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Phytochemical investigation of the cytotoxic latex of *Euphorbia cooperi* N.E.Br.

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ABSTRACT

Background: Many investigations have been performed on the cytotoxic activity of different *Euphorbia* species and proved to possess moderate to strong cytotoxic effect on different human cancer cell lines. **Objective:** Current study aim is to determine the cytotoxic activity of the chloroform fraction derived from *Euphorbia cooperi* N. E. Br. latex methanolic extract on three human cancer cell lines, namely, breast cancer (MCF7), hepatocellular carcinoma (HepG2), and cervix cancer (HELA) cells in comparison to normal human melanocyte (HFB4) using Sulforhodamine B (SRB) assay. In addition, isolation and identification of the chemical constituents that might be responsible for the cytotoxic effect will be carried out. **Results:** The chloroform fraction showed potent cytotoxic activity against MCF7 cell line (IC_{50} = 4.23 ± 0.08 µg/ml), moderate activity against HepG2 (IC_{50} = 10.8 ± 0.74 µg/ml) and weak activity against HELA (IC_{50} = 26.6 ± 2.1 µg/ml) compared to standard doxorubicin (IC_{50} = 3.3 ± 0.1, 4.8 ± 0.14, 4.2 ± 0.3 µg/ml, respectively). The chloroform fraction indicated its possible selectivity against cancer cells rather than normal cells (IC_{50} = 15.6 ± 1.15 µg/ml on HFB4) compared to doxorubicin (IC_{50} = 4.0 ± 0.21 µg/ml on HFB4). A triterpene, euphol **(1)**, one steroid, obtusifoliol **(2)** together with one diterpenoid with tigliane skeleton, 12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate **(3)** were isolated for the first time from the chloroform fraction of the latex of the plant under investigation. The structures of the isolated compounds were established on the basis of physical and spectroscopic analysis, including 1D and 2D NMR experiments and by comparison with the literature data. **Conclusion:** The present study demonstrated that the chloroform fraction of the latex of *E. cooperi* N. E. Br. showed promising cytotoxic activity especially against breast cancer.

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INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and spreading further with continuance and increasing incidence in 21st century (Seffrin, 2000). The International Agency for Research on Cancer (IARC), had estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008 and predict a substantive increase to 19.3 million new cancer cases per year by 2025 (Jemal, 2012) .

Euphorbiaceae is a large family of the flowering plants, including 300 genera and over 10000 species (The plant list, 2013). The genus *Euphorbia* is the largest in the family, with diterpenoids and tritepenoids as characteristic secondary metabolites (Giner *et al*., 2000). It comprises about 2000 species, mostly those originating in Africa and Madagascar, are succulent (Jassbi, 2006). The Euphorbias contain white milky latex which has been used to treat cancers, tumors, and warts, from the time of Hippocrates (Fernandez-Arche *et al*., 2010). Modern studies have highlighted the wide spread use of several of these plants to treat cancerous conditions in the traditional medicine (Ravikanth *et al*., 2002).

Euphorbia cooperi N. E. Br. is native to Eastern South Africa, South Zimbabwe, Mozambique, and Zambia. It is a succulent spiny tree, up to 5m in height. *Euphorbia cooperi* N. E. Br. is planted as an ornamental tree in succulent gardens or rock gardens in South Africa and the United States (Coates Palgrave,1983 and Eggli, 2002). Reviewing the current literature, several studies have been found concerning the cytotoxic activity of several *Euphorbia* species (Abo and Evans, 1981; Baloch *et al*., 2006; Baloch and Baloch, 2010; Fatope *et al*., 1996; Lu, 2008; Shi *et al*., 2005). Reviewing the current literature, only one report was traced dealing with the isolation of a diterpene ester named 12-deoxyphorbol-16-isobutyrate-13-tigliate from the latex of *E. cooperi* N. E. Br. (Gundidza *et al*., 1992),

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However, no data was traced concerning the cytotoxic activity of the plant. Hence, the present paper deals with the isolation of major constituents and investigation of the cytotoxic activity of the chloroform fraction of the latex.

