*In Vitro* Cytotoxic Study of *Euphorbia grantii* Oliv. Aerial Parts against MCF-7 and MCF-7<sup>ADR</sup> Breast Cancer Cell Lines: A Bioactivity-Guided Isolation

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**ABSTRACT:** The development of highly efficient and low-toxicity anticancer drugs is one of the most critical problems in the medical field. *Euphorbia grantii* is commonly reported as an antiviral plant; a dilute solution of its latex is used for intestinal worms and to promote blood clotting and tissue healing. Our study evaluated the antiproliferative activity of the total extract, its respective fractions, and the isolated compounds from *E. grantii* aerial parts. A phytochemical study was done by several chromatographic methods, and the cytotoxic activity was assessed using the sulforhodamine B assay. The dichloromethane fraction (DCMF) exhibited promising cytotoxic activity against breast cancer cell lines (MCF-7 and MCF-7<sup>ADR</sup>), with an IC<sub>50</sub> of 10.31 and 10.41 μg/mL, respectively. Chromatographic purification of the active fraction revealed the isolation of eight compounds. Among the isolated compounds, euphylbenzoate (EB) exhibited a promising effect with an IC<sub>50</sub> of 6.07 and 6.54 μM against MCF-7 and MCF-7<sup>ADR</sup>, respectively, while other compounds showed no activity. Euphol, cycloartenyl acetate, cycloartenol, and epifriedelinyl acetate showed moderate activity (33.27−40.44 μM). Euphylbenzoate has smartly tackled both apoptosis and autophagy programmed cell death mechanisms. These results demonstrated that *E. grantii* aerial parts yield active compounds with significant antiproliferative potential.

1. **INTRODUCTION**

Cancer is a worldwide health problem impacting all regions and community groups. Breast cancer is the most commonly diagnosed malignancy in women being the main reason why people die from cancer. According to the GLOBOCAN’s 2018 estimates, more than 18 million new cases of cancer and 9.2 million more cancer-related deaths were reported. By 2030, it was estimated that the number of cases will reach more 21 million cases worldwide. Non specific chemotherapy, less effective generic cytotoxic agents, the toxicity related to the available drugs, and high frequency of tumor recurrence are common drawbacks of cancer treatments. Natural products have been developed as a key basis of several new drugs in the last decades.

The genus *Euphorbia* (family Euphorbiaceae) has been reported to have some promising activities as anti-inflammatory, anticancer, immunoregulation, and fibrinolytic actions. Phytochemical investigation of the family Euphorbiaceae showed the presence of saponins, diterpenes, triterpenes, flavonoids, lectins, phorbol esters, and glycoproteins. *Euphorbia grantii* Oliv. was reported for its antiviral activity against several reported viruses. The activity was correlated to its content of 3-methoxylavones such as methylquercetin and methylkaempferol.

Triterpenes are biologically active metabolites classified into tetracyclic and pentacyclic triterpenes and have recently caught broad attention. They have been reported as anti-inflammatory, antiviral, antibacterial, sedative, immunoregulatory, blood sugar regulatory, blood pressure-lowering, and antitumor agents. Recent reports indicated that triterpenes could directly inhibit the tumor growth, both *in vivo* and *in vitro* via several mechanisms.

Our study evaluated the antiproliferative activity of the total methanol extract and fractions/subfractions of *E. grantii* aerial parts as well as the isolated compounds from the most active fraction. The most active one was investigated for its mechanisms of action, providing a new insight for further research and investigations on the development of anticancer drugs.

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2. MATERIALS AND METHODS

2.1. General Experimental Procedures. Solvents were supplied by Piochem Company (Cairo, Egypt) and are analytical grade. ¹H NMR (100 MHz) and ¹³C NMR (400 MHz) spectra were performed on a Bruker high-performance digital NMR spectrometer (Bruker, Karlsruhe, Germany). The mechanisms of cytotoxicity were characterized using an ACEA Novocyte flowcytometer (ACEA Biosciences Inc., San Diego, CA), and the data were calculated using ACEA NovoExpress software (ACEA Biosciences Inc., San Diego, CA).

