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Isolation and identification of the primary toxin in the smoke of the Namibian milk bush, *Euphorbia damarana*

Mmankeko P. Degashu[a,b,*], J.J. Marion Meyer[a], Paul S.F. Alberts[a], Nicole L. Meyer[a], Monique Blignaut[a], Masixole Makhaba[c], Ahmed A. Hussein[c]

[a] Department of Plant and Soil Sciences, University of Pretoria, Pretoria 0002, South Africa
[b] Department of Pharmacology, University of Pretoria, Pretoria 0002, South Africa
[c] Department of Chemistry, Cape Peninsula University of Technology, Bellville 7535, South Africa

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ABSTRACT

Euphorbia damarana L.C.Leach, also known as Damara milk bush, grows only in the north-western desert areas of Namibia and southern Angola. It is often the only vegetation in these desert regions and the local inhabitants know very well that it is extremely poisonous to humans and animals. During the 1960s, twenty-seven migrating mineworkers used its dead branches as firewood for their barbecue with deadly consequences. They all passed away after consuming meat that was infused with toxic volatiles in the smoke. We report in this study, on the isolation of the toxic triterpenoid, euphol that is present in high concentrations in the stems and smoke of *E. damarana*. Small concentrations of compounds with phorbol ester skeletons were also detected in this species. The cytotoxicity of euphol in the smoke and stem extracts of *E. damarana* was determined by the sulfo-rhodamine-B stain (SRB) assay on eight human cell lines (A549, PC-3, HeLa, HepG2, MCF-7, MCF-12A, MRC-5 and HaCaT). Significant cytotoxic activity was observed from the purified euphol, stem and smoke extracts with IC_{50} values ranging from 1.99 to 3.99 μg/mL, 5.00 to 20.00 μg/mL and from 11.75 to 40.00 μg/mL respectively, on all the tested human cell lines. Since euphol is the primary compound (concentration 10.3 mg/g) in the smoke extract and toxic to all cell lines tested at a significantly low IC_{50} level, it can be considered as the main toxin in *E. damarana* and responsible for the deaths of the miners.

1. Introduction

Although plants are indispensable as a critical food source and provide many of our medicines, some of them are extremely poisonous and regularly cause human deaths. Numerous species within the genus *Euphorbia* L. are widely recognised for their poisonous nature. This fact was substantiated by El-Hawary et al. (2020) who conducted toxicity experiments on 15 *Euphorbia* species. The study revealed that all tested species demonstrated toxicity against human cell lines and reported significant cytotoxic IC_{50} values of 7.2 μg/mL and 9.1 μg/mL from *E. officinarum* L. and *E. royleana* Boiss. respectively, on the human colon adenocarcinoma (CACO2) cell line. The IC_{50} values of *E. lactea* Haw. against human hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (MCF-7) cell lines were 5.2 μg/mL and 5.1 μg/mL respectively. *E. grandialata* R.A.Dyer had an IC_{50} value of 7.5 μg/mL against MCF-7 and *E. obesa* Hook.f. IC_{50} value against HepG2 was 6.3 μg/mL. Moderate to low cytotoxic activity was reported with the other species.

Since all the *Euphorbia* milk bushes have not yet been studied for toxicity, it is probably safe to consider them all as being toxic. There have been several reports from newspapers and scientific journals (Meyer et al., 2020), as well as from people living in the areas where the Namibian Damara milk bush, *Euphorbia damarana* grows, that it is very toxic, but no scientific toxicology studies have yet been published on this species to confirm its toxicity. Several reports have been published of the traditional use of toxic *Euphorbia* spp. latex being used in arrow poisons by northern Namibian tribes like the San, Herero, Hei||om, Ju|wasi etc. (Bradfield et al., 2015). It should be noted that *E. damarana* was named and described for the first time only in 1975 (Leach, 1975) and was probably only referred to as *Euphorbia* milk bush previously. A recent study by Isaksson et al. (2023) on the poison residues from nineteenth and twentieth-century arrowheads collected from northern Namibia and the Kalahari showed that *Euphorbia* plants were the main ingredient of many of the samples analysed. Examples of *Euphorbia* latex found in arrowheads from Namibia were also presented by Schapera (1925), Watt and Breyer-Brandwijk (1962) and Bradfield et al. (2015).

E. damarana grows only in the north-western desert areas of Namibia and southern Angola (Fig. 1) and is often the only vegetation

* Corresponding author.
E-mail address: petunia.degashu@up.ac.za (M.P. Degashu).

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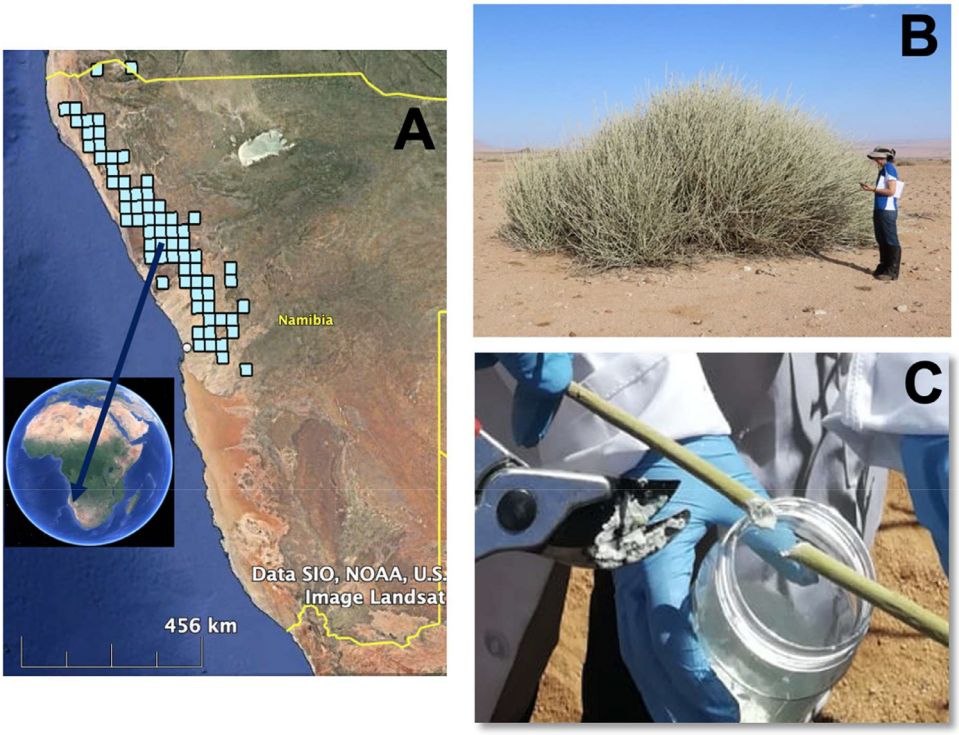


Fig. 1. Distribution map of *Euphorbia damarana* (A), an example of the species (B) and a stem showing the white milky latex (C) (Photographs: Marion Meyer).

one can find in these dry desert regions (Meyer et al., 2020). The local inhabitants know very well how extremely poisonous it is to humans, domesticated cattle and goats, as well as dogs and probably other animals.