MATERIAL AND METHODS

General

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 400MHz spectrometer with standard pulse sequences, operating at 400 and 100 MHz, respectively using tetramethylsilane (TMS) as an internal standard. Column chromatography was carried out on silica gel 60 (70-230 mesh, Merck). TLC analyses were conducted on pre-coated silica gel 60 F_{254} plates (0.25 mm thickness, Merck). Localization of spots was detected by spraying with *p*-anisaldehyde followed by heating at 110 °C. HPLC analysis was performed on an Agilent 1200 Series HPLC coupled with a photodiode array detector (DAD). The column was an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm x 150 mm, 5 µm), was used at 30 °C. The mobile phase used A: water and B: methanol as gradient elution (0–20 min, 50% B; 20–30 min, 100% B) with a constant flow rate of 1.0 ml/min. All the solvents used were of HPLC grade.

Plant Material

The aerial parts of *Euphorbia cooperi* N. E. Br. (Euphorbiaceae) were collected from Toukh, Qalubia, Egypt in March 2012 and identified by Professor Ibrahium El-Garf, Department of Botany, Faculty of Science, Cairo University. The voucher specimen (No. 432015) is kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extraction and Isolation

The latex (500 ml) of *Euphorbia cooperi* N. E. Br. was collected from the aerial part (5 kg) by incision and was suspended in methanol (200 ml) then freeze. Evaporation of the solvent in vacuum gave a whitish, oily material (35 g). The residue was suspended in distilled water (200 ml) and extracted with chloroform (4 x 200 ml) yielding a chloroform fraction (11 g) residue. The chloroform fraction was subjected to flash silica gel column chromatography (8 x 25 cm) eluted with *n*-hexane (100%) through *n*-hexane-ethyl acetate mixtures to ethyl acetate (100%). Similar fractions were pooled together yielding, two crude fractions A and B. Fraction A (2 g) was further separated by repeated silica column chromatography (3 x 17 cm) using 100% *n*-hexane / *n*-hexane : ethyl acetate (70:30 v/v) and *n*-hexane : ethyl acetate (98:2 / 96:4 v/v) to obtain two subfractions. Subfraction I (200 mg) was purified on silica column (1 x 20 cm) using *n*-hexane : ethyl acetate (96:4 v/v) to afford compound **1** (25 mg) and subfraction II (100 mg) was chromatographed on silica column (1 x 20 cm) and eluted with *n*-hexane : ethyl acetate (98:2 / 96:4 v/v) to afford compound **2** (75 mg). Fraction B (1.5 g) was chromatographed on repeated silica column (3 x 17 cm) eluted with *n*-hexane : ethyl acetate (90:10 v/v) then *n*-hexane : ethyl acetate (80:20 v/v) and finally, HPLC (C18, 0–20 min, 50% B; 20–30 min, 100% B) to afford compound **3** (76 mg).

Spectral data

Euphol (1)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 5.12 (1H, t, *J*=6.8 Hz, H-24), 3.25 (1H, dd, *J* = 4.4, 11.6 Hz, H-3), 1.71 (3H, s, H-27), 1.63 (3H, s, H-26), 1.02 (3H, s, H-19), 0.97 (3H, s, H-28), 0.89 (3H, s, H-30), 0.88 (3H, d, *J* = 6.2 Hz, H-21), 0.82 (3H, s, H-29), 0.78 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 15.54 (C-29), 15.62 (C-18), 17.69 (C-27), 18.92 (C-21), 18.95 (C-6), 20.14 (C-20), 21.53 (C-11), 24.47 (C-30), 24.76 (C-23), 25.75 (C-26), 27.67 (C-7), 27.93 (C-2), 28.05 (C-28), 28.15 (C-16), 29.76 (C-15), 30.90 (C-12), 35.26 (C-22), 35.42 (C-20), 35.88 (C-1), 37.26 (C-10), 38.93 (C-4), 44.11 (C-13), 49.63 (C-17), 50.02 (C-14), 50.96 (C-5), 78.98 (C-3), 125.22 (C-24), 130.85 (C-25), 133.54 (C-8), 134.02 (C-9).

