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Flavonol-Glycoside and Rare Triterpenoid Derivatives Isolated from Leaves of *Combretum glutinosum* Perr. Ex Dc. with In Vitro Cytotoxic Activity

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Abstract: Combretaceae plants are used traditionally by many cultures, especially in Sudanese patients for the treatment of diverse ailments such as anti-inflammatory, antimicrobial, antitumor, and antioxidant disorders. Of these plants, the genus *Combretum* are traditional medicinal plants. Thus, they are formed from the non-polar or polar extracts of many isolated phytochemicals. Of these necessities, the use of *Combretum* extracts for their medicinal properties can be found in the earliest of myths and traditions used to document the plants' ability to treat diseases. *Combretum glutinosum* Perr. Ex Dc. is a common shrub native to the African continent, especially Sudan. Currently, there are no published data regarding its cytotoxic activity. Additionally, there are few chemical and biological reports of *C. glutinosum*. Therefore, the current study aimed to isolate the chemical bioactive compounds (**1**–**6**) from the ethyl acetate (EtOAc) extract of *C. glutinosum*. A new flavonoid compound, namely, glutosinumoside (**4**), was afforded, and five known compounds were obtained: three oleanane-glycosides (**1**–**3**) and two phenolic acids (**5**,**6**). The structures of the six compounds were determined by spectroscopic analysis, including one- and two-dimensional (1D and 2D) NMR, mass spectrometry, and chromatographic analysis. Moreover, an in vitro cytotoxic evaluation of the successive extracts and the bioactive EtOAc fractions of *C. glutinosum* against MCF7 (breast), HT29 (colon), HepG2 (liver), and MRC5 (normal lung) cell lines was performed. The isolated compounds showed comparable cytotoxic activities with the crude EtOH extract and doxorubicin against the tested cell lines. Compounds (**1**) and (**6**) exhibited the highest cytotoxicity against MCF7 (1.37 ± 0.21 and 1.48 ± 0.34 µg/mL, respectively) and HepG2 (3.30 ± 0.02 and 2.10 ± 0.22 µg/mL, respectively) in the MTT assay. In addition, compounds (**1**) and (**3**) demonstrated a significant upregulation of cancer's two important hallmarks (caspase 3 and bax genes) by inducing apoptosis and perturbing the MCF7 cell cycle.

Keywords: *Combretum glutinosum*; flavonol-glycoside; triterpenoid saponin; chromatography; cytotoxicity

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1. Introduction

Plants of the family Combretaceae are used significantly for therapeutic applications because they contain a distinct and wide range of secondary metabolites. The family consists of approximately twenty genera with six hundred species. The genus *Combretum* is considered one of the largest genera, having 370 species, and is distributed in Africa and Brazil (tropical and subtropical areas) [1]. Traditionally, in African and Asian countries, the *Combretum* species are extensively used for herbal remedies for the treatment of different ailments, including abortifacient, antibacterial, anticancer, antifungal, antimicrobial, antischistosomal, antitussive, antiviral, and molluscicidal activities [2,3].

Phytochemicals of everal species of the genus *Combretum* have been reported, including alkaloids, triterpenes, flavonoids, lignans, phenanthrenes, saponins, and stilbenoids. Considerable attention has been given to the screening of the plants' extracts for present and possible relationships with their reported biological activities, such as anticancer, antihyperglycemic, anti-inflammatory, antimalarial, and antioxidant activities [1].

Combretum glutinosum Perr. ex DC. is one of approximately 140 species belonging to the largest genus within the *Combretaceae* family, "*Combretum*" [3]. It is a small tree or bushy shrub distributed in tropical Africa and is popularly named "Habil" [1,4]. A systematic phytochemical screening of the *C. glutinosum* leaves revealed the presence of alkaloids [5], benzene dicarboxylic acids [6], flavonoids [4,7], phytosterols such as β-sitosterol and β-sitosterol-glycoside [8], polyphenols [4,5], stilbenes, tannins [4], and triterpenoids such as lupeol, oleanolic acid, betulinic acid, β-amyrin, and 3 β-acetoxydammar-21(22)-ene [8,9]. Among these, oleanane glycosides have a unique structure, and their wild distribution in the genus demonstrated relationships between chemical structure and cytotoxic activity [10]. In previous in vitro biological investigations, *C. glutinosum*'s antibacterial, antifungal, antimalarial, antischistosomal, and molluscicidal activities were significantly demonstrated due to the presence of several bioactive components, such as polyphenols (gallic and ellagic acids) [4,5], flavonoid glycosides (such as quercitrin and rutin) [4,5,11], and tannins such as combreglutinin, 2,3-(S)-hexahydroxydiphenoyl-D-glucose, punicalagin, and punicalin [4]. Likewise, β-amyrin, isolated from the stem bark of *C. glutinosum*, was used as an Ayurvedic formula for the treatment of flu and cold symptoms [9]. The phenolic compound 2,4-di-tert-butylphenol (DTBP) was used as a raw drug for the manufacture of antioxidants [9]. In most African savannahs, the leaves or stembark of the *C. glutinosum* are used to treat microbial infections, anemia, anorexia, diarrhea, fever, malaria, liver failure, respiratory disorders such as bronchitis and cough, as antitussives, and for wound healing [11,12].

One of the major public health diseases is cancer, which causes global death. There are 277 different types of cancer. Breast and prostate cancer are considered the major distributed types in women and men, respectively, in addition to colon, rectum, lung, urinary, and bladder cancer, and cancer of the uterine corpus in one of the sexes [13,14].

To the best of the authors' knowledge, there is no study that focused on the anticancer properties of *C. glutinosum* leaves. This study aimed to isolate, elucidate compounds, and perform an in vitro cytotoxic evaluation of *C. glutinosum*. The chemical investigation was assessed using different chromatographic techniques, and the in vitro cytotoxic activity against MCF7 (breast), HT29 (colon), and HepG2 (liver) cell lines and its selectivity were assessed using MRC5 (normal lung) cell lines in an MTT assay.

2. Materials and Methods

2.1. Plant Material and Extraction

C. glutinosum (leaves) were obtained from their natural habit in Aldbibate Town (12°29′55.2″ N 29°47′48.8″ E), South Kordofan state, Sudan, in March 2018. The plant material was identified by a taxonomist, Dr. Yahya Sulman Mohamed, in the herbarium of Medicinal and Aromatic Plant Research Institute (MAPRI), National Research Center (NCR), Khartoum, Sudan. A voucher specimen (No. MAPRI/2018/03) was deposited in the MAPRI herbarium. The leaves were dried in the shade and ground manually at room temperature. The powdered leaves of *C. glutinosum* (1923 g) were extracted at room

temperature, as described by [15], using 96% ethanol until complete exhaustion of the plant material. The EtOH yield (212.9 g) was partitioned between dichloromethane (DCM) and H_2O (1:1, *v*/*v*). The resulting DCM-soluble fraction (120 g) was subsequently partitioned between MeOH-H_2O, 8:2, and *n*-hexane, according to the NCI protocol, to provide the corresponding EtOH (212.9 g), *n*-hexane (7.9 g), 80% MeOH (8 g), DCM (120 g), and remaining aqueous (8 g) extracts. The aqueous-soluble fraction (25.3 g) was partitioned with ethyl acetate to produce EtOAc extract (1.8 g) (Flowchart S1).

