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Cycloartane from *Tabernaemontana coronaria* (Jacq) Willd flowers with their cytotoxicity against MCF7 and HCT116 cancer cell lines

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Abstract
The n-hexane fraction of *Tabernamontana coronaria* (Jacq) Willd flowers afforded two cycloartane type triterpenes, cycloart-25-en-3β, 24 diol and cycloart-23-en-3β, 22α, 25triol for the first time from this plant, in addition to lupeol acetate, α-amyrin, cycloeucalenol, stigmasterol. Study of cytotoxic activity of methanol extract and n-hexane fraction as well as isolated compounds against breast (MCF7) and colon carcinoma (HCT116) cell lines using Sulforhodamine-B assay revealed that, cycloart-25-en-3β, 24 diol and cycloart-23-en-3β, 22α, 25 triol possess significant activity against MCF (IC_{50}= 4.28µg mL^{-1} and 3.83 µg mL^{-1}, respectively). Meanwhile both methanol (MTC) and n-butanol (BTC) fractions at dose of 100 mg/kg body weight showed significant hepatoprotective and antioxidant potentials in liver damaged and diabetic rats (injected with CCl_4 and alloxan respectively). Treatment with MTC&BTC markedly attenuated the increases in ALT, AST levels caused by CCl_4 intoxication and restored the decreased glutathione level in alloxan-induced diabetic rats.

Keywords: Cycloartane, Tabernaemontana coronaria, cytotoxicity, MCF7, HCT116 cancer cell

Introduction
T. coronaria (Jacq) Willd family Apocynaceae is a glabrous, evergreen, dichotomously branched shrub, grows to a height of 6 - 10 feet tall and 5- 8 feet wide. It has oblong leaves with wavy margins. Previous researchers reported the presence of alkaloids, phenolics, triterpenes and sterols in *T.coronaria* organs. [1-2] the plant proved several pharmacological activities as anthelmintic. [2-3], nephroprotective. [4] in-vitro antioxidant, anti-inflammatory [5-6]. Antimicrobial [1] and cytotoxic potential [7] Karawya and Aboutabl, 1982 [8] investigated the flavonoids and alkaloids of the plant grown in Egypt. Herein we evaluate the *in-vivo* hepatoprotection and antioxidant potentials. Furthermore, we report for the first time the isolation of cytotoxic cycloartens from *T.coronaria* flowers.

Experimental
Plant material
Fresh flowers of *T. coronaria* were collected during 2010-2011 from the plant cultivated in the Experimental Station of Medicinal plants, Pharmacognosy Department, Faculty of Pharmacy, and Cairo University. The plant was kindly identified by Prof. Dr. Wafaa M. Amer Prof. of Taxonomy and Flora, Cairo University Herbarium. A voucher specimen was deposited at Cairo University Herbarium.

Extraction and separation
The fresh flowers of *T. coronaria* (1750 g) were extracted with methanol at room emperature, till exhaustion. The methanol extract was evaporated under reduced pressure to afford a dark residue (80g) which was suspended in water and successively extracted with n-hexane, chloroform, ethyl acetate and nbutanol. The n-hexane fraction (8g) was chromatographed over Silica gel 60 H column (5 cm x 7.5 cm, 30 g) successively eluted with *n*-hexane, *n*-hexane-chloroform, and chloroform-ethyl acetate in increasing order of polarity. Fractions of 200 ml each were collected and monitored by TLC. The spots were visualized after spraying with *p*-anisaldehyde/H_2SO_4 reagent followed by heating. Fractions with similar TLC profile were combined to give four main fractions. Purification of the fraction eluted with n-hexane/chloroform (80:20) showed one major spot which then purified to yield compound **1** (78mg). Fraction eluted with n-hexane/chloroform (50:50) showed 2 major spots it was further

subjected to column chromatography (flash silica), using n-hexane/ethyl acetate (98:2) as Eluent to afford compounds 2 (90 mg) & 3 (29mg). Fraction eluted with n-hexane/ chloroform (45:55) was rechromatographed, gradually eluted with n-hexane/ ethyl acetate (98:2) to yield pure compound 4 (85mg). Fraction eluted with n-hexane/ chloroform(20:80) was rechromatographed over silica gel column, eluted with n-hexane/ ethyl acetate (90:10) to yield compounds 5(47 mg) and **6** (52mg).