Obtusifoliol (2)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 4.57 (1H, brs, H-28a), 4.52 (1H, brs, H-28b), 2.96 (1H, dt, *J* = 4.9, 10.6 Hz, H-3), 0.89 (3H, d, *J* = 6.8 Hz, H-27), 0.87 (3H, d, *J* = 6.8 Hz, H-26), 0.86 (3H, d, *J* = 6.4 Hz, H-21), 0.82 (3H, s, H-19), 0.78 (3H, d, *J* = 6.4 Hz, H-29), 0.74 (3H, s, H-30), 0.56 (3H, s, H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 15.07 (C-29), 15.74 (C-18), 18.23 (C-21), 18.74 (C-19), 20.72 (C-6), 21.77 (C-27), 21.87 (C-11), 22.00 (C-26), 24.44 (C-30), 25.55 (C-12), 28.20 (C-7), 30.79 (C-23), 31.07 (C-2), 31.17 (C-15), 31.30 (C-16), 33.81 (C-25), 35.00 (C-1), 35.01 (C-22), 36.34 (C-10), 36.50 (C-20), 39.22 (C-4), 44.53 (C-13), 47.06 (C-5), 49.86 (C-14), 50.41 (C-17), 76.54 (C-3), 105.94 (C-28), 133.60 (C-8), 134.64 (C-9), 156.91 (C-24).

12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate (3)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 7.60 (1H, brs, H-1), 5.70 (1H, d, *J* = 4.8 Hz, H-7), 5.62 (s, 9-OH), 4.45 (2H, dd, *J* = 12.3, 7.3 Hz, H-20 a,b), 4.25 (1H, d, *J* = 11.4 Hz, H-16a), 4.05 (1H, d, *J* = 11.4 Hz, H-16b), 3.31 (1H, br.s, H-10), 3.07 (1H, t, *J* = 5.7 Hz, H-8), 2.52 (1H, d, *J* = 20.7 Hz, H-5a), 2.38 (1H, d, *J* = 19.1 Hz, H-5b), 2.11 (1H, br.s, H-12a), 1.99 (m, H-11), 1.79 (3H, br.s, H-19), 1.53 (1H, br.s, H-12b), 1.14 (1H, br.s, H-14), 1.19 (3H, s, H-17), 0.89 (3H, d, *J* = 6.3 Hz, H-18), Acetate: 2.05 (3H, s, H-2`), Isobutyl: 2.52 (1H, m, H-2``), 1.13 (3H, d, *J* = 6.9 Hz, H-3``), 1.14 (3H, br.s, H-4``), Angelyl: 6.09 (1H, q, *J* = 7.2 Hz, H-3```), 1.99 (3H, d, *J* = 7.2 Hz, H-

4```), 1.91 (3H, br.s, H-5```); ^{13}C NMR (100 MHz, $CDCl_3$) δ_c: 10.16 (C-19), 11.55 (C-17), 15.75 (C-4```), 18.45 (C-3``), 18.49 (C-4``), 18.58 (C-18), 20.63 (5```), 20.95 (C-2`), 26.43 (C-15), 31.09 (C-14), 31.65 (C-12), 34.22 (C-2``), 36.25 (C-11), 38.82 (C-8), 39.12 (C-5), 55.67 (C-10), 62.84 (C13), 69.35 (C-16), 69.48 (C-20), 73.55 (C-4), 76.01 (C-9), 127.84 (C-2```), 132.89 (C-7), 132.99 (C-2), 135.24 (C-6), 137.58 (C-3```), 161.20 (C-1), 168.00 (C-1```), 170.75 (C-1`), 178.99 (C-1``), 208.07 (C-3).

Cytotoxicity Study

Sulforhodamine-B (SRB) colorimetric assay was used to evaluate the *in vitro* cytotoxic activity (Vichai and Kirtikara, 2006). Three human cancer cell lines, namely, breast cancer (MCF7), hepatocellular carcinoma (HepG2) and cervix cancer (HELA) cells were used to test the cytotoxicity. Human normal melanocyte (HFB4) was used as normal nonmalignant cells to check the cytotoxic selectivity of the tested extract. Doxorubicin, a broad-spectrum anticancer drug, was used as the positive control. Cytotoxicity is expressed as the concentration of test drug kills cells by 50% (IC_{50}). All tests and analysis were run in triplicate and mean values recorded. The cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). The cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial sub-culturing.

RESULTS AND DISCUSSION

The results of the *in vitro* cytotoxicity study on the chloroform fraction (**Table 1**) revealed that it possessed potent cytotoxic activity against MCF7 cell line (IC_{50} = 4.23 ± 0.08 µg/ml), moderate activity against HepG2 cell line (IC_{50} = 10.8 ± 0.74 µg/ml) and weak activity against HELA cell line (IC_{50} = 26.6 ± 2.1 µg/ml) compared to doxorubicin (IC_{50} = 3.3 ± 0.1, 4.8 ± 0.14, 4.2 ± 0.3 µg/ml, respectively).