2.2. General Chemistry

An NMR (1D and 2D, Bruker UltraShield Plus 500 MHz) spectrometer was utilized at the College of Pharmacy, Prince Sattam bin Abdulaziz University, KSA(Bruker, Fällanden, Switzerland, using deuterated $CDCl_3$, pyridine, and CD_3OD at room temperature (Cambridge Isotope Laboratories, Andover, MA, USA). HR-ESI-MS data were acquired by direct injection with electrospray ionization in negative or positive ion modes on an UPLC RS Ultimate 3000—Q Exactive (Thermo Fisher Scientific, Waltham, MA, USA) hybrid quadrupole-Orbitrap mass spectrometer combined with high-performance quadrupole precursor selection with high resolution and accurate mass Orbitrap™ detection. Open column chromatography was performed using Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) and silica gel 60/230–400 mesh (EM Science). The thin layer chromatography (TLC) analysis was performed on Keisel gel 60 F_{254} plates (Merck, Darmstadt, Germany). The reagents and solvents were purchased from Sigma-Aldrich, Loba Chemie Pvt. Ltd. (St. Louis, MO, USA), and SD Fine Chem. Ltd. (Mumbai, India).

2.3. Isolation and Purification of Compounds from the Ethyl Acetate Fraction

The EtOAc fraction (1.8 g) was chromatographed on a Sephadex LH-20 CC and eluted with $CHCl_3$ (100%), a mixture of $CHCl_3$-MeOH (1% increments of methanol in $CHCl_3$ until 10%) to achieve 8 fractions (A-H). The fractions were monitored by TLC on Si gel GF_{245} plates using a spraying reagent, 10% vanillin-sulphuric acid, for visualization. Fraction B (631 mg) was further separated by CC on a silica gel and eluted with a mixture of $CHCl_3$-MeOH (1% to 10%) to furnish 10 subfractions (B.1-B.10). Subfraction B.5 (60.9 mg) was subjected to Si gel CC with isocratic elution (*n*-Hex-EtOAc, 22%) to afford sub-subfraction (SB.5-2, 34.6 mg). The latter (34.6 mg) was purified on a Sephadex LH-20 CC and eluted with a mixture of $CHCl_3$-MeOH (0% to 3%) to obtain compound (**1**) (8 mg). The subfraction B.8 (41 mg) purified by using a Sephadex LH-20 CC, eluted with $CHCl_3$-MeOH (0% to 7%) to afford compounds (**4**) (3 mg) and (**2**) (3.8 mg), while (**3**) (10 mg) was recrystallized from subfraction B.9 ($CHCl_3$-MeOH 92:8, 34 mg). After subfraction B.4 (13.4 mg) was repeatedly chromatographed on Si gel CC, using a step gradient elution with *n*-Hex-EtOAc (5% to 25%), compounds (**5**) (3 mg) and (**6**) (3.1 mg) were obtained (Flowchart S2).

2.4. In Vitro Cytotoxicity Assay

2.4.1. Cell Lines

The *cytotoxic* activity was evaluated against MCF7 (breast), HT29 (colon), and HepG2 (liver) cell lines. In addition to MRC5 (a normal fibroblast), all were obtained from the ATCC, USA. The three cancer cells were sub-cultured in RPMI-1640 media (10% FBS), while MRC5 was maintained in Eagle's minimum essential medium (EMEM, 10% FBS), all at 37 °C and 5% CO_2 for a maximum of 5–10 passages [16,17].

2.4.2. MTT Assay

Five extracts, six compounds (**1**–**6**, Figure 1), and doxorubicin were subjected to an MTT cytotoxicity assay, according to a previous report [18]. Each cell line was separately cultured in a 96-well plate (3×10^3/well) and incubated with either the extract at final concentrations of 0–100 µg/mL or each of the compounds at a final concentration of 0–50 µM at 37 °C overnight (DMSO 0.1%; $n = 3$, three independent experiments). After three days of incubation, the cytotoxicity of each extract or compound was evaluated by

MTT (0.5 mg/mL added/well for 3 h) assay. Next, the supernatant was removed, the DMSO was added, and the absorbance values were measured using a multi-plate reader (BIORAD, PR 4100, Hercules, CA, USA). The selectivity index (S.I.) of the EtOH extract, each of the six compounds, and doxorubicin was calculated according to a previously described method [19] by dividing the IC_{50} of the corresponding extract/compound against MRC-5 cells/ IC_{50} of the same component against either MCF7, HT29, or HepG2 cells.

