Anthelmintic activity and non-cytotoxicity of phaeophorbide-a isolated from the leaf of *Spondias mombin* L.

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SHORT TITLE: Anthelmintic phaeophorbide-a from *Spondias mombin*

Abstract

Ethnopharmacological relevance:

Helminthosis (worm infection) is a disease of grazing livestock, with significant economic implications. Increasing resistance to existing synthetic anthelmintics used to control helminthosis and the unwanted presence of residues of the anthelmintics reported in meat and dairy products present a serious global health challenge. These challenges have necessitated the development of novel anthelmintics that could combat drug resistance and exhibit better safety profiles. *Spondias mombin* L. (Anacardiaceae) is a plant that has been used traditionally as a worm expeller.

Aim of study:

The aim of the work reported herein was to isolate and characterise anthelmintic compound(s) from *S. mombin* leaf, establishing their bioactivity and safety profile.

Materials and methods:

Adult *Haemonchus placei* motility assay was used to assess anthelmintic bioactivity. Bioassay-guided chromatographic fractionation of acetone extract of *S. mombin* leaf was carried out on a silica gel stationary phase. The structure of the compound was elucidated using spectroscopy (^{1}H and ^{13}C NMR) and Liquid Chromatography-Mass Spectrometry (LC-ESI-MS). Screening to exclude potential cytotoxicity against mammalian cells (H460, Caco-2, MC3T3-E1) was done using alamar blue (AB) and CellTitreGlo (CTG) viability reagents.

Results:

The acetone extract yielded an active fraction 8 (Ethyl acetate: methanol 90:10; anthelmintic LC_{50}: 3.97 mg/mL), which yielded an active sub-fraction (Ethyl acetate: Methanol 95:5; anthelmintic LC_{50}: 53.8 µg/mL), from which active compound **1** was isolated and identified as phaeophorbide-a (LC_{50}: 23.0 µg/mL or 38.8 µM). The compound was not toxic below 200 µM but weakly cytotoxic at 200 µM.

Conclusions:

Phaeophorbide-a **(1)** isolated from *S. mombin* leaf extract and reported in the plant for the first time in this species demonstrated anthelmintic activity. No significant toxicity to mammalian cells was observed. It therefore represents a novel anthelmintic pharmacophore as a potential lead for the development of novel anthelmintics.

Keywords*: Spondias mombin*; Medicinal Plants; Anthelmintics; Phaeophorbide a; Cytotoxicity

Spondias mombin

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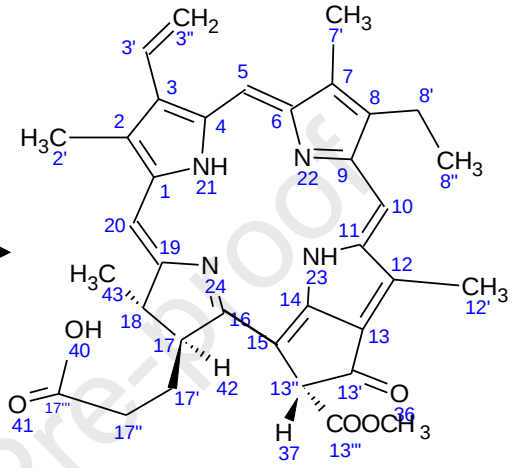
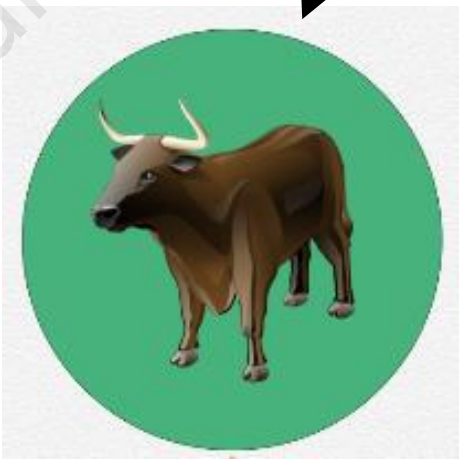


Phaeophorbide-a

Anthelmintic **AGAINST** *Haemonchus placei* worm in



1. Introduction

Helminthosis is a common and a serious disease of grazing livestock. It is an economically important disease because it usually leads to great losses in productivity for the livestock farmer (Soetan *et al*., 2011). Using Australia and Ethiopia as case studies for the devastation caused by parasitic infections, McLeod (1995) and Sackett *et al*. (2006) reported that the losses accrued by livestock farmers in Australia alone is estimated at 1 billion dollars annually, while Ethiopia as a major livestock producer in Africa records a loss of 81.8 million USD annually, due to parasitic diseases of livestock (Biffa *et al*., 2006).

