4.65 | 242 | C ₁₅ H ₃₀ O ₂ |
| 3 | Methyl 13-methyltetradecanoate (C ₁₆) | 21.20 | 0.99 | 256 | C ₁₆ H ₃₂ O ₂ |
| 4 | Ethyl 9-tetradecenoate (C _{16:1}) | 21.56 | 0.52 | 254 | C ₁₆ H ₃₀ O ₂ |
| 5 | Methyl isohexadecanoate (C ₁₇) | 23.75 | 33.13* | 270 | C ₁₇ H ₃₄ O ₂ |
| 6 | 9-Hexadecenoic acid, methyl ester (C _{17:1}) | 24.32 | 0.33 | 268 | C ₁₇ H ₃₂ O ₂ |
| 7 | 11-Hexadecenoic acid, methyl ester (C _{17:1}) | 24.54 | 0.51 | 268 | C ₁₇ H ₃₂ O ₂ |
| 8 | Heptadecanoic acid, methyl ester (C ₁₈) | 26.12 | 1.51 | 284 | C ₁₈ H ₃₆ O ₂ |
| 9 | Methyl stearate (C ₁₉) | 28.45 | 3.75 | 298 | C ₁₉ H ₃₈ O ₂ |
| 10 | 9-Octadecenoic acid, methyl ester (C _{19:1}) | 29.06 | 10.35* | 296 | C ₁₉ H ₃₆ O ₂ |
| 11 | 12-Octadecenoic acid, methyl ester (C _{19:1}) | 29.25 | 1.30 | 296 | C ₁₉ H ₃₆ O ₂ |
| 12 | 8,11-Octadecadienoic acid, methyl ester (C _{19:2}) | 30.38 | 25.70* | 294 | C ₁₉ H ₃₄ O ₂ |
| 13 | 9,12,15-Octadecatrienoic acid, methyl ester (C _{19:3}) | 31.96 | 14.45* | 292 | C ₁₉ H ₃₂ O ₂ |
| 14 | 11-Eicosenoic acid, methyl ester (C _{21:1}) | 33.41 | 0.58 | 324 | C ₂₁ H ₄₀ O ₂ |
| Total identified components | | | 100% | | |
| Total identified saturated fatty acid | | | 46.26 | | |
| Total identified unsaturated fatty acid | | | 53.74 | | |