It also grows naturally in the rustic, small, desert town Uis on the edge of the Namib Desert (Namibia). The town is well known for its tin (and recently discovered lithium) mines, which attract mineworkers from outside the region. Twenty-seven of these migrating mineworkers used the dead branches of the Damara milk bush as firewood for their barbecue in the 1960s with deadly consequences. They all passed away that same night because of the toxic volatiles in the smoke of the milk bush that infused their meat, and they were buried at the exact same location, next to a dead *E. damarana* (Fig. 2). This tragic incident was confirmed recently by one of the elderly inhabitants who was present when these events unfolded.

In this study we report on the toxicity of the stem and smoke extracts of *E. damarana* and the isolation of its main toxin, euphol by using column chromatography and Gas chromatography-mass spectrometry (GC–MS) and Nuclear magnetic resonance (NMR) analyses. Cytotoxicity bioassays were conducted on eight human cell cultures. Smoke was collected and extracted to identify the toxic compound(s) present, that most likely led to the tragic deaths of the 27 mineworkers.

Fig. 2. Graves of the 27 mineworkers who died in Uis, Namibia after consuming meat cooked on the dry stems of a dead *E. damarana* shrub. The dead plant is circled in red (Photographs:Marion Meyer).

2. Materials and methods

2.1. Plant material

Stems of *E. damarana* were randomly collected from mature plants growing on the Giribes Plains (S19°11.416′/ E13°17.557′) in Namibia during March 2018. A voucher specimen was prepared (PRU no: 122,229), identified and preserved at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria. Following plant collection and transport to the University of Pretoria, the stems were freeze-dried and stored at −20 °C in the dark.

2.2. Plant extraction

2.2.1. Stem extracts

The dried *E. damarana* stems (17 g) were cut into 10−15 cm long sections and placed in 40 mL stainless steel vessels (5 stems per vessel) for pressurised solvent extraction (PSE) in a speed extractor (Buchi E916, Switzerland). The following solvents (all from Merck (Pty) Ltd, South Africa) were used for extraction; hexane, ethyl acetate, acetone, methanol and distilled water (Elga LabWater US, Labotec, (Pty) Ltd, South Africa). The speed extractor's temperature was set at 50 °C and pressure at 100 bars. Following extraction, the crude extracts collected from the speed extractor were dried using a Genevac Series EZ-2 solvent evaporator (United Scientific (Pty) Ltd, South Africa). The crude stem extracts were stored in the dark at 4 °C until needed for further analysis.

2.2.2. Smoke extracts

A combustion chamber was used to collect smoke from burning dried *E. damarana* stems (Swart et al., 2024). The apparatus consisted of an airtight combustion chamber with controlled positive airflow supplied by a pressurised air cylinder, and a chamber that contained an electrical resistance coil to ignite dried material. The temperature and duration of the supplied heat were controlled by a current regulator and timer. The following four solvents were used for extraction in glass flasks; hexane, ethyl acetate, acetone and methanol (Fig. 3).

Dried *E. damarana* stems (3.0 g) were placed onto the electrical resistance coil to cover it completely. The combustion chamber was sealed and the pressurised air (approximately 78.08 % nitrogen, 20.95 % oxygen, 0.93 % argon, 0.04 % carbon dioxide) was allowed to flow from the gas cylinder through the air pressure regulator and into the underside of the combustion chamber. Air pressure was set between 3.0 and 4.0 × 1000 cm^3/min. A positive and constant airflow was established before the electrical current control and timer were switched on. The electrical current was set at the maximum (8.75 A) for the ignition phase and smoke was visible in the chamber after 20 s. After 1 min, the electrical current was switched off. The system was allowed to combust and produce smoke for another 9 min, the total burning time was 10 min. During the burning phase, smoke flowed from the combustion chamber and bubbled through a sequential solvent series of hexane, ethyl acetate, acetone, and finally methanol (70 mL each). Following extraction, the crude smoke extracts collected from the combustion chamber were dried carefully in a fume hood and stored in the dark at 4 °C until needed for further analysis.

2.2.3. Stem extraction for euphol isolation and identification

The dried *E. damarana* stems (800 g) were ground (IKA, United Scientific (Pty) Ltd, South Africa) to small pieces and shaken sequentially in acetone and methanol for three days on an orbital shaker (Labotec, (Pty) Ltd, South Africa), decanted and the process repeated twice. The filtrates were combined and concentrated to dryness at 40 °C using a rotary evaporator (Buchi, Germany). The extract was stored in the dark at 4 °C until needed for further analysis.

2.3. Chromatographic isolation and identification of euphol

2.3.1. Column chromatography

A glass column (50×8 cm) was packed with activated silica gel powder (Merck (Pty) Ltd, South Africa), 60 g of the extract was applied to the column and initially eluted with 100 % hexane and then with gradually increased polarity with ethyl acetate to 40 % ethyl acetate. The column was washed with 100 % acetone followed by 100 % methanol. A total of 195 fractions were collected from the silica gel column and similar fractions, as observed on the (Thin layer chromatography) TLC plates, were combined to obtain 33 subfractions. Subfraction 10A was further fractionated on a 5 % (silver nitrate) $AgNO_3$ impregnated silica gel column. A total of 32 fractions were collected and fractions 13B to 15B were combined and further fractionated on another 5 % $AgNO_3$ impregnated silica gel column. A total of 52 fractions were collected and fractions 29C to 34C were combined and further purified on a Sephadex LH-20 (Merck (Pty) Ltd, South Africa) column (40×2 cm). The column was eluted with 10 % methanol. A total of 36 fractions were collected and fraction 29D was analysed on NMR.

2.3.2. Thin layer chromatography (TLC)

Collected fractions were spotted on TLC plates (10×7 cm) pre-coated with silica gel 60 F254 (Merck (Pty) Ltd, South Africa) and

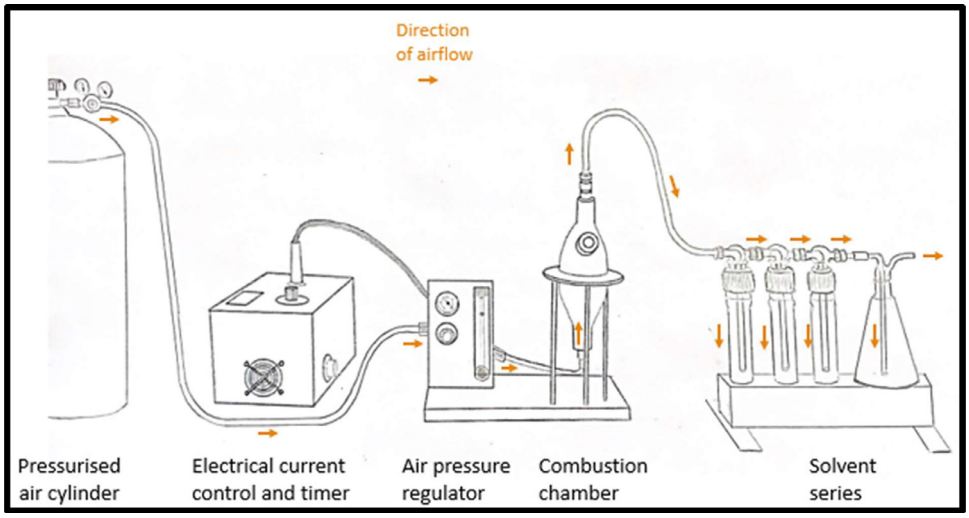


Fig. 3. Illustration of the smoke generating and capturing apparatus (Swart et al., 2024).

developed in hexane: acetone (90:10). Developed plates were visualised by UV light at 254 and 366 nm followed by heating after spraying with vanillin solution (250 mL ethanol, 5 mL sulphuric acid and 7.5 g vanillin powder) (Merck (Pty) Ltd, South Africa).