HPLC analysis of phenolic acids and flavonoids [9-10]

5g of the sample powder were mixed with methanol and centrifuged at 10000 rpm for 10 minute and the supernatant was filtered through a membrane filter (0.2 µ) and 20 µl of filtrate was injected into the HPLC. Chromatography was performed using Shimadzu HPLC (Model SPD-10A UV-VIS Detector) and Inertsil ODS-3 (200 mm × 4.50 mm × 5µm) column with mobile phase consisting of methanol, acetonitrile. Flow rate was maintained at 1.0 mL/minute and the compounds were read at 280 nm. The total run time was 40 minutes and run temperature 35 ^{o}C. The filtered methanol extract was injected under these conditions as well as a mixture of authentic samples of phenolic acids and flavonoids from Sigma Co. Retention time and peak area were used to calculate the polyphenolic compounds concentration by the data analysis of Hewllet packared software.

Animals

Adult male rats of Sprague-Dawley strain [130 -150 g body weight (b.wt)] and Male albino mice, 25-30 g were obtained from the animal house of the National Research Centre, Giza, Egypt. Ethical issue was followed [11] and doses of the drugs were calculated according to Paget and Barner, 1964 [12]

Estimation of antioxidant activity [13]

The antioxidant activity was estimated by determination of blood glutathione level in the blood of alloxan-induced diabetic rats. Thirty rats were divided into 5 groups (6 animals each). One group received 1mL saline and kept as a negative control, while diabetes was induced in 24 rats [14] using a single dose of 150 mg alloxan/Kg body weight by a single intraperitoneal injection. Hyperglycemia was assessed after 72 hours by measuring blood glucose level. [15] Diabetic rats were divided into four groups (6 animals each). First group received 1mL saline, second group received the reference drug, 7.5 mg/kg b.wt vitamin E (positive control). Third and fourth groups received 100mg\kg b.wt of methanol and butanol fractions. Rats were kept one week before the determination of glutathione in blood

Estimation of hepatoprotective activity [16]

Hepatoprotective activity of both methanolic and butanol extracts of the flowers was evaluated by measuring Alanine Amino Transferase (ALT), Aspartate Amino Trasferase (AST) and Alkaline Phosphatase (ALP) levels in blood of CCl_4 damaged liver in rats at a dose of 100 mg/kg.b.wt. The test samples were administered daily for 7 days before induction of liver damage by intraperitoneal injection (I.P) of 5 mL/kg of 25% carbon tetrachloride (CCl_4) in liquid paraffin using silymarin (25 mg/kg.b.wt.) as a reference drug. The test samples as well as the reference drug were further administered to the rats for another 7 days after liver damage. Blood samples were collected of each group at zero time, 7 days after receiving the test sample, 72 hours after induction of liver damage and 7 days after treatment with the test samples. The blood samples were allowed to clot, centrifuged at 1000 xg for 40 minutes and the separated sera were used for the estimation of the levels of AST, ALT. [17] and ALP. [18]

The data obtained for antioxidant and hepatoprotective assays were presented as mean ± standard error and the significance of difference between test and control groups was statistically analyzed using student's t-test. P values of 0.05 or less was considered as criteria for significance. [19]

Cytotoxicity Study [20]

Breast carcinoma cell line (MCF7) and human colon carcinoma cell line (HCT116) were obtained from National Cancer Institute, Kasr El Ainy, and Cairo, Egypt. Cytotoxic activity was tested using Sulforhodamine-B assay. The methanol extract, the n-hexane fraction, compound 3, compound 5 and compound 6 were tested at concentrations of 0, 5, 12.5, 25 and 50 µg/mL in DMSO. Doxorubicin was used as a positive control at the same concentrations. Each assay was done in triplicate. The IC_{50} value (the concentration that reduces the survival of the cancer cells to 50%) was calculated for each tested sample as well as the reference drug doxorubicin.