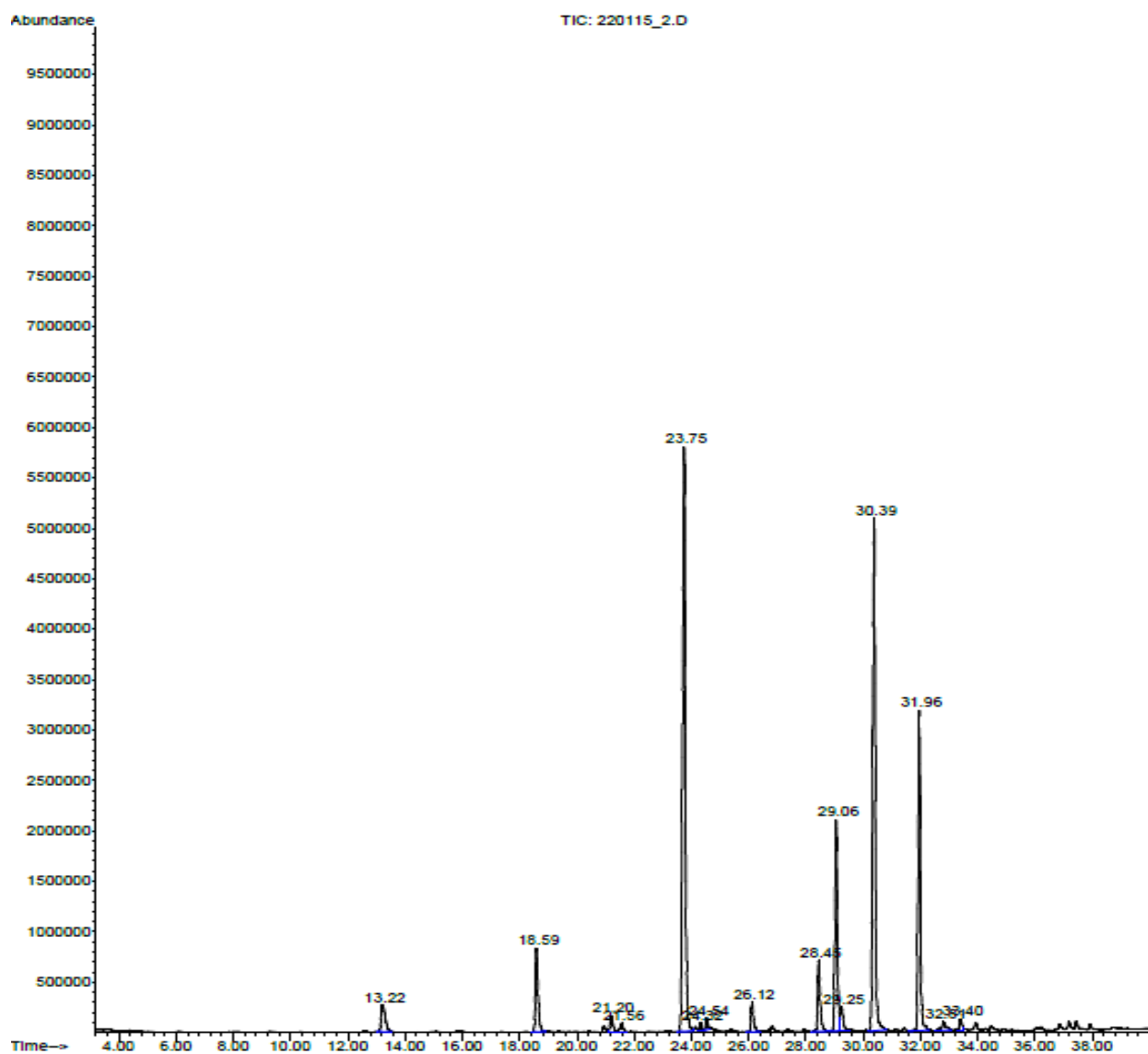
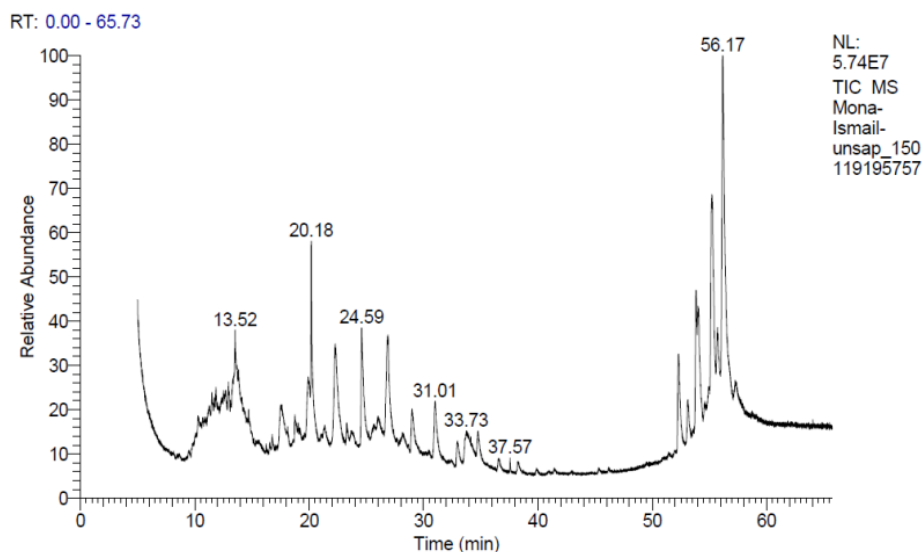
**Fig. 1:** GC/MS chromatogram of the saponifiable matters of *Euphorbia grandialata* R. aerial parts.

Table 2: GC/MS analysis of the unsaponifiable matters of the *n*-hexane extract of *Euphorbia grandialata* R.aerial parts.