The climate in most parts of Africa, including Nigeria, is naturally hot and humid, which encourages the infective larvae of *Strongyloides* sp. to thrive on grazing pasture, thereby infecting livestock almost all year round. Livestock farmers are, therefore, forced to rely on the frequent use of anthelmintics to control the infection in their cattle (Ademola *et al*., 2007). However, after decades of the use of synthetic anthelmintics, reports of resistance to these drugs started to come up, as early as in 1957 (Leland *et al*., 1957), with many other reports following subsequently (Drudge *et al*., 1964; Jambre *et al.*, 1979; Mejia *et al*., 2003; Garretson *et al*., 2009; Demeler *et al*., 2013). In spite of these reports, no new class of anthelmintic drugs had been developed as at 2007 (Brown-Beiser, 2007). Since then, monepantel (2009) and derquantel in combination with abamectin (2010) were introduced into the market. Drug resistance reports are already beginning to surface on monepantel (Mederos et al., 2014; Van den Brom et al., 2015). Therefore, the claim that multiple drug resistance to existing anthelmintics is a new reality (Kaplan and Vidyashankar, 2012) is a valid one. Anthelmintic drug residues are also being reported in meat and dairy products. Because of this, there is now an emphasis on organically produced meat and dairy products as alternatives to those produced by animals treated with synthetic drugs (El-Makaway *et al*., 2006; Cooper *et al*., 2012; Tsiboukis *et al*., 2013).

Phytomedicines are veritable alternatives to synthetic drugs, as their metabolites are generally considered safe if their extracts are not toxic. In veterinary medicine, long before the advent of modern synthetic medicines, plants have been used to treat animals, so the concept of phytotherapy in veterinary medicine is not new. One plant that has shown promise in the development of new anthelmintics is *Spondias mombin* (hog plum in English, "ijikara" in Igbo Language and "Iyeye" in Yoruba Language), a tall tree growing up to 30 metres in height, with yellow, pulp-like fruits. It belongs to the family Anacardiaceae and though native to tropical Americas, it has been naturalized in parts of Africa and Asia (Morton 1987; Burkill 1995).

Kerharo and Bouquet in 1947 first reported the indigenous use of *S. mombin* as an anthelmintic remedy in Ivory Coast. According to their report, the dry powdered leaves of *S. mombin* boiled with citron (*Citrus medica*) was an effective traditional remedy for helminthosis in both humans and animals (Kerharo and Bouquet, 1947). Ademola *et al.* (2005) investigated the efficacy of *S. mombin* leaf extracts *in vivo* and *in vitro* against gastrointestinal parasites of sheep. *In vitro*, they found the ethanolic and aqueous extracts of the leaf to be effective against the *Haemonchus contortus* worm (Aq. extract LC_{50}: 0.907 mg/mL; ethanolic extract LC_{50}: 0.456 mg/mL). *In vivo*, sheep with a naturally acquired mixed gastrointestinal nematode infection (the *Haemonchus* spp. included), were drenched with doses of 125, 250 and 500 mg/kg body weight of the ethanolic *S. mombin* extract. The group administered the highest dose of the extract had a mean *Haemonchus* spp. egg reduction of 11.6%; this was a milder level of bioactivity compared to effects against the other *Strongyloides* spp. studied. Their study has inspired and underpinned further investigation into the anthelmintic potential of *S. mombin*.

Other studies have gone on to show that the plant possesses anthelmintic activity. Gbolade et al. (2008) showed that *S. mombin* leaf extracts possessed *in vitro* activity against earthworms, while Rahman and Pay (2014) reported that its leaves and fruits showed activity against Trichostrongylid nematodes in goats. It was also reported to have activity against *Strongyloides venezuelensis*, a parasitic infection of rodents (Bastos *et al*., 2017).

While these studies have demonstrated the anthelmintic properties of the *S. mombin* extracts, they did not identify the bioactive compounds underpinning the activity. Our study therefore advances current knowledge by seeking to identify active compounds that may be responsible for the observed anthelmintic activity of the plant. We used a biological assay to guide the isolation of anthelmintic compounds from *S. mombin* leaf extract and thereafter evaluated whether those compounds would be safe to use by screening them for potential cytotoxicity against mammalian cell lines.