Results and Discussion

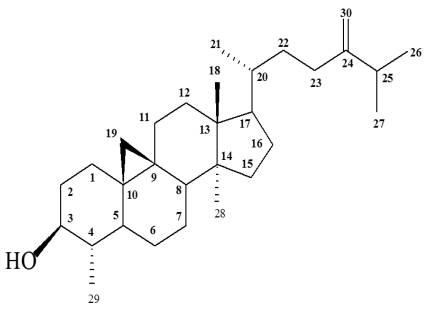
Identification of isolated compounds (Table 1&Figure 1)

Compounds 1, 2 and 4 were identified as lupeol acetate, α-amyrin and stigmasterol, respectively. The identification was based on the melting point, mixed melting point, IR spectrum, and co-chromatography with authentic samples. ^{1}H NMR and ^{13}CNMR data of compounds 3, 5 and 6 are listed in table 1.Compound 3 was isolated as a white amorphous powder. The ^{1}H NMR spectrum of 3 revealed the presence of six methyl protons (4 doubles and 2 singlets). A signal at δ_H 3.20 indicated the proton germinal to a hydroxyl group at position 3, confirmed with the signal at δ_C 76.54. The presence of the exomethylene group was indicated by the signals at δ_H 4.67 and 4.72 (brs., 2H, 28) assigned for the exomethylene protons, confirmed by the presence of one exomethylene carbon at δ_C 105.9 and one olefinic quaternary carbon at δ_C 156.84. The cyclopropyl group was indicated by the the two signals at δ_H 0.29 and 0.51assigned for the two methylene protons of the cyclopropyl group. Through comparison of these data with the previously published data in the literature [21-24], compound 3 was identified as cycloeucalenol. Compound 5 was isolated as a white amorphous powder. The ^{1}H NMR spectrum of 5 revealed the presence of six methyl protons (1doublet, 5 singlets), a side chain terminating in an isopropyl group, which was indicated from the signals two olefinic protons at δ_H 4.70 and 4.83, and from the methyl signal at δ_H 1.62. It was confirmed by the presence of the exomethylene carbon and the olefinic quaternary carbon at δ_C 109.44 and 148.19, respectively. A secondary OH group at position 24 was indicated from the downfield allylic proton signal at δ_H 3.81. [23] Similar to compound 3, the presence of the cyclopropyl group was confirmed by the two signals at δ_H 0.30 and 0.50 assigned for H-19$_{a, b}$. By comparison of these data with the previously published data in the literature, compound 5 was identified as cycloart-25-en-3β, 24 diol. [25-27] Compound 6 was

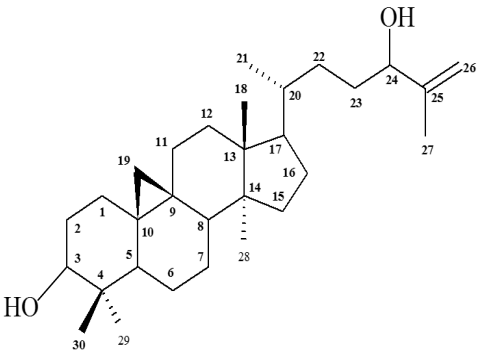
isolated as a white amorphous powder. The ^{1}H NMR spectrum of 6 showed signals due to seven methyl groups (6s and 1d). Similar to compound 3 and 5, the presence of the cyclopropyl group was confirmed by the two characteristic signals at δ_H 0.29 and 0.51 assigned for H-19$_{a, b}$. Also, the presence of a signal at δ_H 3.20 indicated a proton germinal to a hydroxyl group. Another signal at δ_H 4.15 indicated another proton attached to a carbon bearing another hydroxyl group. The presence of two olefinic protons was indicated by the broad multiplet at δ_H 5.50 and confirmed by the two signals at δ_C 123.04 and 140.58. The presence of a hydroxyl group at position 25 was evident from the presence of two methyl signals at δ_H 1.24 assigned for Me-26, 27, and confirmed by the signals at δ_C 68.62. Based on the NMR data of compound 6 and previously published data [28] compound 6 was identified as cycloart-23-en-3β, 22α, 25 triol.

Table 1: ^{13}CNMR of compounds 3, 5 and 6

Carbon No	Compound 3	Compound 5	Compound 6
1	30.76	31.45	31.36
2	34.78	30.10	30.40
3	76.54	76.69	76.68
4	44.57	40.06	40.34
5	43.31	46.63	47.11
6	24.64	21.95	20.48
7	25.13	28.62	28.21
8	46.81	47.32	48.23
9	23.54	20.59	19.19
10	29.66	25.97	25.78
11	26.96	25.82	27.35
12	32.87	32.43	35.77
13	45.33	44.68	44.67
14	48.88	48.24	49.23
15	35.32	35.27	32.29
16	28.07	26.50	25.40
17	52.19	51.69	51.19
18	17.74	17.66	18.83
19	27.18	29.21	29.95
20	36.09	35.33	36.07
21	18.32	18.80	18.28
22	35.01	31.36	76.53
23	31.30	28.97	123.04
24	156.84	74.49	140.58
25	33.92	148.19	68.62
26	21.84	109.44	30.04
27	21.96	17.44	29.01
28	105.90	19.18	25.40
29	18.10	14.06	19.19
30	14.36	25.55	14.0