In the present study, doxorubicin, used for the treatment of a great variety of cancer diseases, was the standard. However, this drug not only demonstrated cytotoxicity against all tested cancer cell lines, but also on the human normal cell line. Due to its high toxicity on human normal cell lines, the continuous use of doxorubicin can cause major adverse effects such as cardiotoxicity, neutropenia and gastrointestinal toxicity (Khanna *et al*., 1998). The chloroform fraction indicated its possible selectivity against the cancer cells rather than the normal cells (IC_{50} = 15.6 ± 1.15 µg/ml on HFB4) compared to doxorubicin (IC_{50} = 4 .0 ± 0.21 µg/ml on HFB4).

The chromatographic separation of the chloroform fraction, led to the isolation of three compounds namely, euphol **(1)** (Zhang *et al*., 2012), obtusifoliol **(2)** (Shamsabadipour, 2013) and 12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate **(3)** (Popplewell, 2010) (**Fig. 1**). Identification of the isolated compounds was carried out on the basis of physical and spectroscopic analysis, including 1D and 2D NMR experiments and by comparison with the literature data.

Euphol (1)

It was obtained as white needle crystals, m.p. 116°C, soluble in chloroform; R_f = 0.37 (*n*-hexane:ethyl acetate = 9:1 v/v). It gave a positive salkowski's test indicating a triterpenoid and/or steroid skeleton. It gave a violet colour with *p*-anisaldehyde/sulphuric acid spray reagent. ^{1}H NMR spectrum showed seven singlet signals assigned for the protons of tertiary methyl groups δ_H 0.78 (H-18), 0.82 (H-29), 0.89 (H-30,), 0.97 (H-28), 1.02 (H-19) and a terminal isopropylidene group at δ_H 1.63 (H-26) and 1.70 (H-27). An oxymethylene proton δ_H 3.24 (dd, *J* = 4.7, 11.6 Hz) assigned to H-3, the large coupling constant of H-3 indicated that the hydroxyl group was oriented equatorially (*β*) at C-3 (Jiang *et al*., 1997). The ^{1}H NMR showed a sharp doublet signal (δ_H 0.88, *J* = 6.3 Hz) assigned to a secondary methyl group (H-21) indicated that **1** belonged to the euphane rather than the tirucallane series (Arai *et al*. 1989, Abe and Rohmer, 1994, Mamta *et al*., 2000). ^{1}H NMR and ^{13}C NMR exhibited signals for a trisubstituted double bond with olefinic signals assigned to δ_H 5.10 (brt, *J* = 6.3 Hz) at H-24 and δ_c 125.22 (C-24) and 130.85 (C-25). ^{13}C NMR spectrum showed presence of another double bond with quaternary carbon at δ_c 133.54 (C-8) and 134.02 (C-9). These data suggested that **1** had a tetracyclic triterpene alcohol skeleton possessing a $\Delta^{8,9}$ ene system and a C8-side chain containing an isopropylidene group. From the aforementioned results and by comparison with published data (Zhang *et al*., 2012), compound **1** could be identified as **euphol**.

Obtusifoliol (2)

It was obtained as white amorphous powder, soluble in chloroform; R_f = 0.44 (*n*-hexane:ethyl acetate, 9:1 v/v). It gave a positive salkowski's test indicating a triterpenoid and/or steroid skeleton. It gave a violet colour with *p*-anisaldehyde/sulphuric acid spray reagent. ^{1}H NMR spectrum showed three singlet methyls, δ_H 0.82 (H-19), 0.74 (H-30) and 0.56 (H-18) with a signal at δ_H 2.96 (dt, *J* = 4.9, 10.6Hz) assigened to H-3, indicating a hydroxyl group at C-3, along with one pair of olefinic protons at δ_H 4.57 (brs) and 4.52 (brs) assigned to H-28(a and b), related to exocyclic terminal methylene at C-28. ^{13}C NMR spectrum showed two signals at δ_c 133.60 and 134.64 corresponding to C-8, C-9 and other two signals at δ_c 105.94 and 156.91 assigned to C-28 and C-24 respectively, characteristic for an ergosta-8,24(28)-dien skeleton. A signal at δ_c 76.54 was characteristic for a hydroxylated C-3. Reviewing the above data and by comparison with published ones

(Shamsabadipour, 2013), compound **2** could be identified as **obtusifoliol**.