2.4. Characterisation of euphol

2.4.1. Gas chromatography-mass spectrometry (GC–MS)

The crude extracts were subjected to GC–MS for the identification and concentration determination of compounds using a Shimadzu QP2010 instrument (Shimadzu South Africa (Pty) Ltd) with a Rtxi-5MS column (30 m x 0.25 mm x 0.25 μm). Helium was used as the carrier gas and the temperature was programmed with an initial oven temperature of 80 °C, then held for 2 min at 200 °C and the final temperature was 300 °C and held for 5 min. 1 μL sample was injected in splitless mode, the ionisation potential set to 70 eV and a scan range of 50 to 550 amu used. The total runtime for a sample was 40 min. The GC–MS data was processed and analysed using MestReNova 11.0.1 (Mestrelab Research, Spain) and the database of the National Institute of Standards and Technology vers. 14 (NIST) was used for compound identification.

2.4.2. Nuclear magnetic resonance (NMR) spectrometry

NMR analysis was done on a Bruker Ascend 400 MHz spectrometer (Bruker, Germany). The compounds were analysed in 5 mm NMR tubes (5 mg/mL in $CDCl_3$) at 1 024 scans for ^{1}H NMR and 10 000 scans for ^{13}C NMR. The temperature was kept constant at 25 °C and all the spectra were referenced to the internal standard, manually phased and baseline corrected.

2.5. Human cell lines and culturing for cytotoxicity bioassay

The human cell lines HepG2 (liver cancer cells), HeLa (cervical cancer cells), A549 (lung cancer cells), PC-3 (prostate cancer cells), MCF-7 (breast cancer cells), MRC-5 (lung fibroblast cells), HaCat (keratinocytes cells) and MCF-12A (breast epithelial cells) were grown in culture flasks with Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, South Africa) and incubated at 37 °C in a humidified atmosphere under 5 % CO_2 in an incubator (Labotec, (Pty) Ltd, South Africa) for about 2 weeks. Subculturing was done every 2–3 days using phosphate buffer saline (PBS) (Merck (Pty) Ltd, South Africa). A seeding density of 1×10^4 cells/mL was prepared and 100 μL of the cell culture suspension was added to 96 well plates.

2.6. Cytotoxicity bioassay

The cytotoxic activity was determined in vitro using the sulforhodamine-B stain (SRB) (Merck (Pty) Ltd, South Africa) assay according to Skehan et al. (1990) and Vichai and Kirtikara (2006) with some modifications.

Stock solutions of the stem and smoke extracts (1 mg/mL) were prepared and dissolved in 0.2 % dimethylsulphoxide (DMSO) (2 μg/mL) (Merck (Pty) Ltd, South Africa). The stem extracts were tested at concentrations of 20.0 μg/mL to 0.625 μg/mL while smoke extracts were tested from 40.0 μg/mL to 1.25 μg/mL. Following 24 h incubation of the cell cultures, 100 μL of the samples, 100 μL of 1 % (10 μg/mL) saponin solution and 100 μL of cisplatin (positive control, Merck (Pty) Ltd, South Africa) and 100 μL of 0.2 % DMSO were added to the 96 well plates and incubated for 48 h at 37 °C under 5 % CO_2 and 95 % humidity.

Following 72 h incubation of the treated cell culture plates, 50 μL of a 10 % trichloroacetic acid (TCA) (Merck (Pty) Ltd, South Africa) solution was added to each well to fix the cells. Plates were then incubated at 4 °C overnight. After the incubation period the plates were washed three times under running water and dried in an oven at 30 °C. Once the plates have dried, 0.057 % SRB stain solution made up in 1 % acetic acid (100 μL) was added to each well and the plates incubated at room temperature for 30 min in the dark. The plates were washed three times to remove unbound SRB stain by adding 150 μl of a 1 % acetic acid solution to each well and the plates were then dried in an oven at 30 °C. 10 mM TRIS buffer (Merck (Pty) Ltd, South Africa) at pH 10.5 (200 μL) was added to each well. The plates were left on a shaker for approximately 1 hour to allow the dye to solubilise. The absorbance of the plates was determined using a spectrophotometer (BioTek ELx800, Analytical Solutions, South Africa) at 540 nm with a reference wavelength of 630 nm.

Data was blank-adjusted and expressed as the mean percentage of the average of the untreated controls using Microsoft Excel. The calculated results were used to generate a cell density curve (non-linear regression method, normalised-variable slope) to determine the IC_{50} with GraphPad Prism (GraphPad Software, Inc).

3. Results

3.1. Plant extraction

The highest yields of stem extract were with methanol and ethyl acetate, followed by acetone, then hexane, with water as the lowest. The yields of the four smoke extracts were very similar with a slightly higher yield obtained with methanol and ethyl acetate.

3.2. Phorbol esters

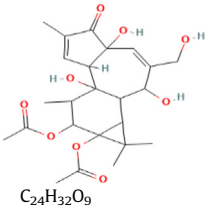
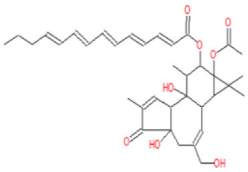
Since phorbol esters are often the major toxic compounds of *Euphorbia* species, this study set out to determine if they are also present in *E. damarana*. The presence of only small concentrations of two compounds in the stem and smoke extracts with phorbol ester skeletons, were indicated in the GC–MS analysis by the NIST database (Table 1). The NIST similarity index of these two compounds were notably low (50–60 %). The two phorbol esters were suggested to be 5h-cyclopropa-(3,4)-benz(1,2-e)azulen-5-one,9,9a-bis (acetyloxy)–1,1a,1b,2,4a,7a,7b,8,9,9a-decahydro-2,4a,7b-tri-hydroxy-3-(hydroxymethyl)–1,1,6,8-tetramethyl and 2,4,6,8,10-tetradeca-penta-enoic acid,9a-(acetyloxy)–1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxy-methyl)–1,1,6,8-tetramethyl-5-oxo-1H-cyclopropa[3,4]benz[1,2-e]az.