Compound 3



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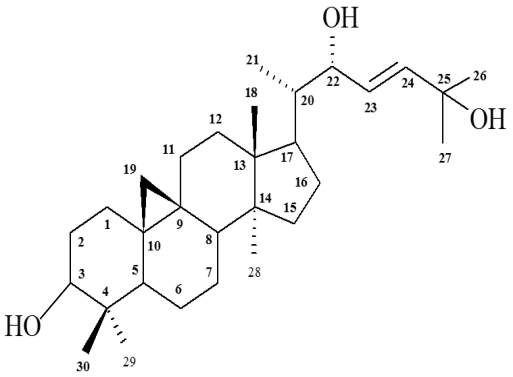


Compound 6

Fig 1: Structures of the isolated compounds (3, 5, 6)

HPLC analysis

The major detected phenolics are pyrogallol (447. 5 mg/100g) and catechin (173.1 mg/100g). Chlorogenic, salicylic, ellagic, ferulic, caffeic, p-coumaric and vanillic acids are detected as 56.8, 10.4, 9.5, 2.1, 1.25, 0.77, 0.73 mg/100g respectively. The detected flavonoids are hesperidin (2.93 mg/100g) and kaempferol (0.35 mg/100g).

Antioxidant activity

Results in Table 2 revealed that the level of glutathione in diabetic rats (21.9 ± 0.6) was restored after the oral administration of methanol and butanol extracts (35.2 ± 0.9&35.4 ± 1.2 respectively).

Table 2: Antioxidant activity of the methanol and n-butanol extracts of *T.coronaria*, flowers

Group	Blood glutathione (mg %)	
	Mean ± S.E.	% of difference from control
Control (saline)	36.2 ± 1.4	-
Diabetic non-treated	21.9 ± 0.6	39.50
Diabetic vitamin E.(35mg/Kg)	35.8 ± 1.1	1.10
Methanol ext.	35.2 ± 0.9	2.76
Butanol ext.	35.4 ± 1.2	2.21

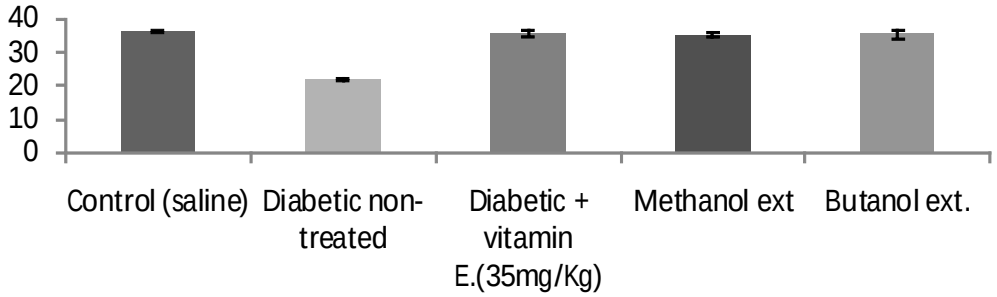


Fig 2: Effect of *T. coronaria*, flowers methanol and butanol extracts on serum glutathione in alloxan-induced diabetic rats.

Hepatoprotective activity (Tables 3-5)

The tested extracts reduced the elevated activities of serum AST, ALT and ALP in liver damaged rats comparable to that of reference drug, silymarin. The effect of both extracts is more or less similar. This finding may relate the hepatoprotective activity to the phenolic constituents extracted by both solvents.