12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate (3)

It was isolated as colourless oil, soluble in chloroform; R_f = 0.55 (*n*-hexane:ethyl acetate, 7:3 v/v). The ^{1}H and ^{13}C NMR spectra showed typical signals of phorbol esters with tigliane-type diterpene skeleton (Marco *et al*., 1999). As the ^{1}H NMR displayed signals for three methyl groups δ_H 0.89 (d, *J* = 6.3 Hz, H-18), 1.19 (s, H-17), 1.79 (brs, H-19) and two olefinic protons δ_H 7.60 (brs, H-1) and 5.70 (d, *J* = 4.8 Hz, H-7). The broad singlet at δ_H 1.14 was attributed to H-14. A two oxymethylene groups were detected at δ_H 4.45 (2H, dd, *J* = 12.3 Hz, H-20a, b) and another one at δ_H 4.25 (1H, d, *J* = 11.4 Hz, H-16a) and 4.05 (1H, d, *J* = 11.4 Hz, H-16b). A large deshielded singlet at δ_H 5.62 that showed no correlation in the HSQC spectrum, was assigned to the hydroxyl group 9-OH. The appearance of two broad singlet centered at δ_H 2.11 (1H, brs, H-12a) and 1.53 (1H, brs, H-12b) indicated that compound **3** is an ester derivative of 12-deoxyphorbol (Miana *et al*., 1985).

The ^{13}C NMR spectrum showed the existence of an α, β-unsaturated ketone at δ_c 208.07, 161.20, 132.99 and one trisubstituted double bond at δ_c 135.24, 132.89 and five oxygenated carbons at δ_c 76.01, 73.55, 69.48, 69.35 and 62.84 (C-9, C4, C-20, C-16 and -C13, respectively).

Additionally, the presence of typical signals of acetyl, isobutyl and angelyl groups was observed. The position of these groups was determined as follow: Long range correlations observed in the HMBC spectrum between H-20 (δ_H 4.45) and acetyl carbonyl carbon signal at δ_c 170.7. Additionally, the same proton showed correlation with carbons at δ_c 132.89 (C-7) and 135.24 (C-6), which confirmed that the acetyl group is present on C-20.

The ^{13}C NMR spectrum showed an ester carbonyl (δ_c 168.00), an olefinic methine (δ_c 137.58), an olefinic quaternary carbon (δ_c 127.84), a strongly deshielded methylene (δ_c 69.35), and two methyl carbons (δ_c 15.75 and 20.63). The disappearance of one of the two methyl signals at C-15 normally appearing at δ_H (1.16-1.2) in the ^{1}H NMR spectrum indicated the esterification of one of them. An HMBC correlation of H-14 (δ_H 1.14) with the deshielded methylene carbon (δ_c 69.35, C-16) supported the esterification of one of the C-15 methyl groups. Moreover, HMBC correlations of both methylene proton signals (H-16a, b, δ_H 4.05 and 4.25) to the carbonyl of the ester group (C-1```: δ_c 168.00) provides further confirmation to the site of esterification. The structure of the unsaturated angelate ester functionality at C-15 was detected from series of HMBC correlations between the olefinic methane quartet (H-3```, δ_H 6.09) and the vinylic methyl carbon (C-4```, δ_c 15.75) and H-4``` (δ_H 1.99) to both C-3``` (δ_c 137.58) and the olefiic quaternary carbon C-2``` (δ_c 127.84). Finally, HMBC correlations between the methyl singlet H-5``` (δ_H 1.91) and both C-1``` (δ_c 168.00) and C-3``` (δ_c 137.58) confirmed the presence of angelate ester group.

A typical resonance for isobutyrate ester group [δ_H 2.52 m (1H), 1.13 d (3H), 1.14 d (3H); δ_c 178.99, 34.22, 18.45 and 18.49] were appeared in the spectrum. The location of the isobutyl group at C-13 was determined on the basis of the chemical shift value of C-13 (δ_c 62.84), which was in the usual range of 13-acyl-substituted phorbol esters (δ_c 62.7-63.6).