3.3. Euphol isolation and identification

Chemical characterisation of the triterpenoid, euphol (Fig. 4) was achieved by NMR (^{1}H, ^{13}C, and distortionless enhancement by polarisation transfer-135 (DEPT)), 2D-NMR experiments (correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC)), Fourier Transform Infrared Spectroscopy (FTIR) and MS, as well as comparison to the available literature data (Nyigo et al., 2016; Silva et al., 2018). The spectroscopic information of the isolated compound is as follows:

Euphol (fraction 29D of column chromatography) was isolated as clear crystals (7.5 mg). The ^{1}H NMR (400 MHz) spectrum showed several overlapping signals in the shielded region, which is a common trend observed in triterpenes (Silva et al., 2018). Nevertheless, the signals δ_H, that were identified include: 5.05 (1H, *brt*, 7.0, H-24), 3.20 (1H, *dd*, 11.6, 4.2, H-3), 1.64 (3H, *s*, H-27), 1.57 (3H, *s*, H-26), 1.49 (1H, *m*, H-17), 0.96 (3H, *s*, H-29), 0.91 (3H, *s*, H-19), 0.84 (3H, *s*, H-28), 0.82 (3H, *d*, 6.2, H-21), 0.76 (3H, *s*, H-18), and 0.71 (3H, *s*, H-30). The ^{13}C (100 MHz) and DEPT-135 NMR data showed thirty signals. The most apparent signals were the olefin carbons at δ_C 125.4 (C-24), 131.0 (C-25), 133.7 (C-9), and 134.2 (C-8). Very intense signals also appeared upfield on the ^{13}C NMR spectrum and were assigned to the eight methyl groups: δ_C 15.7 (C-18), 15.8 (C-30), 17.8 (C-26), 19.1 (C-21), 20.7 (C-19), 24.6 (C-28), 25.9 (C-27), 28.2 (C-29). In addition, the signal of the methine δ_C, which is characteristic of a carbon bearing a hydroxyl group (Nyigo et al., 2016), appeared at 79.2 (C-3).

Table 1
Compounds with phorbol ester skeletons in the methanol smoke extract as indicated by the NIST database during GC–MS analysis.

NIST suggested structure, molecular weight and chemical formula	Retention time (min.)	Similarity index (%)	Suggested similar compound	Area on chromato-gram (%)
 $C_{24}H_{32}O_9$ Molecular weight: 464.2	23:07	54.4	5H—Cyclopropa-(3,4)-benz(1,2-e)azulen-5-one, 9,9a-bis(acetyloxy)–1,1a,1b,2,4a,7a,7b,8,9,9a-decahydro-2,4a,7b-trihydroxy-3-(hydroxymethyl)–1,1,6,8-tetramethyl	1.06
 $C_{36}H_{46}O_8$ Molecular weight: 606.3	18.81	59.4	2,4,6,8,10-Tetradecapentaenoic acid, 9a-(acetyloxy)–1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)–1,1,6,8-tetramethyl-5-oxo-1H-cyclopropa(3,4)benz(1,2-e)az	1.22

3.4. Cytotoxicity of E. damarana extracts

The morphology and growth of cells appeared normal in the culture medium and the 0.2 % DMSO solution. *E. damarana* stem and smoke extracts exhibited very good cytotoxic activity against all tested human cell lines (Table 2). The hexane stem extract exhibited the highest cytotoxic activity, followed by acetone, methanol, ethyl acetate, and then water extracts. The overall IC_{50} values of organic solvent stem extracts on cancerous cell lines ranged between 5.53 and 20.00 μg/ml, while for the non-cancerous human cell lines it ranged between 5.16 and 20.00 μg/ml. The highest smoke cytotoxic activity was exhibited by the methanol extract, followed by the hexane, acetone and then ethyl acetate extracts. The overall IC_{50} values of smoke extracts against cancerous cell lines was between 11.75 and 40.00 μg/mL, while for the non-cancerous human cell lines it ranged between 20.00 and 40.00 μg/mL.

3.5. Cytotoxicity and concentration of euphol

The isolated euphol exhibited significant cytotoxic activity (Table 2) on all the tested cancerous human cell lines with the PC-3 cell line being the most sensitive to it, followed by the A549, MCF-7, HeLa and HepG2 cell lines. Very high toxicity was also observed on the non-cancerous cell lines (MRC-5, MCF-12F and HaCaT). The overall IC_{50} value for all cancerous human cell lines was between 1.99 and 3.77 μg/mL, while for the non-cancerous human cell lines it was between 2.76 and 3.99 μg/mL.

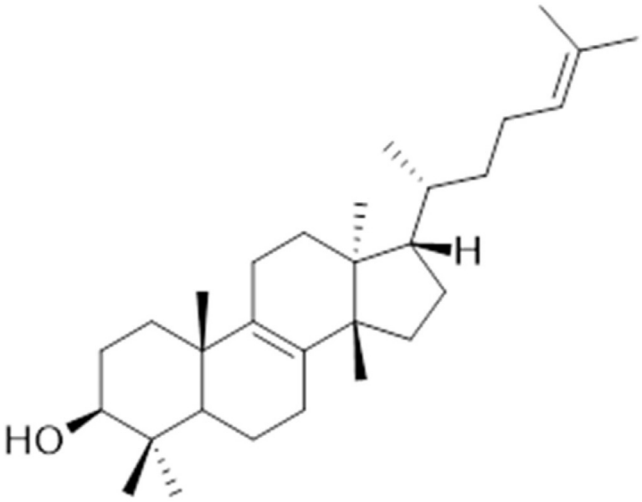


Fig. 4. Chemical structure of euphol.

The concentration of euphol in the different *E. damarana* extracts was determined by GC–MS analysis (Fig. 5 and Table 3). It was found that euphol is the major compound in the smoke extracts and present in high amounts (Table 3). By burning 3.0 g of *E. damarana* twigs for 10 min., 30.89 mg euphol was transferred in total to the smoke, a significantly high amount.

4. Discussion

Since the *E. damarana* compounds with phorbol ester skeletons identified by GC–MS analysis were present in such small quantities, they couldn't be isolated and their chemical structures be elucidated. They might contribute in a small degree to the toxicity of *E. damarana,* but this needs further investigation. Toxic phorbol esters were isolated and reported in other *Euphorbia* species such as in *Euphorbia fischeriana, Euphorbia frankiana, Euphorbia cocrulescence, Euphorbia ticulli* and *Euphorbia bothae* (Goel. et al., 2007; Popplewell et al., 2010; Wang et al., 2013a, Wang et al., 2015; Tostes et al., 2021) and possess beneficial effects e.g., being tumour inhibitors and having antileukemic activity (Fürstenberger and Hecker, 1986; Jassbi, 2006; Haba et al., 2007; Goel. et al., 2007; Baloch et al., 2008; Popplewell et al., 2010; Wang et al., 2015, 2020; Tostes et al., 2021). Exposure to these toxic compounds occurs during consumption of animal products polluted with toxic phorbol esters. These animal products may include honey collected from bees, meat contaminated by toxic plants during primitive hunting as well as meat and milk from animals that were fed feeds contaminated with the toxic plants (Haas et al., 2002; Silinsky and Searl, 2003; Goel. et al., 2007; Wang et al., 2015; Vega-Quirós et al., 2022).