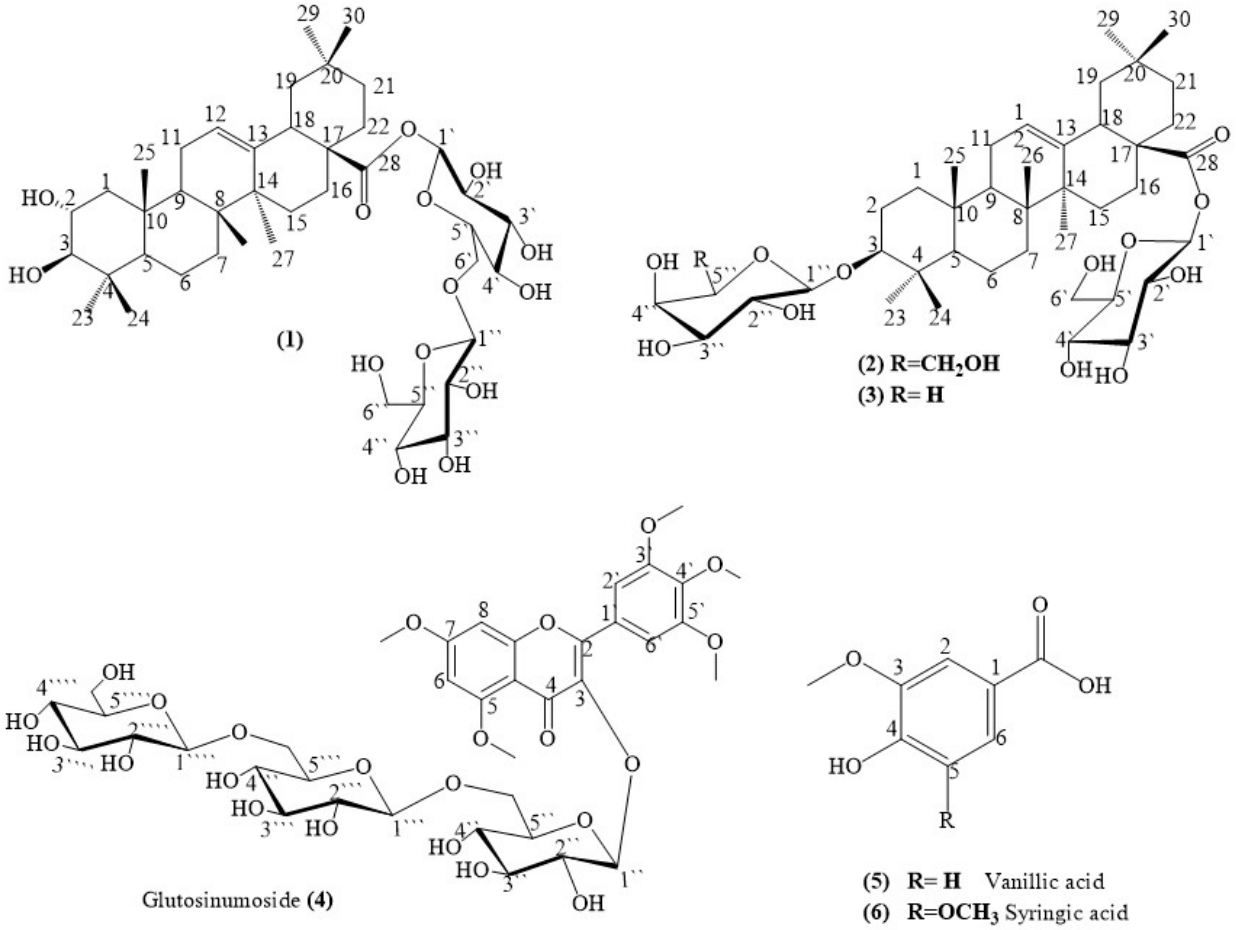

Figure 1. Structures of isolated compounds from *Combretum glutinosum* (**1**–**6**).

2.4.3. Quantitative Real-Time PCR

The quantification of apoptosis-related (caspase 3, bax, or surviving) or cell cycle-related (PCNA and CCND1) gene expression was performed in MCF7 cells using a real-time PCR (Applied Biosystems 7500 Fast Real-Time PCR System, Waltham, MA, USA) for two compounds (**1**,**3**), according to their resulting yield, though the yield of compound (**1**) was not high enough for the RT-PCR analysis. Briefly, MCF cells (1×10^6) were cultivated in 6-well plates. Treatments: vehicle control (0.1% DMSO), doxorubicin (0.1 µM), compound (**1**) (0.1 µM), and compound (**3**) (2 µM) for 24 h. The manufacturer's instructions were used to isolate the total RNA using an isolation kit and the SYBR Green Master mix (Thermo Fisher Scientific, Waltham, MA, USA). The synthesis of the complementary DNA (cDNA) using the isolated RNA (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Waltham, MA, USA) was performed. The mixture of cDNA, 2X SYBR Green I Mastermix, PCR-grade water, and forward and reversed human primers of the selective genes were used for the RT-PCR (Table 1) [18].

Table 1. Sequence of primers used in the RT-PCR.

Gene	Sequence
Caspase 3	F: ACATGGAAGCGAATCAATGGACTC R: AAGGACTCAAATTCTGTTGCCACC
Bax	F: GCCCTTTTGCTTCAGGGTTT R: TCCAATGTCCAGCCCATGAT
Survivin	F: TTGCTCCTGCACCCCAGAGC R: AGGCTCAGCGTAAGGCAGCC
PCNA	F: CACCAAGGAGGGTGTCAAGT R: GATCTTGGGGTGCCAGATAA
CCND1	F: GGATGCTGGAGGTCTGCGA R: AGAGGCCACGAACATGCAAG
GAPDH	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA

2.4.4. Statistics

An ANOVA (one-way analysis of variance) and Tukey's post-hoc were employed to determine statistical differences using Graphpad Prism software (version 9).

3. Results and Discussion

3.1. Identification of the Isolated Compounds

The phytochemical investigation of the *C. glutinosum* leaves showed the isolation of six compounds. The spectral data of the isolated compounds are recorded in Table 2, Table 3 and Tables S1–S4 and Figures S1–S43 using 1D-, 2D-NMR, and HR-ESI-MS data. The isolated compounds included three oleanane glycosides (**1**–**3**), one flavonol-glycoside (**4**), and two known phenolic compounds (**5**,**6**). It is worth noting that this is the first report on the isolation and identification of compound (**4**) from nature, although the four other compounds were isolated for the first time from the genus [*viz*. (**1**–**3**), and (**6**)], and compound (**5**) was previously identified from *C. glutinosum* by HPLC [20].

3.1.1. Identification of Compound (**1**)

From the HR-ESI-MS data in the negative mode, the pseudo-molecular formula of compound (**1**) was determined to be $C_{43}H_{70}O_{15}$, m/z = 843.4384 [M + HCOOH, 31%, calcd. 843.4697. The molecular formula of (**1**) was dedicated as $C_{42}H_{67}O_{13}$, m/z = 795.4777 [M–H^+, 10%, calcd. 795.4531], and the most abundant peaks were found at m/z = 727.3915 [M–H^+– 4OH^-, 100%, calcd. 727.4421] and 304.2999 [M– $C_{22}H_{36}O_{12}{}^{2\bullet}$, 100%, calcd. 304.2402] in positive mode (Table S1, Figures S11 and S12), identified as yellow amorphous. The ^{1}H-APT, DEPT-NMR, and HSQC spectral data of compound (**1**) (Table 2, Figures S1–S7) were consistent with the basic skeleton of oleanane triterpenes with the presence of the hydroxyl groups of the A-ring and sugar moieties attached to the 28-carboxylic group. The ^{1}H-NMR spectrum of (**1**) displayed seven tertiary methyl groups (each *s*) at δ_H: 1.35, 1.24 (2-CH_3), 0.98, 0.92, and 0.91(2-CH_3), and a broad olefinic proton doublet for H-12 at δ_H: 5.31 (*d*, *J* = 8.3 Hz); and two broad singlets for H-3 and H-18 at δ_H: 3.25 (1H, *s*) and 3.03 (1H, *s*), respectively. The two characteristic downfield protons of two methine groups at δ_H: 4.34 (*br.s*) and 3.24 (*br.s*) and the two anomeric signals at δ_H: 5.31 (*d*, *J* = 8.3 Hz) which were correlated in the HSQC spectrum (Figures S6 and S7) with the carbon signals at δ_C 69.3, 82.9, and 96.4, respectively, suggest that this compound has characteristic two hydroxy groups of a glycosidic-triterpenoid type. The ^{13}C-NMR data showed thirty-three carbon signals categorized as seven methyls, ten methylenes, five methines, and seven quaternary carbon atoms. In addition, the data from the C-2, C-3, and C-28 were downfield at δ_C 69.3, 82.9, and 179.1, respectively, indicating that the aglycone of (**1**) is maslinic [21,22]. The COSY spectrum data (Figure S8) established the triterpene skeleton as an oleanane type (maslinic acid), and the sugars are two moieties of D-glucose (Glc) [23,24]. The HMBC

spectrum data (Figures S9 and S10) establish the attachment of the sugar moieties with the triterpene skeleton at the C-28 position [25]. In the HMBC spectrum of (**1**), long-range couplings were observed between H-1′ of the Glc moiety (δ_H 5.31) and C-28 of an oleanolic acid moiety at δ_C 179.1 and between H-5′ (δ_H 3.31) of Glc and C-1″ of the terminal Glc moiety at δ_C 96.4. The anomeric configuration of the Glc moieties was established to be *β*-based on the relatively large $^3J_{H\text{-}6'\text{-}H\text{-}1''}$ values (8.3 Hz). Thus, (**1**) was assigned as 28-O-[*β*-D-glucopyranosyl]-(1″→6′)-[*β*-D-glucopyranosyl]-maslinic acid (Figures 1 and 2) [21–25].