2.2. Plant Material. *E. grantii* Oliv. aerial parts were collected from El-Orman Botanical Garden, Giza, Egypt, in January 2020. The plant material was kindly authenticated by Therese Labib, a Botanical Specialist and Consultant at El-Orman Botanical Garden. A voucher specimen (No. 4.01.2023) was deposited at the herbarium of the pharmacognosy department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

2.3. Extraction, Fractionation, and Isolation. The air-dried powdered plant material (600 g) was extracted with methanol (3 L × 5) by maceration for 3 days. The collected extract was filtered and evaporated under reduced pressure to give 81.5 g of dry residue. A part of the total methanol extract (52 g) was fractionated in methanol, water, and dichloromethane (DCM) with a ratio of 1:1:1, to yield two fractions: DCMF (30 g) and the remaining mother liquor (MLF, 22 g).

The DCMF (11 g) was fractionated by chromatography on a Si gel column using n-hexane/ethyl acetate (EtOAc) mixtures as an eluent (10–70% EtOAc/n-hexane) to get five subfractions as follows: Fr. 1 (2.4 g), Fr. 2 (3 g), Fr. 3 (2 g), Fr. 4 (1.4 g), and Fr. 5 (eluted with 100% methanol, 1.8 g). Fr. 1 was the most active fraction among the five tested fractions as indicated by results of *in vitro* cytotoxic assay on breast cancer cell lines.

Therefore, part of Fr. 1 (2 g) was chromatographed on a Si gel column (25 × 2.5 cm²) using n-hexane/EtOAc (95:5 v/v) as a mobile phase and fractions of 50 mL each were collected to afford three main subfractions (Subfr 1a–c). Subfraction 1a (350 mg) was further purified using a medium-pressure liquid chromatography (MPLC) column (3 × 15 cm², normal-phase silica) using n-hexane/EtOAc (18.5:1.5 v/v) as a solvent system and 5 mL fractions were collected. The column afforded compound 1 (110 mg), compound 2 (11 mg), and compound 3 (8 mg). Subfraction 1b (850 mg) was purified by chromatography on a Si gel column (1.5 × 20 cm²) using 5% EtOAc in n-hexane, to yield compound 4 (50 mg), compound 5 (19.8 mg), and compound 6 (25 mg). Subfraction 1c (760 mg) yielded compound 7 (10.8 mg) and compound 8 (20 mg) upon chromatography on a Si gel column (3 × 20 cm²) using n-hexane/EtOAc (99:1 v/v) as a solvent system.

2.4. Cytotoxic Activity. The samples were tested for cytotoxic activity against breast adenocarcinoma (MCF-7) and doxorubicin-resistant breast cancer (MCF-7ADR®) cell lines. The two cell lines were obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cell viability was assessed by sulforhodamine B (SRB) assay. The absorbance was measured at 540 nm using a BMGLABTECH-FLUOStar Omega microplate reader (BMGLabtech, Ortenberg, Germany). All assays adopted the method mentioned before.

2.5. Analysis of Cell Cycle Distribution. To assess the effect of EB on the cell cycle distribution of MCF7 and MCF7ADR®, cells were treated with the predetermined IC₅₀ EB or drug-free media for 48 h. All procedures were described before.

2.6. Apoptosis Assessment. Apoptosis and necrosis cell populations were characterized for EB using an annexin V-FITC apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK), adopting a previously described method.

2.7. Autophagy Assessment. To clarify the method of cell death by which cells are killed in response to therapy, autophagic cell death was quantitatively determined using acridine orange coupled with cytometric analysis. Cells were treated with the predefined IC₅₀ of EB for 24 h. All procedures were carried out as described before.

2.8. Statistical Analysis. Data are presented as mean ± SD. Analysis of variance (ANOVA) with the LSD test post hoc was used for testing significance using SPSS for windows, version 17.0.0. *P* < 0.05 was taken as being statistically significant.

3. RESULTS AND DISCUSSION

The genus *Euphorbia* is an important source of natural product with a wide range of therapeutically important biological activities: antitumor, cytotoxic, anti-HIV, and anti-inflammatory activity. Terpenoids (diterpenes and triterpenes) represent the most important classes with their interesting structural diversity. Several classes of compounds were identified from the genus *Euphorbia*, in addition to terpenoids such as polyphenolic compounds, organic acids, alkaloids, and fatty acids. These compounds are responsible for the diverse activities reported for the plants such as the antiproliferative, anti-inflammatory, immunoregulation, antiangiogenic, antiparasitic, and antiasthma activities.

Cancer is well known of being the second cause of death worldwide. Each year, cancer is diagnosed in about 11 million people, ending with death in 10 million; the number estimated for the year 2030 will top up to 13.1 million cases. Today, about 50% of the clinically available anticancer drugs are derived from natural sources or inspired by natural compounds.