Table 3: Effect of methanol and n-butanol extracts of *T.coronaria*, on the serum AST level in liver damaged rats

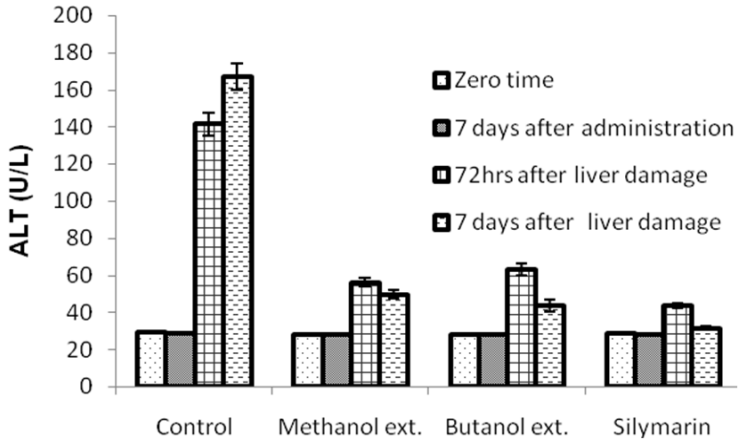
Group	AST (U/L)			
	Zero time	7 days after administration	72hrs after liver damage	7 days after liver damage
	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
Control	33.4±1.2	31.8±1.1	134.2±6.4	156.3±6.2
Methanol ext.	32.6±0.9	31.4±1.2	71.2±2.3	46.4±2.9
Butanol ext.	29.8±0.7	30.1±1.1	74.3±4.1	51.2±3.2
Silymarin	31.9±1.1	30.8±0.9	46.2±1.7	33.4±1.4

Table 4: Effects of methanol and butanol extracts of *T.coronaria*, the serum ALT level in liver damaged rats (n=6).

Group	ALT (U/L)			
	Zero time	7 days after administration	72hrs after liver damage	7 days after liver damage
	Mean±S.E	Mean±S.E.	Mean±S.E.	Mean±S.E.
Control	29.1±0.6	28.8±0.7	141.3±6.3	166.8±7.3
Methanol ext.	28.2±0.6	27.9±0.4	56.2±2.3	49.6±2.4
Butanol ext.	27.8±0.3	28.2±0.5	62.9±3.3	43.7±3.1
Silymarin	28.7±0.4	28.1±0.5	43.7±1.5	31.5±1.1

Table 5: Effects of methanol and n-butanol extracts of *T.coronaria*, Flowers on the serum ALP level in liver damaged rats (n=6).

Group	ALP (U/L)			
	Zero time	7 days after administration	72hrs after liver damage	7 days after liver damage
	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
Control	7.1±0.1	7.2±0.1	64.8±2.1	75.3±2.4
Methanol ext.	7.4±0.1	7.1±0.1	36.3±0.6	23.8±0.4
Butanol ext.	7.2±0.1	7.30.1	29.8±1.4	19.6±1.3
Silymarin	7.3±0.1	6.9±0.1	18.2±0.3	7.4±0.1