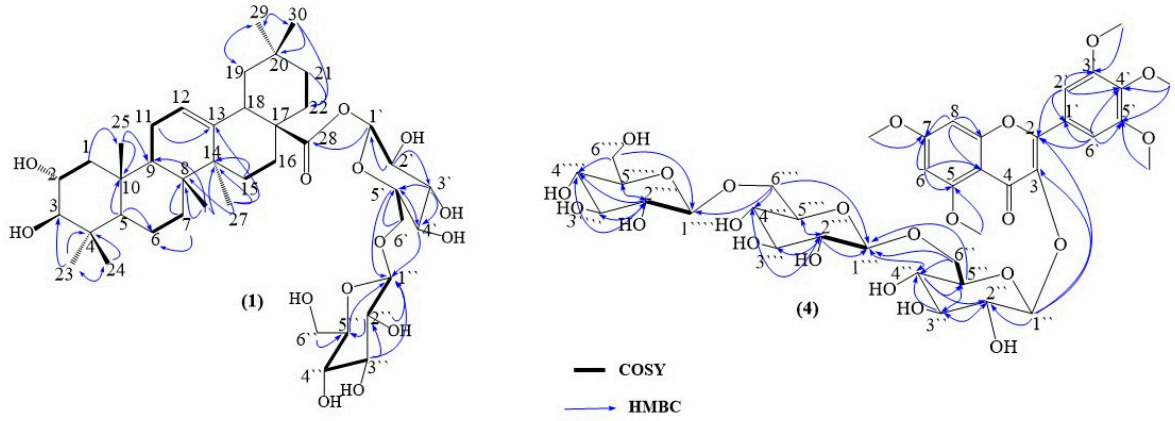

Figure 2. COSY and HMBC correlations of isolated compounds (**1**,**4**).

Table 2. NMR Spectroscopic data for the compounds (**1**) in deuterated methanol.

Position	Type	HSQC		COSY	HMBC (H→C)			Maslinic acid [a/b]	
		* δ_H (*J* in *Hz*)	** δ_C	(^{1}H-^{1}H)	J^2	J^3	J^4	δ_H (*J* in *Hz*) [a/b]	δ_C [a/b]
				Aglycone					
1	CH_2	1.74, 1.46, *m*	41.6	C2		C25		0.91, 2.00	46.3
2	CH	4.34, *br.s*	69.3	C1				3.70, *m*	68.9
3	CH	3.24, *br.s*	82.9					3.01, *d* (9.5)	83.2
4	C		36.4						39.4
5	CH	1.25, *m*	49.8		C6, C10			0.85, *bd* (11.7)	55.4
6	CH_2	0.98, *br.s*, 1.74, *m*	29.8	C7				1.40, *ddd*, 1.55, *m*	18.4
7	CH_2	1.25, 1.73, *m*	33.7	C6	C6, C8			1.32, *dt*, 1.46, *ddd*	32.8
8	C		40.5						39.3
9	CH	1.81, *m*	50.1	C11				1.62, *m*	47.7
10	C		39.1						38.0
11	CH_2	1.23, 1.96, *m*	25.1	C9, C12		C13		1.90, 1.95, *ddd*	23.5
12	CH	5.31, *d* (8.3)	125.4	C11				5.30, *t* (3.65)	121.7
13	C		144.2		C11				144.4
14	C		43.6						41.7
15	CH_2	0.98, *br.s*, 1.69, *m*	29.8	C16	C14			1.09, *dt*, 1.71, *ddd*	27.8
16	CH_2	2.29, 1.67, *m*	28.9	C15				1.62, *m*, 2.00, *dt*	23.3
17	C		47.6						46.2
18	CH	3.03, *br.s*	45.4					2.83, *dd*	41.5
19	CH_2	1.84, 0.85, *m*	50.3					1.63, *m*, 1.67, *ddd*	46.0

Table 2. *Cont.*

Position	Type	HSQC		COSY	HMBC (H→C)			Maslinic acid [a/b]	
		* δ_H (*J* in *Hz*)	** δ_C	(^{1}H-^{1}H)	J^2	J^3	J^4	δ_H (*J* in *Hz*) [a/b]	δ_C [a/b]
20	C		39.1						30.5
21	CH_2	1.26, 1.70, *m*	33.7	C22	C22			1.22, *m*, 1.35, *ddd*	33.8
22	CH_2	1.75, 0.98, *m*	29.9	C21				1.59, *m*, 1.78, *td*	32.7
23	CH_3	0.91, *s*	29.1		C4	C3, C24		1.03, *s*	28.9
24	CH_3	0.92, *s*	25.6		C4	C3, C23		0.83, *s*	17.1
25	CH_3	1.35, *s*	19.3		C9, C10			0.99, *s*	16.4
26	CH_3	0.98, *s*	19.1		C8	C9, C14		0.82, *s*	17.2
27	CH_3	1.24, *s*	25.4		C14	C8, C13		1.14, *s*	25.7
28	C		179.1						179.0
29	CH_3	0.91, *s*	29.1			C19,C30		0.91, *s*	32.8
30	CH_3	1.24, *s*	25.4		C20	C29	C22	0.93, *s*	23.3
				***β*-D-Glucose [c–e/f]**					
1′	CH	5.31, *d* (8.3)	96.4	C2′	C28			6.31, *d* (8.0)	96.0
								5.37, *d* (8.1)	95.7
2′	CH	3.29, *br.s*	74.4	C1′	C1′,C3′			4.23	74.2
								3.33, *m*	73.9
3′	CH	3.36, *d* (8.8)	78.7		C4′	C5′		4.30	79.1
								3.35, *d* (8.5)	78.2
4′	CH	3.32, *br.s*	71.6				C1″	4.34	71.3
								3.39, *m*	71.1
5′	CH	3.31, *br.s*	79.1	C6′	C4′	C1″		4.04	79.5
								3.42, *m*	78.5
6′	CH_2	3.54, *d* (11.1); 3.41, *d* (11.1)	66.4	C5′		C4′		4.97 *dd* (1.9; 11.5) 4.70 *dd* (5.5; 11.5)	67.1
1″	CH	5.31, *d* (8.3)	96.4	C2″				6.31, *d* (8.0)	96.0
								5.37, *d* (8.1)	95.7
2″	CH	3.29, *br.s*	74.4	C1″	C1″	C5″		4.23	74.2
								3.33, *m*	73.9
3″	CH	3.27, *br.s*	78.7	C4″	C2″	C1″		4.30	79.1
								3.35, *d* (8.5)	78.2
4″	CH	3.71, *br.s*	70.1	C3″,C5″				4.34	71.3
								3.39, *m*	71.1
5″	CH	3.26, *br.s*	78.7	C4″	C1″			4.04	79.5
								3.42, *m*	78.5
6″	CH_2	3.78, *d* (11.8); 3.65, *d* (11.7)	62.8		C5″			4.43, 4.48, *d* (11.8)	62.4
								3.80; 3.71 *m*	62.5

* Measured at 500 MHz, ** Measured at 125 MHz, [a/b] [21,22], [c–e] [23–25], [f] [26]. Assignments were based on the HMBC, HSQC, COSY, and DEPT experiments.