The crude extract and its respective fractions of *E. grantii* (MeOH, DCMF, and MLF) were evaluated for the half-maximal inhibitory concentration (IC₅₀) effect in hormone
receptor-positive breast cancer (MCF-7) and doxorubicin-resistant breast cancer (MCF-7ADR) cell lines. Of particular relevance is that the nonpolar fraction (DCMF) showed a potent cytotoxic effect, with IC₅₀ values of 10.31 and 10.41 μg/mL against MCF-7 and MCF-7ADR, respectively (Table 1).

Triterpenes are divided into linear ones: tetracyclic and pentacyclic. They are the major secondary metabolites detected in the genus including tirucallane, euphane, cycloartane, and oleanane subclasses and dominated in the DCMF active fraction. They were previously assessed for in vitro cytotoxic activities against estrogen-sensitive cells (MCF-7). In particular, 3β,25-dihydroxyxycloart-23-(E)-ene, 3β,25-dihydroxyxycloart-23-(Z)-ene, (24 R)-tirucalla-8,25-diene-3β,24-diol, lupenone, and 11β-hydroperoxyeupha-8, 25-diene-3β-ol exhibited significant activities against MCF-7 cells.

The phytochemical studies of the DCMF of the methanolic extract of E. grantii led to the isolation of eight triterpenes (Figure 1). The isolated compounds were identified as friedelin (1), 3β-friedelanol (2), epifriedelanol (3), euphol (4), cycloartenyl acetate (5), cycloartenol (6), epifriedeliny acetate (7), and euphylbenzoate (8) using ¹H and ¹³C NMR (see the Supporting Information). The structures of the

Figure 1. Structures of the isolated compounds.

Figure 2. Effect of euphylbenzoate on the cell cycle distribution of MCF7 (A) and MCF7ADR (B) cells. The cells were exposed to euphylbenzoate for 48 h. Cell cycle distribution was determined using DNA cytometry analysis and different cell phases were plotted as percentage of total events. Data is presented as mean ± SD; n = 3.


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isolated compounds were elucidated by comparison of their physical and spectral data (\(^1\)H and \(^{13}\)C NMR) with those mentioned in the literature.\(^{18-24}\)

The \(^{13}\)C NMR showed 30 carbons, indicative of a triterpenoid typical of the friedelane skeleton in compounds 1, 2, 3, and 7, which were reliable with the reported values of friedelin, 3-\(\beta\)-friedelanol, epifriedelanol, and epifriedelanyl acetate, respectively. The C-3 signal of the carbon bearing a carbonyl group at \(\delta_{\text{C}}\) of 213.25 in compound 1 was observed. Furthermore, the C-3 signal of the carbon bearing a hydroxyl group at \(\delta_{\text{C}}\) 72.22 and 72.77 could easily be correlated with the chemical shift of H-3 at \(\delta_{\text{H}}\) 3.76c and 3.40 in compounds 2 and 3, respectively. Furthermore, the C-3 signal of the carbon bearing a hydroxyl group at a \(\delta_{\text{C}}\) 74.61 in addition to carbon signals of the acetyl group (\(\delta_{\text{C}}\) 21.12, 170.92) could be correlated with the chemical shift of H-3 at \(\delta_{\text{H}}\) 2.04 (OAc, 1H, s). In the four compounds, the interpretation nearly matched and is characteristic of the tertiary methyl 8 carbons resonated at \(\delta_{\text{C}}\) 11.31—35.03 (C-23-30); these carbons exhibited coupling with protons at \(\delta_{\text{H}}\) 0.75—1.15.

Compound 4 is a tetracyclic triterpene identified as euphol, where compound 8 was identified as its benzoyl ester. Both compounds exhibited signals mostly appearing at the upfield region and are typical of the triterpenoid signals. The more peculiar ones in the H NMR spectra are an olefinic proton (\(\delta_{\text{H}}\) 5.02, 1H, m) and an axial proton on an oxygen-bearing carbon (\(\delta_{\text{H}}\) 3.14—3.18, m). Furthermore, both compounds exhibited seven singlet signals characteristic of tertiary methyl groups (\(\delta_{\text{H}}\) 0.77—1.61) and a secondary methyl group, \(\delta_{\text{H}}\) of 0.82 (H-21, 3H, d, J = 6), which form the eighth methyl groups in the skeleton. The \(^{13}\)C NMR spectrum of 4 exhibited four carbon signals (\(\delta_{\text{C}}\) 134.02, 133.54, 125.21, and 130.88) characteristic of four olefinic carbons of C-8, C-9, C-24, and C-25, respectively, indicating that compound 4 contains two double bonds. The carbon signal (\(\delta_{\text{C}}\) 79.01) was identified as carbon bearing a hydroxyl group of C-3. These data are in agreement with the structure of euphol,\(^ {21}\) where compound 8 showed protons and carbons of the benzoyl moiety.\(^ {24}\)