2. Materials and methods

2.1 Chemicals and reagents

n-hexane, ethyl acetate, acetone (analytical grade) and methanol (HPLC grade) (Sigma-Aldrich, UK); methanol and chloroform (BDH, England); sodium chloride and potassium dihydrogen phosphate (Kermel); TLC grade silica gel Kieselgel 60 G (Merck, Germany), Polysorbate 80 (pharmaceutical grade, Viswaat Chemicals Ltd., India, Batch number 0651/11); silica gel CT without additives (Reeve Angel Scientific - chromatographically tested materials); deuterated chloroform (Cambridge Isotope Laboratories Inc., MA, USA); compressed nitrogen gas (BOC, Linde Group, UN1066); CellTitreGlo viability reagent (Promega Incorporation, WI, USA), MC3T3 cell line (from existing stock in the lab); Caco-2 cell line (ECACC, UK); H460 cell line (a kind gift from the Institute of Therapeutics, University of Bradford, UK); Dulbecco's Minimum Essential Medium (DMEM), Foetal Bovine Serum (FBS), L-glutamine, Non-Essential Amino Acids (NEAA), Phosphate-Buffered Saline (PBS), trypsin (TrypLE™ Express), antibiotic-antimycotic solution (anti-anti), and Alamar Blue viability reagent (Thermo Fisher Scientific MA, USA); Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich, UK.

2.2. Plant material

The leaves of *S. mombin* were collected from the Botanical Garden, University of Ibadan, Nigeria, in August 2010. It was identified at the Forest Research Institute Nigeria's (FRIN) Herbarium, Idi-Ishin, Ibadan, Oyo state, Nigeria, by Omolola Ajayi, the curator in charge. A voucher specimen (number: FHI 108820) was deposited at the FRIN where it was identified.

2.3. Extraction, bioactivity-guided fractionation and isolation

S. mombin leaves (4 kg) were air-dried under shade at room temperature (up to 30°C) for 3 weeks and then ground to a fine powder. This plant material was exhaustively and successively extracted, first by n-hexane, then by acetone using a soxhlet extractor. The resulting acetone extract was dried under vacuum to give an extract weighing 150 g. This extract was stored in a -80°C freezer until needed. The plant

residue left from the acetone extraction was macerated at room temperature for 24 h with distilled water, and then lyophilized to obtain 30 g of aqueous extract.

The acetone extract (150 g), found to be the most active extract, was fractionated using Vacuum Liquid Chromatography (VLC) and 1.2 g of the active fraction was accumulated after several runs of the VLC. The stationary phase was TLC grade silica gel Kieselgel 60 G, and mobile phase mixtures of *n* hexane, ethyl acetate and methanol, with increments of 10%, were used for elution, i.e., to *n* hexane was added ethyl acetate in increments of 10%, then methanol was added to ethyl acetate also in increments of 10%. For the first stage of the VLC, the extract-to-sorbent ratio was 1:50. Twenty-one fractions were generated from this first stage of chromatography and after TLC, 10 pooled fractions were obtained. Each fraction was screened using an assay that was a modification of the Sharma *et al*. (1971) adult worm motility assay (described in a later section) and, based on its activity, the eighth fraction was selected for further chromatography.

A second stage of chromatography was carried out with the same stationary phase as above and an analyte-to-sorbent ratio of 1:70, using a long column (length 100 cm x diameter 3 cm) with medium pressure liquid chromatography. Fraction 8 (0.5 g) was applied to a column loaded with 70 g of silica gel, with an isocratic elution of 100% ethyl acetate (1 L) to yield 10 sub-fractions, with fraction 2 (200 mL) being the fraction containing an impure mixture of compound **1**. The same procedure was repeated to accumulate a purer sub-fraction containing mainly compound **1** with traces of impurities present.

A final chromatographic clean-up of this sub-fraction was carried out using another column (length 75 cm, diameter 2.9 cm), positive pressure and an isocratic mobile phase of 100% ethyl acetate (1 L). 105 g of the same stationary phase as above was loaded onto this column and the impure mixture of compound **1** (0.2 g) was chromatographed on the column as quickly as possible. The first greenish-black band (eluted out as the 2nd fraction; 50 mL) contained the active compound **1** (60 mg), after about 4 h. The compound

was dried *in vacuo*, flushed with nitrogen gas and stored in amber bottles under nitrogen at -80˚C. All chromatographic steps were carried out in a dim room and at cool temperatures (< 16˚C) because compound **1** was observed to be light-sensitive.