| Peak no | Identified components | R _i | % Area | Molecular weight | Molecular formula |
|--|--|----------------|---------------|------------------|---|
| 1 | 9-Borabicyclo[3.3.1]nonane-9-ol | 10.28 | 0.83 | 138 | C ₈ H ₁₅ BO |
| 2 | 9-n-Dodecylperhydrophenanthrene | 11.49 | 0.96 | 360 | C ₂₆ H ₄₈ |
| 3 | Spiro[4.5]decane-6,10-dione | 11.84 | 0.81 | 166 | C ₁₀ H ₁₄ O ₂ |
| 4 | Docosane | 12.94 | 1.25 | 310 | C ₂₂ H ₄₆ |
| 5 | 17-Pentatriacontene | 13.32 | 1.02 | 490 | C ₃₅ H ₇₀ |
| 6 | 2,6-Dimethylundecane | 13.52 | 4.66 | 184 | C ₁₃ H ₂₈ |
| 7 | Isocolchifoline | 17.49 | 3.45 | 415 | C ₂₂ H ₂₅ NO ₇ |
| 8 | 3-Ethyl-5- (2-ethylbutyl)octadecane | 18.75 | 1.34 | 366 | C ₂₆ H ₅₄ |
| 9 | Tridecanol | 19.91 | 3.36 | 200 | C ₁₃ H ₂₈ O |
| 10 | Methyl 5-hydroxy-5-methyl-5,6,7,8-tetrahydronaphthalene-1-carboxylate | 20.18 | 5.56 | 220 | C ₁₃ H ₁₆ O ₃ |
| 11 | Curan-17-oicacid,19- (acetyloxy)-2,16-didehydro-20-hydroxy,methylester | 21.35 | 0.94 | 398 | C ₂₂ H ₂₆ N ₂ O ₅ |
| 12 | 2,2,4,9,11,11-Hexamethyldodecane | 22.28 | 4.47 | 254 | C ₁₈ H ₃₈ |
| 13 | Heneicosane | 23.28 | 1.12 | 296 | C ₂₁ H ₄₄ |
| 14 | Nonacosane | 24.59 | 4.83 | 408 | C ₂₉ H ₆₀ |
| 15 | 1-Nonanol | 26.83 | 7.23* | 144 | C₉H₂₀O |
| 16 | Colchifoleine | 28.99 | 2.20 | 401 | C ₂₁ H ₂₃ NO ₇ |
| 17 | Gibberellic acid | 32.93 | 1.30 | 346 | C ₁₉ H ₂₂ O ₆ |
| 18 | Antiriline | 33.70 | 0.81 | 296 | C ₁₉ H ₂₄ N ₂ O |
| 19 | Isooctylvinyl Ether | 34.76 | 1.65 | 156 | C ₁₀ H ₂₀ O |
| 20 | (3β,15α,16α,21β,22α)-Olean-12-ene-3,15,16,21,22,28-hexol | 36.56 | 0.89 | 506 | C ₃₀ H ₅₀ O ₆ |
| 21 | 4,4-Dimethyl-3-oxacholest-5-en-7-one | 52.28 | 4.47 | 414 | C ₂₈ H ₄₆ O ₂ |
| 22 | O-methyl-13-benzoyldehydroalamaridine | 53.09 | 2.18 | 412 | C ₂₆ H ₂₄ N ₂ O ₃ |
| 23 | Lanosta-8,24-dien-3-ol | 53.82 | 4.48* | 426 | C₃₀H₅₀O |
| 24 | 9,19-Cyclolanostane-3,7-diol | 54.04 | 2.44 | 444 | C ₃₀ H ₅₂ O ₂ |
| 25 | 17- (1,5-Dimethylhexyl)-10,13-dimethyl-4 vinylhexadecahydrocyclopenta[a]phenanthren-3-ol | 54.58 | 0.75 | 414 | C ₂₉ H ₅₀ O |
| 26 | α-Amyrin | 55.20 | 5.39* | 426 | C₃₀H₅₀O |
| 27 | Glycocholic acid methyl ester | 55.70 | 3.15 | 695 | C ₃₆ H ₆₉ NO ₆ Si ₃ |
| 28 | A'-neogammacer-5-en-3-ol | 56.14 | 14.35* | 426 | C₃₀H₅₀O |
| 29 | 6,9-Dihydroxy-7,8-dichloro-2-methyl-2- (4methyl3pentenyl)-2H-naphtho[2,3b]pyran-5,10-dione | 57.25 | 1.13 | 408 | C ₂₀ H ₁₈ Cl ₂ O ₅ |
| Total identified components | | | | 87.02% | |
| Total identified hydrocarbons | | | | 51.1 % | |
| Total identified sterols and triterpenes | | | | 35.92% | |

**Fig. 2.** GC/MS chromatogram of the unsaponifiable matters of *Euphorbia grandialata* R. aerial parts.

CONCLUSION

The analysis of the aerial parts of *E. grandialata* using GC/MS displayed that the saponifiable matter revealed the presence of 14 components. The major saturated and unsaturated fatty acids were methyl isohexadecanoate and 8, 11-octadecadienoic acid respectively, while USM showed 29

components; 1-nonanol was the major hydrocarbon, lanosta-8, 24-dien-3-ol was the major sterol and A'-neogammacer-5-en-3-ol and α-amyrin were the major triterpenes. *Euphorbia grandialata* could be counted as a valuable exporter for natural antioxidants. These antioxidant phytochemicals may be helpful in treatment or prevention of many degenerative diseases such as cancer and other

human diseases related to oxidative stress. Further work is needed for full characterization of its active principles.

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