The concentration of euphol present in *E damarana* smoke extracts was determined with GC—C-MS analysis. The highest concentration of euphol in the smoke extracts was found in the methanol extract and the lowest in ethyl acetate. Euphol was also reported as a major compound in other *Euphorbia* species (Gewali et al., 1990; Uchida et al., 2010; Cruz. et al., 2018; Silva et al., 2018; Athmouni et al., 2019; De Oliviera et al., 2021; Zhu et al., 2023). The current

Table 2
The percentage growth inhibition and log IC_{50} values of stem and smoke extracts, euphol and cisplatin (μg/mL) of *E. damarana* (mean of four replicates).

Solvent		A549	HeLa	HepG2	MCF-7	PC-3	MRC-5	HaCat	MCF-12A
Stem extracts									
Methanol	50 % inhibition	7.61	9.36	8.28	12.63	12.72	8.54	14.68	9.86
	Log IC_{50}	0.88	0.97	0.91	1.10	1.10	0.93	1.17	0.99
Acetone	50 % inhibition	7.07	8.08	5.00	12.42	12.68	5.16	8.29	4.47
	Log IC_{50}	0.84	0.91	0.70	1.09	1.10	0.71	0.92	0.65
Ethyl acetate	50 % inhibition	6.33	8.08	7.78	>20.00	9.65	5.77	9.15	5.43
	Log IC_{50}	0.80	0.91	0.89	>1.30	0.98	0.76	0.96	0.73
Hexane	50 % inhibition	5.53	7.17	6.74	8.54	8.31	5.77	8.29	4.07
	Log IC_{50}	0.74	0.86	0.83	0.93	0.91	0.76	0.92	0.60
Water	50 % inhibition	>20.00	>20.00	>20.00	>20.00	>20.00	18.48	>20.00	>20.00
	Log IC_{50}	>1.30	>1.30	>1.30	>1.30	>1.30	1.26	>1.30	>1.30
Smoke extracts									
Methanol	50 % inhibition	32.06	11.75	31.17	29.50	30.50	20.00	40.00	37.71
	Log IC_{50}	1.50	1.07	1.49	1.46	1.48	1.30	1.60	1.57
Acetone	50 % inhibition	33.81	16.00	33.39	34.11	40.00	27.40	>40.00	34.55
	Log IC_{50}	1.53	1.20	1.52	1.53	1.60	1.44	>1.60	1.54
Ethyl acetate	50 % inhibition	>40.00	37.69	>40.00	>40.00	>40.00	>40.00	>40.00	28.16
	Log IC_{50}	>1.60	1.58	>1.60	>1.60	>1.60	>1.60	>1.60	1.44
Hexane	50 % inhibition	33.86	14.00	31.17	32.58	39.88	40.00	>40.00	>40.00
	Log IC_{50}	1.53	1.14	1.49	1.51	1.60	1.60	>1.60	>1.60
Isolated euphol									
Euphol	50 % inhibition	2.14	2.22	3.77	2.33	1.99	2.76	3.99	3.38
	Log IC_{50}	0.33	0.34	0.58	0.37	0.29	0.44	0.60	0.53
Cisplatin (positive control)									
Cisplatin	50 % inhibition	1.04	1.00	1.90	1.90	0.62	0.31	2.07	0.31
	Log IC_{50}	0.02	0.00	0.28	0.28	- 0.21	- 0.51	0.32	- 0.51

study confirmed through GC–MS analysis that euphol is the major compound present in the smoke produced by burning *E. damarana*'s dried stems.

Based on the spectroscopic evidence provided herein, the identity of the isolated compound was confidently determined to be euphol. In addition, the experimental data is consistent with the previously published data (Nyigo et al., 2016; Silva et al., 2018). Euphol is an alcoholic tetracyclic triterpene (Fig. 4) with a wide range of pharmacological properties, including anticancer and anti-inflammatory activities (Dutra et al., 2011; Lin et al., 2012; Vuong et al., 2015; Silva et al., 2018; De Oliviera et al., 2021). Euphol was previously isolated from the following *Euphorbia* species: *E. antiquorum* L., (Mallavadhani et al., 2004; Akihisa et al., 2002) *E. broteroi* Daveau (Gewali et al., 1990; Lin et al., 2000), *E. kansui* Liou ex S.B.Ho (Lin et al., 2000; Zhang et al., 2012), *E. fusiformis* Buch.-Ham. ex D.Don. (Natarajan et al., 2005), *E. tirucalli* (Mallavadhani et al., 2004; Uchida et al., 2010), and *E. resinifera* O.Berg (Fattorusso et al., 2002). Studies of the bioactivity of euphol have proved it to have good anti-inflammatory, antinociceptive, antiarthritic, fibrinolytic and anticancer activity amongst others (Yasukawa et al., 2000; Dutra et al., 2011; Wang et al., 2013b; Cruz. et al., 2018; Silva et al., 2018; De Oliviera et al., 2021).

Significant cytotoxic activity to all the tested cancerous as well as non-cancerous human cell lines was found in this study as is reported above. Silva (2018) also reported the cytotoxic activity of euphol, isolated from the latex of *E. tirucalli* against seventy-three human cancerous cell lines with IC_{50} values for MCF-7, HeLa, PC-3 and A549 of 7.74 μg/mL, 7.48 μg/mL, 5.09 μg/mL and 4.69 μg/mL, respectively. No cytotoxic activity of euphol against PC-3 cell line was reported by Romero-Morán et al. (2022). Cruz et al. (2021), studied the *E. umbellata* latex and they reported no or poor cytotoxic activity against MCF-7 from the methanol extract with an IC_{50} value above 200 μg/mL while cytotoxic activity against MCF-7 cell line was reported with the hexane extract, ethyl acetate extract and euphol with an IC_{50} value of 48.21 μg/mL, 35.47 μg/mL and 43.80 μg/mL respectively.

E. damarana stem and smoke extracts exhibited significant cytotoxic activity against all tested human cell lines. The stem extracts were more toxic than the smoke extracts, possibly because of toxic compounds breaking down in the fire or not being volatile enough to be present in the smoke. Several authors reported the cytotoxicity of *Euphorbia* species with IC_{50} values ranging from 0.43 μg/mL to 135.00 μg/mL by *E. caducifolia* Haines, *E. peplus* L., *E. antiquorum, E. mauritanica* L., *E. milli* Des Moul., *E. prostrata* Burch. ex Hemsl., *E. prostrata* Burch. ex Hemsl., *E. tirucalli* on a wide range of human cell cultures (Zirihi et al., 2005; Bano et al., 2017; Al-Emam et al., 2019; Ruangnoo et al., 2017; Choene and Motadi, 2014; Chohan et al., 2020; Khiralla et al., 2016; Kwan et al., 2016; Wang et al., 2011; Benjamaa et al., 2022).

5. Conclusion

The IC_{50} values of all the tested crude plant stem extracts including the smoke extracts, have shown than the plant contains phytochemicals that display good anticancer properties but are equally toxic to the normal cell lines.

Since phorbol esters are commonly found in *Euphorbia* species and are toxic to humans and animals, one of the aims of this study was to determine if they are responsible for the toxicity of *E. damarana.* The GC–MS results obtained in this study has shown that two compounds with phorbol ester skeletons, present in very low concentrations, are present in this species. However, since they are present in such small quantities, they could not be isolated for conclusive structure elucidation.