2.4.Anthelminthic screening

Stock solutions of fraction 8, the impure mixture of compound **1**, and the pure compound **1** were prepared by diluting them with 20% polysorbate-80 in 0.9% sodium chloride solution. Appropriate serial dilutions were made from the stock solutions in standard flasks and transferred to 24 well plates and these were used for the assay. The assay was a modified adult worm motility test assay based on the Sharma *et al.* (1971) assay. Briefly, test solutions of various concentrations were prepared in duplicate and ten *H. placei* worms (isolated from the abomasa of infected cattle) were incubated in duplicate per test concentration for 3 h; there after the worms were washed and incubated again in warm PBS, pH 7.4, at 38.3˚C for 30 min to revive any paralyzed worms. The worms were then examined for death and paralysis. Two separate determinations were done, with levamisole hydrochloride used as the positive control and the diluent as the negative control.

2.5. Characterisation of the isolated compound

Proton Nuclear Magnetic Resonance (NMR) spectra were recorded for the isolated compound using a Bruker DPX 400MHz Spectrometer, while the ^{13}C NMR spectra were recorded for the isolated compound using a JEOL 600MHz DELTA_2 NMR Spectrometer. The compound was dissolved in $CDCl_3$ for the NMR experiments. Electro Spray Ionisation Mass Spectrometry (ESI-MS) was recorded for the isolated compound using a Quattro Ultima Triple Quad Mass Spectrometer (coupled to an HPLC system) and run in the positive mode. The compound was dissolved in methanol and ammonium acetate was added to remove sodium adducts formed by the compound. The sample was further diluted with methanol and run through a Harvard syringe pump at a rate of 10 µL/min through the HPLC into the mass spectrometer.

Infra-red (IR) spectroscopy was carried out using an Attenuated Total Reflectance PerkinElmer Infra-Red Spectrometer (version 10.4.00). A solution of compound **1** was made in chloroform and a film spread across the universal ATR plate allowed to dry, before recording its spectrum.

2.6. Cell culture

Cultures of the human lung cancer cell line (H460), the human epithelial colorectal adenocarcinoma cell line (Caco-2), and the murine osteoblastic cell line (MC3T3-E1) were used in this study, grown using standard media (DMEM or RPMI, supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, 1% antibiotic-antimycotic solution (anti-anti) and, for H460 cells, 1mM sodium pyruvate) and cell culturing techniques as previously described (Fatokun *et al*., 2013; Taiwo *et al*., 2016; Taiwo *et al*., 2017).

2.6.1. Toxicity screening: compound preparation and determination of cell viability

A 50 mg/ml DMSO stock solution of the compound was prepared, from which the following tested serial dilutions were prepared using the growth medium: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.781 (µg/mL). Hydrogen peroxide (H_2O_2), a cytotoxic agent, was used as positive control, at 2, 20 and 200 µM (or at 5, 10 and 20 µM, where indicated). The highest percentage of DMSO to which the cells were exposed was 0.4% (the % of DMSO in the 200 µg/mL concentration of the compound), which was confirmed to be non-toxic to the cells. Cultured cells were examined under the microscope and once any microbial or fungal contamination was excluded the cultures were exposed to the different concentrations of the compound, prepared as earlier described, for 48 h. Separate cultures were also exposed to hydrogen peroxide. Each treatment was done in triplicate.

Following 48 h treatment, the viability of the cultures was determined using two different viability assays: the alamar blue (AB) assay, which is based on fluorescence (black plates used), and the CellTitreGlo (CTG) assay, which is based on luminescence (white plates used). The use of complementary viability

assays relying on different readouts enables the detection of any artefact that might be due to potential confounding factors such as interactions of a test agent with the viability reagent. Assessment of viability was carried out as previously reported ((Fatokun *et al*., 2013; Taiwo *et al*., 2016; Taiwo *et al*., 2017). Briefly, pre-warmed alamar blue was added to treated cultures in black 96-well plates at 10% v/v and the plates were incubated for 3 h at 37°C. The cultures were then left at room temperature for 20 min, after which fluorescence in the wells was determined using a FlexStation 3 plate reader (Molecular Devices, CA, USA) at an excitation wavelength of 530 nm (544 nm used) and an emission wavelength of 590 nm (Fatokun *et al*., 2013).