3.1.2. Identification of Compound (**2**)

The molecular formula of Compound (**2**) [yellow amorphous] was determined to be $C_{42}H_{68}O_{13}$, *m*/*z* 780.4891 ([M]$^-$, calculated 780.4660). It had a pseudo-molecular peak, $C_{43}H_{70}O_{15}$ at *m*/*z* 826.4355 [M+ HCOOH, 1%, calculated 826.4715], the most abundant peak at *m*/*z* 353.0881 [M–H$^+$ –$C_{21}H_{32}O_7{}^{2}$ –2$CH_3{}^-$, 100%, calculated 353.1964], and showed a characteristic fragment at *m*/*z* 701.3679 [M–2H$^+$ –4CH_3 –OH^-, 80%, calculated 701.3537] and 421.1637 [M–H$^+$ –2sugars, 10%, calculated 421.3470] based on HR-ESI-MS data (Figure S20) in the negative mode. Compound (**2**) bore a close similarity to (**1**) in the ^{13}C-NMR and ^{1}H-NMR spectral data (Figures S13 and S14) of the aglycone and the oleanane skeleton [27] except for the absence of a 2-hydroxyl group attached to the ring A and the type/position of one of the sugar moieties. However, except for the lack of signal from the terminal glucose sugar unit, a comparison of the NMR (1D, 2D) spectral data of (**2**) with those of (**1**) indicated the close attachment of the aglycone with the *β*-Glu- at C-28. The presence of the galactose sugar unit in the significant downfield shift of C-3 (δ_C 82.9) and the coupling patterns of the anomeric protons confirmed the presence of *β*-D-glucose and *β*-D-galactose at C-28 and C-3, respectively (Table S3). The characteristic key HMBC correlations (Figures S18 and S19) of δ_H 3.24 (1H, *d*, *J* = 3.5 Hz, H-3) with δ_C 96.3 (galactose C-1″) and δ_H 5.33 (1H, *d*, *J* = 8.3 Hz, H-1′) with δ_C 179.0 (aglycone C-28), assigned in the structure of (**2**) as 3*β*-O-*β*-D-galactopyranosylolean-12-en-28-oic acid 28-O-*β*-D-glucopyranosyl ester, (Figure 1) was confirmed by the 2D-NMR and mass spectral data (Figures S13–S20) [24–26,28]. This is the first report of the isolation of the compound (**2**) from the Combretaceae family by the SciFinder database; it was isolated before from the leaves of *Aralia elata* [29].

3.1.3. Identification of Compound (**3**)

Compound (**3**) was identified as a white amorphous compound. Its molecular formula was determined to be $C_{41}H_{66}O_{12}$, *m*/*z* 750.4072 ([M]$^-$, 4%, calcd. 750.4554), its most abundant peak was at *m*/*z* 711.3965 ([M–3H]$^+$ –2H_2O, 100%, calcd. 711.4108), and it showed a characteristic fragment at *m*/*z* 713.4022 ([M–H]$^+$ –2H_2O, 10%, calcd. 713.4265) based on HR-ESI-MS data (Figure S29) in the negative mode. The comparison of the 1D- and 2D-NMR spectral data of (**3**) with those of (**2**) indicated their close relationship with the aglycone, the *β*-Glu- at C-28, and also in the significant downfield shift of C-3 (δ_C 81.7). The assignments of the spectral data analysis of the sugar moieties, which were established by ^{1}H–^{1}H COSY and HMBC correlations (Figures S27 and S28), suggest that the glycosidic sequence of (**3**) does not correspond to sequence of (**2**). The ^{1}H- and ^{13}C-NMR (Figures S21–S23) demonstrated signals for one terminal *β*-D-glucopyranosyl (Glc) and *α*-arabinose (Ara) units [δ_H 6.37, *d* (8.0), H-1′, δ_C 96.6, 74.9, 79.7, 71.5, 80.1 (each CH), and 62.8 (CH_2) of Glc; and δ_H 6.06, *d* (5.5), H-1″, δ_C 96.6, 71.5, 74.9, 69.6 (each CH), and 67.1 (CH_2) of Ara]. In the HMBC spectrum of (**3**), long-range correlations were observed between H-1′ of Glc and C-28 of the aglycone at δ_C 178.1 and between H-1″ of Ara with C-3 of the aglycone at δ_C 81.7. Thus, (**3**) was assigned as 3*β*-O-*β*-L-arabinopyranosylolean-12-en-28-oic acid, 28-O-*β*-D-glucopyranosyl ester (Table S3 and Figure 1 and Figures S21–S28) [30]. Compound (**3**) is a rare triterpenoid saponin that is being reported for the first time in the Combretaceae family. In accordance with the SciFinder database, this is the second report of (**3**) in nature. It is noteworthy that compound (**3**) was firstly isolated by Geizi et al. from the leaves of *Lafoensia glyptocarpa* [30].

3.1.4. Identification of Compound (**4**)

Compound (**4**) displayed as yellow amorphous. Its chemical formula was determined as $C_{38}H_{50}O_{23}$, Mol. Wt. 874.7950. Its most abundant peak is *m*/*z* 575.2101 [M—7OH^- –$C_9H_8O_4{}^{2-}$, 100%, calcd. 575.2422] in positive mode (Figures S38 and S44, Table S2), and it showed characteristic fragments at *m*/*z* 263.1319 [M–2H—3 sugar moieties –CH_3 –3CH_3O^-, 50%, calcd. 263.0344] and 587.1901 [M–2H –6OH^- –CH_3O^- –$C_8H_8O_3{}^{2}CH_3O^-$, 2%, calcd. 587.1765] based on the HR-ESI-MS data (Figures S39 and S45, Table S2) in negative mode. The results of the spectral data analysis revealed the presence of a flavonol