The isolated major compound of *E. damarana* smoke extracts, irrespective of the solvent used to prepare the extract, was conclusively identified as the triterpenoid, euphol. The cytotoxicity analyses of the current study have shown that it has a very low IC_{50} value of below 5.0 μg/mL against all the cell lines tested. The burning of 3.0 g of dried *E. damarana* twigs resulted in 30.89 mg euphol being detected in the combined smoke extracts. However, the miners from Uis, Namibia would have used many more branches of this toxic milk bush as firewood for barbecuing their meat. Hence, their meat would likely have been exposed to a significantly higher concentration of euphol in the toxic *E. damarana* smoke, compared to the 30.89 mg detected in this

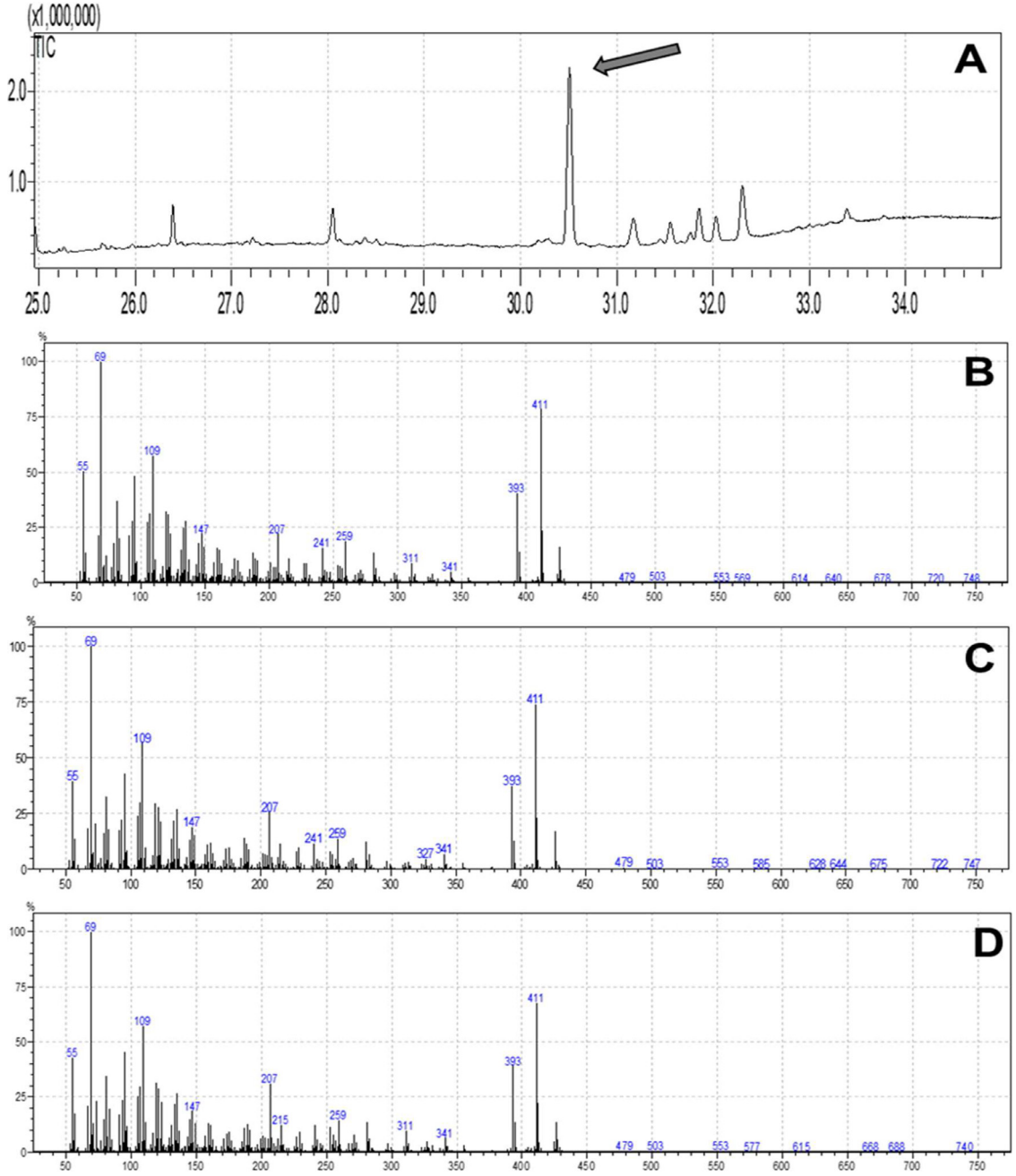


Fig. 5. A) GC–MS chromatogram of the methanol smoke extract showing the euphol peak (arrow) at rt of 30.5 min. Mass spectra of rt peaks at 30.5 min. of B) methanol smoke extract, C) methanol stem extract, D) isolated pure euphol.

Table 3
Euphol quantity and percentage per extract as determined by GC–MS analysis in smoke extracts generated by burning 3.0 g dried *E. damarana* stems for 10 min.

Solvent	Retention time (min.)	Mass of extracts (mg)	Mass of euphol in extracts (mg)	Euphol con-centration (%)
Hexane	30.52	50.0	6.32	12.65
Acetone	30.51	51.0	8.54	16.76
Ethyl acetate	30.51	52.0	4.59	8.83
Methanol	30.50	55.0	11.44	20.81

experimental study from burning only 3.0 g of twigs. This amount far exceeds the IC_{50} value of euphol of less than 5.0 μg/mL.

Hussain et al. (2010) stated that all compounds or extracts with an IC_{50} of below 10 μg/mL is considered as being very toxic. Since the IC_{50} values of the isolated pure euphol was less than 10 μg/mL against all tested human cell lines, it should be considered as being very toxic. It is therefore very likely that the barbecued meat of the 27 mine workers in Uis, Namibia, contained lethal amounts of euphol.

Declaration of Conflicting Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mmankeko P. Degashu: Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft. **J.J. Marion Meyer:** Conceptualization, Supervision, Validation, Writing – review & editing. **Paul S.F. Alberts:** Methodology, Software. **Nicole L. Meyer:** Project administration, Writing – review & editing. **Monique Blignaut:** Methodology, Software. **Masixole Makhaba:** Methodology, Software, Methodology, Software. **Ahmed A. Hussein:** Methodology, Software.

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Data availability

Data will be made available on request.

References

Akihisa, T., Kithsiri Wijeratne, E.M., Tokuda, H., Enjo, F., Toriumi, M., Kimura, Y., Koike, K., Nikaido, T., Tezuka, Y., Nishino, H., 2002. Eupha-7,9 (11),24-trien-3ß-ol ("Antiquol C") and other triterpenes from *Euphorbia antiquorum* latex and their inhibitory effects on epstein-barr virus activation. J. Nat. Prod. 65 (2), 158–162.

Al-Emam, A., Al-Shraim, M., Eid, R.A., Jamil, A.M., Moustafa, M.F., Radad, K., 2019. Cytotoxicity of *Euphorbia peplus* extract on MCF-7 breast cancer cells. Folia Biol. (Kraków) 67 (3), 127–139.