For the CTG assay, the white 96-well plates were removed from the incubator, following treatments, and allowed to cool down at room temperature for 30 min. Re-constituted CTG reagent was then added to each well at a 1:1 (volume) ratio. Plates were put on an orbital shaker for 2 min to ensure complete lysis of the cells. They were then left at room temperature for 10 min to stabilize the luminescence signal, which was later read on the FlexStation 3 plate reader (Fatokun *et al*., 2013).

2.6.2. Assessment of morphological changes

To assess the corresponding effects of the treatments on the morphology of cells, a Nikon Eclipse TS100 inverted microscope was used. Brightfield images were acquired for control cultures treated with growth medium only and for cultures treated with concentrations of the compound or peroxide.

2.6.3. Data analysis and statistics

For the anthelmintic assay, values were expressed as Mean number of dead worms ± SD. Data analysis was performed using GraphPad Prism 5 (GraphPad Inc., CA, USA), with which sigmoidal (variable slope) equation was fitted to the adult worm mortality data with appropriate mathematical constraints (0

for 'no worms dead' and 100 for 'all worms dead,' plotted on the Y-axis), plotting the log of concentration on the X-axis and the mean number of dead worms on the Y-axis. The LC_{50} values were thereafter computed with associated uncertainty at a 95% confidence interval (Ogedengbe *et al*., 2012; Aderibigbe *et al*., 2012; Ogedengbe *et al.*, 2019). For the cytotoxicity experiments, data analysis was carried out by taking the averages of fluorescence or luminescence values for each set of treatments. These averages were then converted to a percentage, with the average for the control wells (that received only DMSO) taken as 100% and every other average (in each of the wells treated with different concentrations of each compound) expressed as a proportion of this percentage value. Statistical significance of treatments was assessed on GraphPad Prism (Version 8) using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test (each treatment compared to the control). Differences between means were considered statistically significant at $P<0.05$.

3. Results

3.1. Bioassay-guided isolation and anthelmintic screening

When tested earlier in the adult worm motility assay we developed and optimised (Ogedengbe et al., 2019), the hexane (AH-H01), acetone (AH-A02) and aqueous (AH-W03) leaf extracts elicited anthelmintic effects, with LC_{50} values of 104.2 mg/ml, 30.6 mg/ml and 56.3 mg/ml, respectively, at 95% Confidence Intervals (CIs). The wide CI for the LC_{50} of the hexane extract was due to the fact that the extract was very difficult to dissolve so its higher concentrations could not be prepared and tested. As the acetone extract was the most potent, it was chosen for the subsequent fractionation reported in this paper. The bioassay-guided chromatographic fractionation of the acetone extract yielded an active fraction (fraction 8, LC_{50} 3.97 mg/mL, Fig. 1), which on further purification and biological screening yielded an impure mixture of compound **1** (LC_{50} 53.8 µg/mL, Fig. 1) and finally the pure compound **1** (LC_{50} 23.0 µg/mL (38.8 µM), Fig. 1), following a final chromatographic step. The LC_{50} of the positive control,

levamisole hydrochloride, was earlier reported (Ogedengbe et al., 2019) as 8.9 ng/ml (37.1 nM, indicated on Fig. 1).

	Fraction 8 (µg/ml)	An impure sample of 1 (µg/ml)	Compound 1 (µg/ml)	Levamisole hydrochloride (ng/ml)
LC_{50}	3970	53.8	23.0 (38.8 µM)	8.9 (37.1 nM)
CI (95%)	3380-4660	49.6-58.5	18.7 - 28.3 (31.6 - 47.8 µM)	7.05 - 11.3 (29.3 - 46.9 nM)