skeleton substituted with penta-methoxy groups, which was identified as a myricetin pentamethyl ether [24,31–33], based on the ^{1}H, ^{13}C, and DEPT-NMR spectra (Table 3, Figures S30–S33). These spectra demonstrated the presence of four aromatic protons, including two *meta*-coupled protons at δ_H 6.38 (*overlapped*, H-6) and 6.52 (*br.s*, H-8) of the A-ring, δ_H 6.69 (*t*, H-2′) and 6.70 (*t*, H-6′) of the B-ring of the flavonol moiety, and five methoxy groups at δ_H 3.80 (*br.s*, $CH_3OCH_3O^-$), 3.76 (*s*, CH_3O^-), 3.75 (*br.s*, $CH_3OCH_3O^-$), 3.70 (*s*, $CH_3OCH_3O^-$), and 3.27 (*s*, CH_3O^-) which were correlated with the five quaternary carbons at δ_C 57.0, 56.8, 57.2, 57.3, and 60.4, respectively. The HSQC (Figure S34) confirmed the presence of 5,7,3′,4′,5′-pentamethyl ether [6,12,24,31–33]. The NMR spectral data of compound (**4**) (Table 3 and Figures S30–S37) showed the possible presence of the three sugar moieties; this was confirmed by the presence of three anomeric proton signals at δ_H 6.37, (*d*, *J* = 8.4 Hz), 4.17, (*d*, *J* = 9.0 Hz), and 3.95, (*d*, *J* = 7.6 Hz), which were correlated with the three anomeric carbons at δ_C 107.3, 105.5, and 105.2, respectively, from the HSQC (Figure S34) [24,27,31–33] and from the presence of a characteristic fragment at *m*/*z* 517.1487 [M–H—2 glucose units—CH_3, 5%, calcd. 517.1346], which was coincident with $C_{25}H_{25}O_{12}^{\bullet}$ (Table S2, Figures S39 and S45) in a negative mode. The anomeric configuration of the three Glu was established to be *β*-based on the relatively large $^{3}J_{H\text{-}1\text{-}H\text{-}6}$ values (7.6–9.0 Hz) (Figures S30–S37). The presence of a flavonol substitutional part was confirmed by the presence of a characteristic peak at *m*/*z* 371.15604 [M–3glucose parts, 1%, calcd. 371.1131], coincident with $C_{20}H_{19}O_7^{\bullet}$ (Table S2, Figure S38 and S45) in positive mode. In addition, the presence of 3-*O*-*β*-glucosyl substitutions was evident from the HMBC correlation between the proton signal at δ_H 6.37 (*d*, *J* = 8.4 Hz, H-1″) and at C-2 and C-3 (δ_C 149.4 and 139.3, respectively) (Figure 2, Figures S36 and S37). Thus, the structure of (**4**) was confirmed to be 3-[*β*-O-D-glucopyranosyl]-(1‴→6″)-O-*β*-D-glucopyranosyl-(1⁗→6‴)-O-*β*-D-glucopyranosyl-5,7,3′,4′,5′-pentamethoxy flavonol, named glutosinumoside (Figures 1 and 2). When compared with the SciFinder database, no reports on the occurrence of (**4**) in the herbal plants have been found in the literature.

Table 3. NMR spectral data of compound (**4**) in CD_3OD (500 MHz) for ^{1}H-NMR and (125 MHz) for ^{13}C-NMR.

Position	Type	HSQC		COSY	HMBC	Myricetin *	
		δ_H (*J* in *Hz*)	δ_C	^{1}H-^{1}H	(H→C)	δ_H (*J* in *Hz*)	δ_C
Aglycone							
2	C		149.4				157.2
3	C		139.3				134.3
4	C		178.5				178.1
5	C		149.5				161.4
6	CH	6.37 (overlapped)	108.2		C5, C7, C9,C10	6.67, *br.s*	98.6
7	C		149.1				165.8
8	CH	6.52, *br.s*	108.2		C7, C9, C10	6.36, *br.s*	92.8
9	C		149.5				157.0
10	C		139.2				105.6
1′	C		123.6				122.1
2′	CH	6.69, *t*	116.3		C2,C3,C1′,C2′,C3′	7.55, *br.s*	116.4
3′	C		149.3				146.5
4′	C		148.0				150.6
5′	C		147.6			7.04 (*d*, *J* = 8.7 Hz)	111.9
6′	CH	6.70, *t*	114.6		C2,C3,C1′,C4′,C5′	7.73 (*br.d*, *J* = 8.7 Hz)	123.0

Table 3. *Cont.*

Position	Type	HSQC		COSY	HMBC	Myricetin *	
		δ_H (*J* in *Hz*)	δ_C	^{1}H-^{1}H	(H→C)	δ_H (*J* in *Hz*)	δ_C
				***β*-Glucose**			
1″	CH	6.37, *d* (8.4)	107.4		C2, C3	4.4, *d* (7.9)	105.6
						5.69, *d* (7.7)	104.9
2″	CH	3.12–3.19, *m*	75.3		C3″, C4″, C5″	3.17, *t* (8.5)	75.5
						4.08	75.7
3″	CH	3.23, *br.s*	78.4		C2″, C4″, C1‴	3.33	78.3
						4.27	78.2
4″	CH	3.27, *m*	71.7		C3″, C5″	3.29	71.5
						4.15	71.4
5″	CH	3.12–3.19, *m*	75.3	C6″	C2″, C4″	3.27	77.7
						4.04	76.2
6″	CH_2	3.76, 3.80, *br.s*	67.5	C5″	C4″	3.67, *dd* (11.9, 4.9) 3.83, *dd* (11.9, 2.1)	62.7
						4.35; 4.65, *brd* (10.7)	69.3
				***β*-Glucose**			
1‴	CH	4.17, *dd* (2.6, 9.0)	105.5	C2″	C1″, C4″	4.4, *d* (7.9)	105.6
2‴	CH	3.12–3.19, *m*	75.3	C1‴	C1‴, C3‴, C4‴, C5‴	3.17, *t* (8.5)	75.5
3‴	CH	3.31, *br.s*	78.5		C2‴	3.33	78.3
4‴	CH	3.31, *m*	71.7		C2‴	3.29	71.5
5‴	CH	3.27, *br.s*	78.5		C2‴	3.27	77.7
6‴	CH_2	3.75, 3.80, *br.s*	67.5		C1⁗, C1‴, C4‴	3.67, *dd* (11.9, 4.9) 3.83, *dd* (11.9, 2.1)	62.7
						4.35; 4.65, *brd* (10.7)	69.3
				***β*-Glucose**			
1⁗	CH	3.95, *d* (7.6)	105.2	C2⁗		4.4, *d* (7.9)	105.6
2⁗	CH	3.22, *br.d*	75.4	C1⁗	C3⁗, C4⁗, C5⁗	3.17, *t* (8.5)	75.5
3⁗	CH	3.31, *br.s*	78.5		C2⁗	3.33	78.3
4⁗	CH	3.31, *m*	71.7		C2⁗	3.29	71.5
5⁗	CH	3.27, *br.s*	78.4		C2⁗	3.27	77.7
6⁗	CH_2	3.70, 3.74, *br.s*	65.5		C1⁗, C4⁗	3.67, *dd* (11.9, 4.9) 3.83, *dd* (11.9, 2.1)	62.7
5-O-CH_3		3.75, *br.s*	57.2		C5	3.86, *s*	56.7
7-O-CH_3		3.80, *br.s*	57.0		C7	3.85, *s*	56.2
3′-O-CH_3		3.70, *s*	57.3		C3′	3.86, *s*	56.7
4′-O-CH_3		3.27, *s*	60.4		C4′, C5′	3.86, *s*	56.7
5′-O-CH_3		3.76, *s*	56.8		C5′	3.86, *s*	56.7

* [24,27,31–33].