Athmouni, K., Feki, A.E., Ayadi, H., 2019. Hepatotoxic effects of Euphol-rich fractions from *Euphorbia bivonae* - Relevance to cytotoxic and antitumor activities. Pathophysiol 26 (1), 69–76.

Baloch, I.B., Baloch, M.K., Saqib, Q.N., 2008. Anti-tumor 12-deoxyphorbol esters from *Euphorbia cornigera*. Europ. J. Med. Chem. 43, 274–281.

Bano, S., Siddiqui, B.S., Farroq, A.D., Begum, S., Siddiqui, F., Kashif, M., 2017. In vitro growth inhibition and cytotoxicity of *Euphorbia caducifolia* against four human cancer cell lines and its phytochemical characterisation. Nat. Prod. Res. 31 (24), 2936–2940.

Benjamaa, R., Moujanni, A., Kaushik, N., Choi, E.H., Essamadi, A.K., Kaushil, N.K., 2022. *Euphorbia* species latex: a comprehensive review on phytochemistry and biological activities. Front. Plant Sci. 1–24.

Bradfield, J., Wadley, L., Lombard, M., 2015. Southern African arrow poison recipes, their ingredients and implications for Stone Age archaeology. Southern Afri. Hum. 27, 29–64.

Choene, M.S., Motadi, L.R., 2014. Abstract 3216: anti-proliferative properties of Euphorbia mauritanica medicinal plant against breast cancer. Am. Ass. Can. Res. 74.

Chohan, T.A., Sarfraz, M., Rehman, K., Muhammad, T., Ghori, M.U., Khan, K.M., Afzal, I., Akash, M.S.H., i, Alamgeer, Malik, A., Chohan, T.A, 2020. Phytochemical profiling, antioxidant and antiproliferation potential of *Euphorbia milii* var.: experimental analysis and in-silico validation. Saudi J. Biol. Sci. 27 (11), 3025–3034.

Cruz, L.S., de Oliveira, T.L., Kanunfre, C.C., Paludo, K.S., Minozzo, B.R., Prestes, A.P., Wang, M., Fernandes, D., dos Santos, F.A., Manda, V.K., Khan, S.I., Ali, Z., de Messias-Reason, I.J., Avula, B., Khan, I.A., Beltrame, F.L, 2018. Pharmacokinetics and cytotoxic study of euphol from *Euphorbia umbellata* (Bruyns) Pax latex. Phytomedicine 47, 105–112.

Cruz, L.S., Kanunfre, C.C., Khan, S., Khan, I., Beltrame, F.L., 2021. Fractions from *Euphorbia umbellata* (Pax) Bruyns sap are cytotoxic to breast and colorectal cells. World J. Pharm. Pharm. Sci. 10 (4), 1577–1585.

De Oliviera, T.L., Bavia, L., Fontana, P.D., Cruz, L.S., Paludo, K.S., Crisma, A.R., Messias-Reason, I.J., Beltrame, F.L., 2021. Immunomodulatory and cytotoxic activities of euphol. Life Sci 280, 1–9.

Dutra, R.C., Claudino, R.F., Bento, A.F., Marcon, R., Schmidt, E.C., Bouzon, Z.L., Pianowski, L.F., Calixto, J.B., 2011. Preventive and therapeutic euphol treatment attenuates experimental colitis in mice. PLoS ONE 6 (11), 1–15.

El-Hawary, S.S., Mohammed, R., Tawfike, A.F., Lithy, N.M., Abouzid, S.F., Amin, M.N., Abdelmohsen, U.R., Amin, E., 2020. Cytotoxic activity and metabolic profiling of fifteen *Euphorbia* species. Metabolites 11 (1), 1–15.

Fattorusso, E., Lanzotti, V., Taglialatela-Scafati, O., Tron, G.C., Appendino, G., 2002. Bis-norsesquiterpenoids from *Euphorbia resinifera* Berg. and an expeditious procedure to obtain resiniferatoxin from its fresh latex. European J. Org. Chem. 1, 71–78.

Fürstenberger, G., Hecker, E., 1986. On the active principles of the Euphorbiaceae, XII. Highly unsaturated irritant diterpene esters from *Euphorbia tirucalli* originating from Madagascar. J. Nat. Prod. 49 (3), 386–397.

Gewali, M.B., Hattori, M., Tezuka, Y., Kikuchi, T., Namba, T., 1990. Constituents of the latex of *Euphorbia antiquorum*. Phytochemistry 29 (5), 1625–1628.

Goel, G., Makkar, H.P.S., Francis, G., Becker, K., 2007. Phorbol esters: structure, biological and toxicity in animals. Int. J. Toxicol. 26, 279–288.

Haas, W., Sterk, H., Mittelbach, M., 2002. Novel 12-Deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J. Nat. Prod. 65, 1434–1440.

Haba, H., Lavaud, C., Harkat, H., Magid, A.A., Marcourt, L., Benkhaled, M., 2007. Diterpenoids and triterpenoids from *Euphorbia guyoniana*. Phytochemistry 68, 1255–1260 .

Hussain, A.I., Anwar, F., Nigam, P.S., Ashraf, M., Gilani, A.H., 2010. Seasonal variation in content, chemical composition and antimicrobial and cytotoxicity activities of essential oils from four *Mentha* species. J. Sci. Food Agric. 90 (11), 1827–1836.

Isaksson, S., Högberg, A., Lombard, M., Bradfield, J., 2023. Potential biomarkers for southern African hunter-gatherer arrow poisons applied to ethno-historical and archaeological samples. Sci. Rep. 13 (1), 1–16.

Jassbi, A.R., 2006. Chemistry and biological activity of secondary metabolites in *Euphorbia* from Iran. Phytochemistry 67, 1977–1984.

Khiralla, A., Mohamed, L.E., Tzanova, T., Schohn, H., Slezack-Deschaumes, S., Hehn, A., André, P., Carre, G., Spina, R., Lobstein, A., Yagi, S., Laurain-Mattar, D., 2016. Endophytic fungi associated with Sudanese medicinal plants show cytotoxic and antibiotic potential. FEMS Microbiol. Lett. 363 (11), 1–8.

Kwan, Y.P., Saito, T., Ibrahim, D., Al-Hassan, F.M.S., Oon, C.E., Chen, Y., Jothy, S.L., Kanwar, J.R., Sasidharan, S, 2016. Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by *Euphorbia hirta* in MCF-7 breast cancer cells. Pharm. Biol. 54 (7), 1223–1236.

Leach, L.C., 1975. *Euphorbia gummifera, E. gregaria* and a new species from Damaraland. Bothalia 11 (4), 495–503.

Lin, J., Ku, Y.R., Lin, Y.T., Teng, S.F., Wen, K.C., Liao, C.H., 2000. Preparative isolation and gas chromatography-mass spectrometry analysis of triterpenoids in *Kansui radix*. J. Food Drug Anal. 8 (4), 278–282.

Lin, M.W., Lin, A.S., Wu, D-C., Wang, S.S.W., Chang, F.R., Wu, Y.C., Huang, Y.B., 2012. Euphol from Euphorbia *tirucalli* selectively inhibits human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis. Food Chem. Toxicol. 50 (12), 4333–4339.