All tigliane diterpenoids discovered in nature up to now show a configuration H-8β, 9α-OH, H-10α and 13α-OR (Ma *et al*., 1997). Additionally, previous ^{1}H NMR data reported that the Δδ of the two H-5 signals in the case of a 4α-OH was larger than that of a 4β-OH. In case of compound 3, two unequivelant methylene protons appeared as two doublets at δ_H 2.52 (H5a, *J* = 20.7 Hz) and 2.38 (H5b, *J* = 19.1 Hz) and the $\Delta\delta_H$ was only 0.14 which agreed with C-4-OH being β (Ma *et al*., 1997). All of the above data are compatible with the structure of **3** being **12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate** (Popplewell, 2010).

Table 1: *In vitro* cytotoxic activity of the chloroform fraction of the latex of *Euphorbia cooperi* N. E. Br. against human cancer (MCF7, HepG2 and HELA) and non-cancer (HBF4) cell lines.

Extracts	IC_{50} values (ug/ml)			
	MCF7	HepG2	HELA	HFB4
Chloroform fraction of latex	4.23 ± 0.08	10.8 ± 0.74	26.6 ± 2.1	15.6 ± 1.15
Doxorubicin*	3.30 ± 0.10	4.8 ± 0.14	4.2 ± 0.3	4.0 ± 0.2

According to NCI, $IC_{50} \leq 20$ µg/mL is generally considered to be active (Boik, 2001).
Tabulated values are mean ± standard deviation (SD) of three replicates; Doxorubicin* was used as the reference compound.

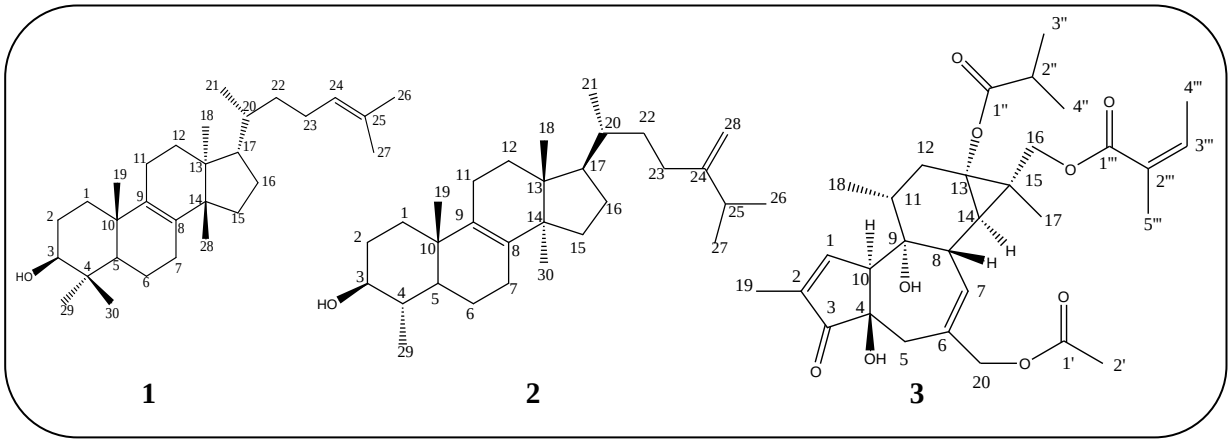

Fig. 1: Compounds isolated from the chloroform fraction of the latex of *Euphorbia cooperi* N. E. Br.

Conclusion

In conclusion, the chloroform fraction of the latex of *Euphorbia cooperi* N. E. Br. showed promising cytotoxic activity especially against breast cancer. Three known compounds, identified as euphol, obtusifoliol and 12-deoxyphorbol-13-isobutyrate-16-angelate-20-acetate were isolated. This study is considered as the first report for the isolation of these compounds from *Euphorbia cooperi* N. E. Br. which could be helpful and can contribute in the chemotaxonomic analysis of this complex genus.

The cytotoxic effect may be attributed to euphol which in accordance with previous reports concerning the cytotoxic activity of euphol (Mazoir *et al*., 2008; Zhang *et al*., 2012; Wang *et al*., 2013; Lin *et al*., 2012). A further investigation of the cytotoxic activity of the isolated compounds against the tested cell lines will be carried out.

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