3.1.5. Identification of Compound (**5**)

The 1D-NMR data (Figures S40 and S41) identified the structure of compound (**5**) as white, amorphous vanillic acid, which was in accordance with the results of an authentic sample and reported data in the literature [34,35]. This compound was previously identified

from *C. glutinosum* by HPLC analysis [20]. ^{1}H-NMR spectrum data (CD_3OD, 500 MHz) are as follows: δ_H: 6.76 (1H, *d*, *J* = 8.0 Hz, H-5), 7.50 (1H, *s*, H-2), 7.48(1H, *br.d*, *J* = 9.0 Hz, H-6), 3.85 (3H, *s*, CH_3O-); ^{13}C-NMR spectrum data (CD_3OD, 125 MHz) δ_C: 169.2 (COOH), 116.2 (C-2), 149.1 (C-3), 152.8 (C-4), 114.3 (C-5), 125.7 (C-6), and 56.8 (CH_3O^-) [34,35].

3.1.6. Identification of Compound (**6**)

Compound (**6**) was yellow and amorphous, identified by ^{1}H and ^{13}C-NMR (CD_3OD, 500 and 125 MHz) spectral data (Figures S42 and S43), as follows: δ_H: 7.27 (2H, *s*, H-2/H-6) and 3.82 (3H, *s*, CH_3O-); δ_C: 171.0 (COOH), 123.0 (C-3/C-5), 142.0 (C-4), 149.3 (C-1), 108.7 (C-2/C-6), 57.2 (CH_3O-) [36]. In addition, co-chromatography with an authentic sample revealed that this compound (**6**) is syringic acid. This compound was isolated from the genus *Combretum* for the first time.

3.2. Biological Activities of Main Fractions and Isolates from C. glutinosum

3.2.1. In Vitro Cytotoxic Activity

Based on the biological, bio-guided procedure, the anticancer activity of the five extracts of *C. glutinosum* was determined to select the most significant extract(s) for further phytochemical investigations (Table 4). The anticancer activity of DCM and the aqueous extracts against MCF7 and HT29 demonstrated a growth inhibition of 62%-93% compared to the control after treatment with 50 µg/mL, indicating that the activity is associated with the natural compounds within the DCM extract. Therefore, the DCM extract was kept for future investigations. The growth inhibition % of the crude EtOH was superior to that of the DCM, reaching 93% against MCF7 (Table 4). Thus, further investigations were carried out on the crude EtOH extract. Table 5 shows the cytotoxic activity of the crude EtOH extract in addition to the six isolated compounds and doxorubicin. The most sensitive cell line was MCF7; the most active compounds were (**1**), followed by (**5**), (**6**), and (**4**) (Table 5).

Table 4. Percent (%) of the growth inhibition compared to control after treatment with each of the five crude extracts of *C. glutinosum* (50 µg/mL, *n* = 3, 72 h).

Extract	Growth Inhibition% ± SD		
	MCF-7	**HT-29**	**MRC-5**
Crude EtOH	93 ± 2.40	90 ± 1.24	85 ± 3.21
DCM	89 ± 2	93 ± 1	94.2 ± 0.6
MeOH 80%	55 ± 3	35 ± 5	47 ± 4
n-Hex	31 ± 5	25 ± 4	48.4 ± 5.5
Re. Aq	81 ± 1	62 ± 2	49 ± 3

EtOH = ethanol; DCM = dichloromethane; MeOH = methanol; *n*-Hex = *n*-hexane; Re. Aq = remaining aqueous.

Table 5. Cytotoxic activity (IC_{50} µM ± SD, *n* = 3, 72 h) of the isolated compounds from *C. glutinosum* and doxorubicin against three cell lines and normal fibroblasts.

Extract */ Compound	MCF7	HT29	HepG2	Average ** IC_{50}	MRC5
EtOH Ext.	3.50 ± 0.74	12.09 ± 2.22	2.99 ± 0.56	6.19	13.84 ± 1.57
1	1.37 ± 0.21	4.54 ± 1.48	3.30 ± 0.02	3.07	10.22 ± 1.46
2	2.40 ± 0.22	18.48 ± 0.78	3.37 ± 0.02	8.08	14.76 ± 0.32
3	2.27 ± 0.19	6.24 ± 0.93	2.42 ± 0.21	3.64	3.54 ± 1.35
4	1.72 ± 0.19	3.99 ± 1.03	3.30 ± 0.93	3.00	15.68 ± 3.02
5	1.48 ± 0.02	6.62 ± 0.67	3.82 ± 0.50	3.97	7.04 ± 0.61
6	1.48 ± 0.34	4.06 ± 0.48	2.10 ± 0.22	2.54	13.85 ± 2.00
Doxo	0.29 ± 0.03	0.13 ± 0.03	2.33 ± 0.19	0.91	3.71 ± 0.02

* IC_{50} of extract in µg/mL, ** average cytotoxicity (IC_{50}) of each extract/ compound against the three cancer cells.

3.2.2. Effect of Compounds (**1**) and (**3**) on Gene Expression

Compared to the vehicle control, both doxorubicin and compound (**1**) caused a significant upregulation of the pro-apoptotic caspase 3 and bax gene expression and the downregulation of survivin in MCF7 cells. The effect of doxorubicin was more significant compared to compound (**1**) with respect to Caspase 3 and survivin, while compound (**1**) showed a greater bax upregulatory effect than doxorubicin, as shown in Figure 3. Moreover, both compounds showed significant cell-cycle effects through the downregulation of PCNA and CCND1 gene expression, while compounds (**1**) and (**3**) surpassed the effect of doxorubicin with respect to CCND1. The effect of doxorubicin was more remarkable compared to compound (**3**) with respect to the caspase 3 and bax genes, while compound (**3**) surpassed doxorubicin in downregulating survivin, as shown in Figure 4. Thus, compounds (**1**) and (**3**) were shown to affect cancer's critical hallmarks by inducing apoptosis and perturbing the MCF7 cell cycle.