Mallavadhani, U.V., Satyanarayana, K.V.S., Mahapatra, A., Sudhakar, A.V.S., 2004. A new tetracyclic triterpene from the latex of *Euphorbia nerifolia*. Nat. Prod. Res. 18 (1), 33–37.

Meyer, J.J.M., Schutte, C.E., Hurter, J.W., Galt, N.S., Degashu, P., Breetzke, G., Baranenko, D., Meyer, N.L., 2020. The allelopathic, adhesive, hydrophobic and toxic latex of Euphorbia species is the cause of fairy circles investigated at several locations in Namibia. BMC Ecol. 20, 45.

Natarajan, D., Britto, S.J., Srinivasan, K., NagamurugaN, C., Mohanasundari, C., Perumal, C., 2005. Anti-bacterial activity of *Euphorbia fusiformis* – A rare medicinal herb. J. Ethnopharmacol. 123–126.

Nyigo, V.A., Peter, X., Fouche, G., Mabiki, F., Malebo, H.M., Mdegela, R.H., 2016. Isolation and identification of euphol and β-sitosterol from the dichloromethane extracts of *Synadenium glaucescens*. J. Phytopharmacol. 5 (3), 100–110.

Popplewell, W.L., Marais, E.A., Brand, L., Harvey, B.H., Davies-Coleman, M.T., 2010. Euphorbias of South Africa: two new phorbol esters from Euphorbia bothae. S. Afri. J. Chem. 63, 175–179.

Romero-Morán, L.J., Ramírez-Apan, M.T., Hernández-Ortega, H., Martínez-Otero, D., Delgado, G., 2022. Trisnor-Euphane-Type triterpenoid and other constituents isolated from Euphorbia tanquahuete Sessé & Moc.: preparation and cytotoxic evaluation of semisynthetic derivatives of Euphol. ACS Omega 7 (39), 35077–35082.

Ruangnoo, S., Kuropakornpong, P., Itharat, A., 2017. Cytotoxic activity of medicinal plants selected from Euphorbiaceae family against Hepatocellular carcinoma. Planta Med. Int. 4 (1) S1-S202.

Schapera, I., 1925. Bushmen arrow poisons. Bantu Stud. 2, 190–214.

Silinsky, E.M., Searl, T.J., 2003. Review: phorbol esters and neurotransmitter release: more than just protein kinase C? British J. Pharm. 138, 1191–1201.

Silva, V.A.O., Rosa, M.N., Tansini, A., Oliveira, R.J., Martinho, O., Lima, J.P., Pianowski, L.F., Reis, R.M., 2018. In vitro screening of cytotoxic activity of euphol from *Euphorbia tirucalli* on a large panel of human cancer-derived cell lines. Exp. Ther. Med. 16 (2), 557–566.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Nat. Can. Inst. 82, 1107–1112.

Swart, C., Yusuf, A.A., Weckerle, C.S., Stafford, G. (2024). Novel combustion apparatus and method to produce and capture plant-derived smoke for chemical and biological analysis. *In press.*

Tostes, J.B., Carvalho, A.L.D., Da Silva, A.J.R., Mourão, P.J.P., Rossi, A.D., Tanuri, A., Siani, A.C., 2021. Phorbol esters from the latex of *Euphorbia umbellata*: bioguided isolation of highly potent HIV-1 latency interrupters in virus reservoir cells. J. Nat. Prod. 84 (5), 1666–1670.

Uchida, H., Ohyama, K., Suzuki, M., Yamashita, H., Muranaka, T., Ohyama, K., 2010. Triterpenoid levels are reduced during *Euphorbia tirucalli* L. callus formation. Plant Biotechnol 27, 105–109.

Vega-Quirós, N., Arnáez-Serrano, E., Moreira-González, I.M., Muñoz-Arrieta, R., Borbón, L., Orozco-Ortiz, C., Guillermo Vargas-Hernández, G., Herrera, F., Araya-Valverde, E., 2022. Single nucleotide polymorphism (SNP) of *Jatropha curcas* associated with the content of phorbol ester. J. Plant Biochem. Biotechnol. 446–452.

Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat. Protoc. 1 (3), 1112–1116.

Vuong, Q.V., Nguyen, V.T., Thanh, D.T., Bhuyan, D.J., Goldsmith, C.D., Sadeqzadeh, E., Scarlett, C.J., Bowyer, M.C., 2015. Optimizing of ultrasound-assisted extraction conditions for euphol from the medicinal plant, *Euphorbia tirucalli*, using response surface methodology. Ind. Crops Prod. 63, 197–202.

Wang, H.B., Chen, W., Zhang, Y.Y., Wang, X.Y., Liu, L-P., Tong, L-J., Chen, Y., 2013a. Four new diterpenoids from the roots of *Euphorbia fischeriana*. Fitoterapia 91, 211–216.

Wang, J.F., Yang, S.H., Liu, Y.Q., Li, D-X., He, W.J., Zhang, X.X., Liu, Y.H., Zhou, X.J., 2015. Five new phorbol esters with cytotoxic and selective anti-inflammatory activities from *Croton tiglium*. Bioorg. Med. Chem. Lett. 25, 1986–1989.

Wang, L., Wang, G., Yang, D., Guo, X., XU, Y., Feng, B., Kang, J., 2013b. Euphol arrests breast cancer cells at the G1 phase through the modulation of cyclin D1, p21 and p27 expression. Mol. Med. Rep. 8 (4), 1279–1285.

Wang, X., Liu, L., Zhu, R., Kang, T., Tong, L., Xie, H., Wang, H., 2011. Cytotoxic activities of some selected medicinal plants of the genus *Euphorbia*. J. Med. Plants Res. 5, 6766–6769.

Wang, X.H., Liu, J.Q., Chen, S., Yanfeng, Y., Liu, Y., Zhang, C., 2020. Hydroxy-octadecenoic acids instead of phorbol esters are responsible for the *Jatropha curcas* kernel cake's toxicity. Commun. Bio. 3, 228.

Watt, J.M., Breyer-Brandwijk, M.G, 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa Being an Account of Their Medicinal and Other uses, Chemical composition, Pharmacological Effects and Toxicology in Man and Animal, 2nd Edition E. & S. Livingstone Ltd, United Kingdom.

Yasukawa, K., Akihisa, T., Yoshida, Z., Takido, M., 2000. Inhibitory effect of euphol, a triterpene alcohol from the roots of *Euphorbia kansui*, on tumour promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. J. Pharm. Pharmacol. 52, 119–124.

Zhang, L., Gao, L., Li, Z., Yan, Z., Yang, Y., Tang, Y., Cao, Y., Ding, A., 2012. Bio-guided isolation of the cytotoxic terpenoids from the roots of *Euphorbia kansui* against human normal cell lines L-O2 and GES-1. Int. J. Mol. Sci. 13, 11247–11259.

Zhu, H., Ren, X., Huang, Y., Su, T., Yang, L., 2023. Chemical constituents of *Euphorbia stracheyi* Boiss (Euphorbiaceae). Metabolites 13 (7), 852.

Zirihi, G.N., Mambu, L., Guédé-Guina, F., Bodo, B., Grellier, P., 2005. In vitro antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. J. Ethnopharmacol. 98 (3), 281–285.