The \(^1\)H NMR spectra of compounds 5 and 6 revealed the presence of a geminal olefinic proton resonate at \(\delta_{\text{H}}\) 5.09 (H-24) and 5.28 (H-24), respectively, a cyclopropyl group at \(\delta_{\text{H}}\) 0.81 (H-30) and 0.78 (H-30), respectively, and two allylic methyl at \(\delta_{\text{H}}\) 1.61 (H-26) and 1.69 (H-26), respectively, with five other methyl groups as well as hydroxy methine at \(\delta_{\text{H}}\) 3.67 (H-3). The presence of a cyclopropyl group of C-19 with C-9 and C-10 was in agreement with the data reported in the literature of both compounds.\(^ {22}\) Thus, the structure of compound 5 as cycloartenol and compound 6 as acetate ester of 5 was elucidated by comparison with the data in the literature;\(^ {22}\) in addition, compound 6 showed signals due to the acetate group. \(^{13}\)C NMR: \(\delta_{\text{C}}\) 21.12, 170.92, and \(^1\)H NMR: \(\delta_{\text{H}}\) 2.05 (3H, s).\(^ {22}\)

Triterpenes are phyto compounds that display a broad spectrum of biological activities, antimicrobial, anti-inflammatory, antioxidative, antiviral, and antifungal properties, in addition to anticancer and chemopreventive actions. They are able to inhibit the life of neoplastic cell lines, in addition to inducing apoptosis of cancer cells and causing their “suicidal” death, without any threats to normal cells of the body.\(^ {15}\) In vitro cytotoxic activity of the isolated compounds revealed that
exposed to euphylbenzoate for 48 h and then were stained with P<0.05.

different from the corresponding single treatment (5-FU or GCB) at euphylbenzoate in MCF7 (A) and MCF7 showed potent activity with an IC from 15.60 to 27.77 compounds 1, 2, and 3 showed no activity in both cell lines, where compounds 4–7 had a moderate activity with IC ranging from 15.60 to 27.77 μg/mL against MCF-7 and 23.73 to 26.25 μg/mL against MCF-ADR, respectively. It is worth noting that compound 8, namely euphylbenzoate (EB), which was isolated for the first time from nature but previously reported as a synthesized compound from euphol (4), showed potent activity with an IC of 3.22 and 3.47 against MCF-7 and MCF-ADR cells, respectively (Table 1).

Cell cycle distribution using DNA flow cytometry was characterized to explain the cytotoxic/antiproliferative effect of EB against MCF7 and MCF7ADR cells. Treating MCF7 cells with the IC of EB significantly increased cell populations in both the S-phase and G2/M-phase from 21.7 ± 1.4 to 27.3 ± 0.7% and from 29.4 ± 1.1 to 33.7 ± 0.3%, respectively. Reciprocally, the nonproliferating cell fraction (G0/G1-phase) significantly decreased from 48.8 ± 0.5 to 39.0 ± 0.4% (Figure 2A). On the other hand, EB marginally enhanced cells in the G2/M-phase from 22.9 ± 1.7 to 22.9 ± 1.7% in the resistant MCF7ADR cells with no other significant influence on any other cell population (Figure 2B). However, EB significantly increased the pre-G cells in both naïve (MCF7) and resistant (MCF7ADR) breast cancer cells from to 1.4 ± 0.1 to 24.4 ± 0.1% and from 1.8 ± 0.1 to 14.0 ± 1.8%, respectively (Figure 2A,B). The increased Pre-G cell population is indicative of cell death rather than antiproliferative properties for EB. Still, the exact mechanism of cell death is not well defined.25

To establish the exact mechanism of cell death (programmed or not programmed), both naïve (MCF7) and resistant (MCF7ADR) cells were evaluated by annexin-V/FITC staining coupled with flowcytometric analysis after exposure to the IC of EB for 48 h. Still, no considerable apoptosis or necrosis was noticed in MCF7 cells after 48 h of exposure to EB (Figure 3A). However, exposure of the resistant MCF7ADR cells to EB for 48 h resulted in significant apoptosis (5-fold) and necrosis (4-fold) compared to the control untreated cells (Figure 3B).