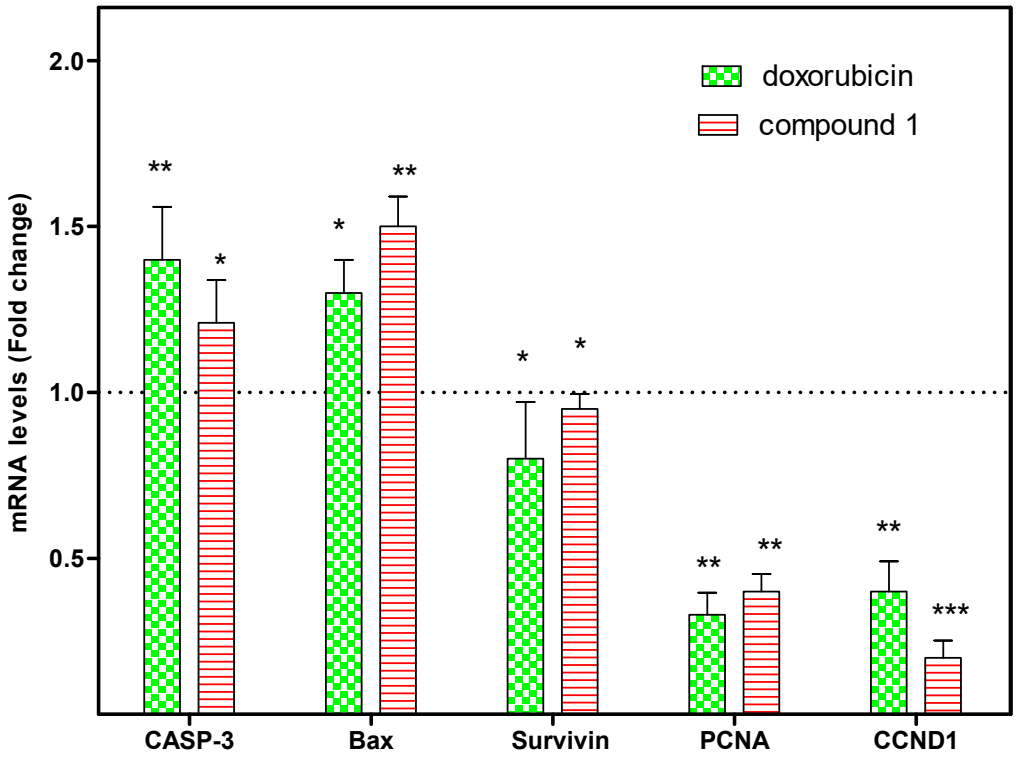

Figure 3. The effect of vehicle control, doxorubicin (0.1 μM), and compound (**1**) (1 μM) on the expression of caspase 3, Bax, survivin, PCNA, and CCND1 in MCF7 cells (24 h). RT-PCR was used for the estimation of the proteins (mean ± S.D., fold change = 1 dashed line, $n = 2 \times 2$ independent experiments). $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant.

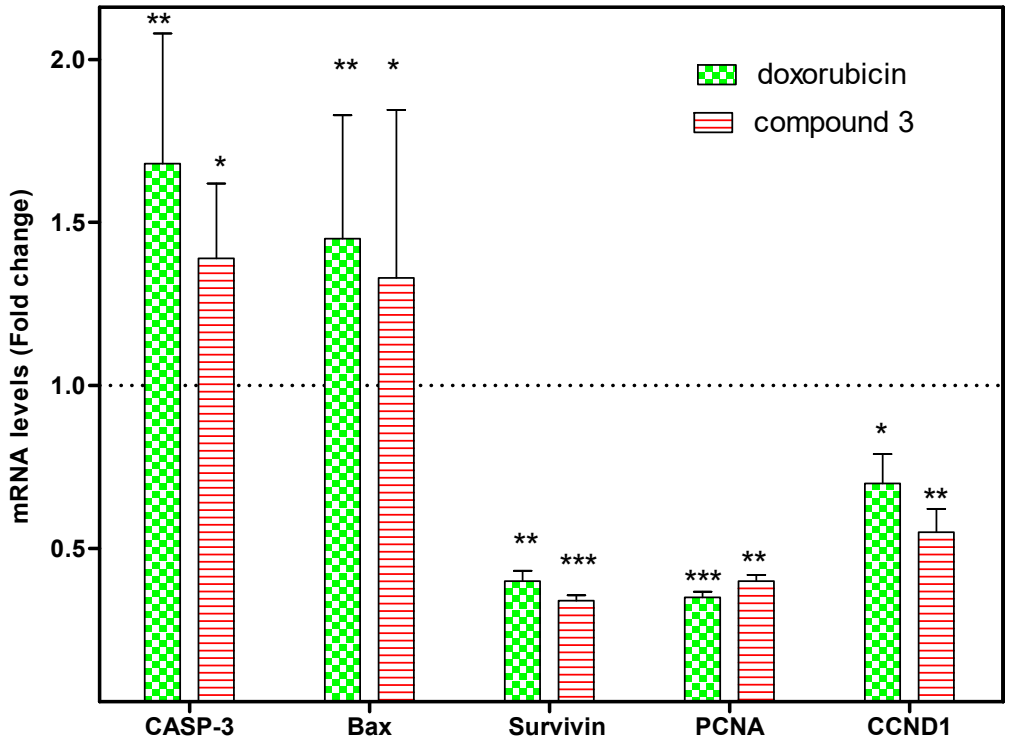

Figure 4. The effect of vehicle control, doxorubicin (0.1 μM), and compound (**3**) (2 μM) on the expression of caspase 3, Bax, survivin, PCNA, and CCND1 in MCF7 cells (24 h) RT-PCR was used for the estimation of the proteins (mean ± S.D., fold change = 1 dashed line, $n = 2 \times 2$ independent experiments). $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant.

4. Conclusions

This study was conducted to evaluate the cytotoxic activity of the polar fraction (ethyl acetate) of the Sudanian plant, *C. glutinosum*, to identify new phytochemicals for effective therapy. The study reported herein the isolation and identification of six bioactive components, including three triterpenoid saponins [28-O-[*β*-D-glucopyranosyl-(1″→6′)-[*β*-D-glucopyranosyl]-maslinic acid (**1**), 3*β*-O-*β*-D-galactopyranosylolean-12-en-28-oic acid, 28-O-*β*-D-glucopyranosyl ester (**2**), and 3*β*-O-*β*-L-arabinopyranosylolean-12-en-28-oic acid, 28-O-*β*-D-glucopyranosyl ester (**3**)], a flavonol-glycoside derivative (**4**), and two phenolic acids (**5**,**6**). One compound of isolates was identified for the first time from nature, viz., glutosinumoside (**4**) [flavonol-glycoside]. This research also showed that four bioactive phytochemicals [(**1**–**3**) and (**6**)] had not before been isolated from *C. glutinosum* nor the Combretaceae family. The biological results of this study demonstrated that the plant has significant cytotoxic activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10030209/s1, Tables S1 and S2: HR-ESI-MS spectral data for the compounds (**1**,**4**); Table S3: NMR spectral data for the compounds (**2**,**3**); Table S4: NMR spectral data for the compounds (**5**,**6**); Figures S1–S43: Spectra of the compounds (**1**–**6**); Figures S44 and S45: Mass fragmentations of the compound (**4**); Flowchart S1: Extraction and Fractionation procedure of the *C. glutinosum* leaves; Flowchart S2: Fractionation and purification of the ethyl acetate extract of *C. glutinosum* leaves. References [22–24,31,36] are cited in the Supplementary Material.

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