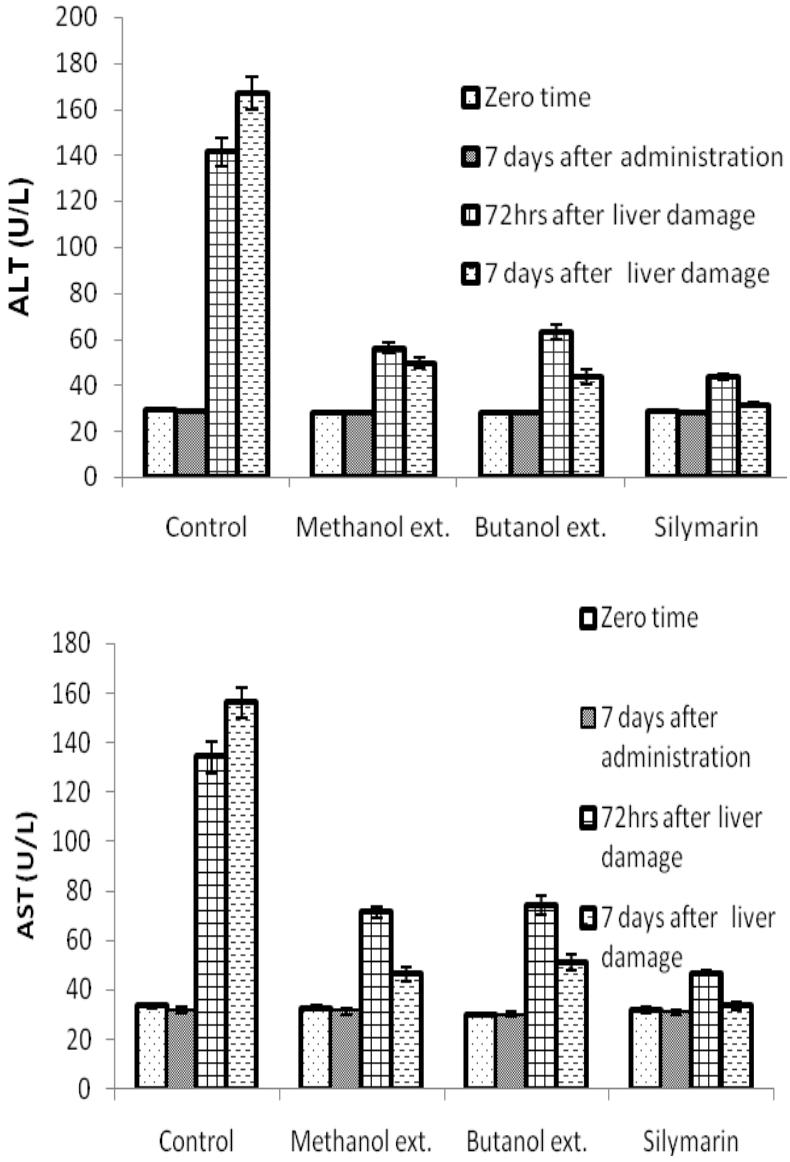


Fig 2: Effect of *T.coronaria*, flowers methanol and butanol extracts on the serum activity of ALT, AST and ALP in liver damaged rats.

Cytotoxic activity (Table 6)

The methanol extract and the n- hexane fraction recorded IC_{50} = 11.6 & 10.1 µg mL^{-1} and 10.9 & 15.7 µg mL^{-1}against breast and colon cancer cell lines respectively. Moreover, it was found that compound 5 possesses significant cytotoxic activity against both breast and colon carcinoma cell lines (IC_{50}= 4.28µg mL^{-1} and 4.88µg mL^{-1}, respectively). Also, compound 6 exhibited a remarkable cytotoxic activity against breast cancer cell line (IC_{50}= 3.83µg mL^{-1}), while it has a moderate cytotoxic activity against colon carcinoma cell line (IC_{50}= 14.9µg mL^{-1}). On the other hand, compound 3 possesses moderate activities against both breast and colon carcinoma cell lines (IC_{50}= 12.5µg mL^{-1} and 14.8µg mL^{-1}, respectively).

Based on a previous study [5] that proved the significant free radical scavenging activity of the plant, the present study evaluated the in vivo antioxidant activity and hepatoprotective potentials using animal models. Pretreatment with methanol and butanol fractions significantly ameliorate the altered enzymes, indicating that both extracts possess significant antioxidant and hepatoprotective potentials, similar effect of both extracts might be attributed to the presence and synergistic effects of phenolic and flavonoid compounds detected by HPLC, besides any other active compounds present. High content of pyrogallol and catechin may contribute as radical scavengers. [29-31] Furthermore, the protective effect of extracts is at least partly due to its antioxidant activity as evidenced by restoring the glutathione level. Recent reports proved that some Cycloartanol are inhibitors for kinases that play a role in cancer onset and progression. The present study led to isolation of cycloart-25-en-3β, 24 diol and cycloart-23-en-3β, 22α, 25 triol for the first time from *T.coronaria* flowers. Both compounds proved significant cytotoxic activity against breast and colon carcinoma cell lines. Cycloart-23-en-3β, 22α, 25 triol proved higher cytotoxicity against MCF7 cell line which may be attributed to free OH groups.[27] The present result considered the first report for cytotoxicity of cycloart-23-en-3β, 22α, 25 triol, furthermore it confirmed the previous reports of cycloart-25-en-3β, 24 diol cytotoxicity.[32-33]

Table 6: Cytotoxic activity of methanol, hexane extracts and isolated compounds 3, 5 and 6

Tested sample	IC_{50}	
	MCF7 cell line	HCT 116 cell line
Alcohol extract	11.6	10.1
Hexane fraction	10.9	15.7
Compound 3	12.5	14.8
Compound 5	4.28	4.88
Compound 6	3.83	14.9
Doxorubicin	2.97 ± 0.05	3.73 ± 0.21

Conclusion

Despite the moderate activity of the n-hexane and methanol fractions, compounds 5&6 revealed higher cytotoxicity which means that plant extracts may be subjected to chemical diversification of their components to increase the activity. Herein we report for the first time the cytotoxic potential of cycloart-23-en-3β, 22α, 25 triol on MCF7 cell line.

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Author Disclosure Statement

No competing financial interests exist

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