MCF7 cells showed minimal or no apoptosis response compared to MCF7ADR cells after treatment with EB. On the other hand, the pre-G cell population in MCF7 was significantly increased, which is indicative of detectable cell death. Still, alternative pathways (rather than apoptosis/necrosis) are expected to result in cell death in the MCF7 cell line. It is worth mentioning that MCF7 cells are defective in the caspase-3 expression, and consequently, apoptosis is expected to participate minimally in the cell death pathway of this cell line.26 Autophagy is an alternative programmed cell death pathway; however, it has complicated roles and is debated in the cancer cell death.27 In this, we performed autophagy using acridine orange lysosomal stain coupled with flowcytometric analysis.

In MCF7ADR cells, no significant autophagy was detected upon treatment with EB for 48 h (Figure 4A). On the other hand, autophagy was the predominating method of cell death in MCF7 cells in response to EB with a significant increase of the acridine orange-induced autophagosome fluorescence signal (Figure 4B). From the literature, it is well known that MCF7 cells are lacking caspase-dependent apoptosis27 and autophagy in this cell line is a pro-death mechanism rather than pro-survival apoptosis escape mode.28,29

To the best of our knowledge, this feature is not reported yet in MCF-7ADR. Still, it is very interesting how a steroidal compound such as euphylbenzoate behaves smartly and tackles both apoptosis and autophagy programmed cell death mechanisms to induce cell killing as noticed in the form of elevated pre-G cell population (Figure 2A,B).

This study suggests that euphylbenzoate may have a unique mode of action compared to other compounds that induce cell death through a single mechanism. The ability of the compound to target multiple pathways may also make it a promising candidate for the treatment of cancer or other diseases where programmed cell death is involved. However, it is important to note that further research is needed to confirm these findings and fully understand the mechanism of action of euphylbenzoate. Further in vitro studies are needed, such as western blot analysis to examine the expression of proteins associated with apoptosis and autophagy or fluorescence markers to visualize the cellular changes associated with these pathways. In vivo experiments on tumor growth and programmed cell death in the animal model could be of benefit to determine whether euphylbenzoate has the same effects in vivo as it does in vitro.

This study further confirmed that the Euphorbia species could be a possible source of bioactive metabolites for exploring new anticancer drugs. Furthermore, EB could be a
lead compound to synthesize structurally related compounds to be examined for their cytotoxicity.

From the results in Table 1 and structures in Figure 1, it was indicated that acylation is required for the activity, as seen for compound 3 (inactive), which upon acylation gave the moderately active compound 7 with an IC\textsubscript{50} of 19.04 and 26.18 μg/mL against MCF-7 and MCF-7\textsuperscript{ADR} cells, respectively. In addition, the activity of compound 5 was marginally increased upon acetylation to compound 7. On the other hand, benzoylation of compound 4 to compound 8 increased the activity by 7.5- and 8.6-fold against MCF-7\textsuperscript{ADR} and MCF-7 cells, respectively.

Oxidation of the OH group at C-3 shows no relation to the activity (see compounds 1, 2, and 3), while the extended double bond conjugation to the carbonyl at C-3 results in an increase of the activity, as seen in quinone-methide triterpenes commonly present in the family Celastraceae.\textsuperscript{30,31} Euphylbenzoate is isolated for the first time in the nature and showed potent cytotoxic activity.

4. CONCLUSIONS

The \textit{Euphorbia} species have a powerful history of ethnomedicinal uses as well as ethnopharmacological uses in drug discovery from ancient times to the present. \textit{E. grantii} Oliv. (Euphorbiaceae) showed potent cytotoxicity against the MCF7 breast cancer cells and their resistant variant MCF7\textsuperscript{ADR} cells, which indicated that it could be a source for novel anticancer drugs to fight breast cancer. The phytochemical investigations of the plant resulted in the isolation of eight triterpenes from its active DCM fraction, where euphylbenzoate (EB) was the most potent isolate. EB resulted in significant apoptosis and necrosis compared to the control untreated cells against MCF7\textsuperscript{ADR}.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02091.

\begin{itemize}
  \item 1H NMR data of the isolated compounds, 13C NMR data of the isolated compounds, and 1H NMR and 13C NMR charts of the isolated compounds (C1–C8) (PDF)
\end{itemize}

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\section*{Notes}

The authors declare no competing financial interest.

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