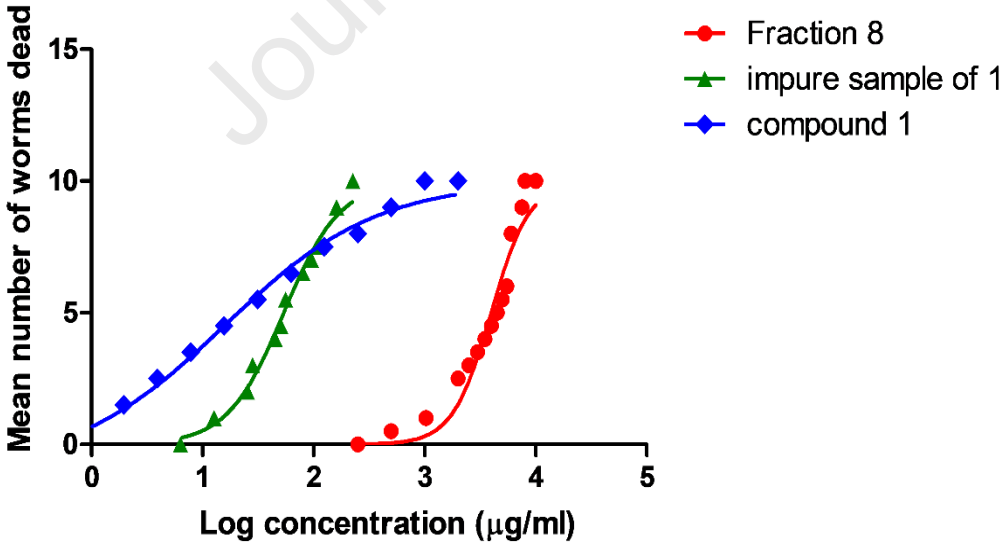


Fig. 1. Concentration-effect plot of the anthelmintic activities of fraction 8, an impure sample of compound 1 and compound 1 against *H. placei* in an adult worm motility assay, with the table above

the plot showing the LC_{50} parameters, including for the positive control levamisole hydrochloride (as reported in Ogedengbe et al., 2019).

*3.2. Yield and structure elucidation of compound **1***

The yield of compound **1** was 0.04% w/w of the original acetone extract and 0.0015% w/w of the dried plant (leaf) material. The structure of Compound **1** (Fig. 2) was determined to be phaeophorbide-a by comparing its spectroscopic data with data from the literature (Van Breemen *et al*., 1991a; Sakata *et al*., 1990; Schwikkard *et al*., 1998; Ohshima *et al*., 1994; Cheng *et al*., 2001 and Endo *et al*., 1982) (see Table 1 and Supplementary Fig. S1-S2).

ESIMS +ve mode: m/z 593 [M+1], $C_{35}H_{36}N_4O_5$ (mass 592.68), 533 [M-CH_3COO-] (Supplementary Fig. S3) (see Van Breemen *et al*., 1991b and Cheng *et al*., 2001).

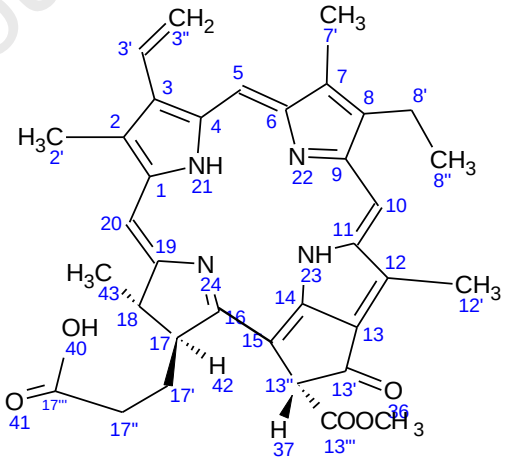


Fig. 2 Structure of phaeophorbide-a (**1**)

Table 1. NMR spectral data of phaeophorbide-a compared with the literature. *Sakata *et al.* (1990). **Oshima *et al*. (1994). ***undetected signal due to small sample size

Results from this study				Results from literature	
C-atom	δC	H-atom	δH	δC*	δH**
1	142.00	1	-	142.04	-
2	132.00	2	-	131.79	-
2′	12.02	2′	3.34(s)	12.01	3.34(s)
3	136.47	3	-	136.47	
3′	129.02	3′	7.90 (dd, J=17.0, 11.4Hz)	129.02	7.89(dd, J=17.8,11.5)
3″	123.00	3″(E)	6.31(dd, J=18.0,1.5)	122.64	6.21(dd,J=17.8, 1.5Hz)
		3″(Z)	6.10(dd, J=11.5, 1.5)		6.11(dd, J=11.5, 1.5Hz)
4	136.16	4	-	136.16	-
5	97.60	5	9.25(s)	97.47	9.25(s)
6	155.00	6	-	155.58	-
7	136.17	7	-	136.16	-

7′	11.10	7′	3.12(s)	11.08	3.13(s)
8	145.00	8	-	145.11	-
8′	19.50	8′	3.58(q, J=7.2)	19.34	3.57(q, J= 7.3)
8″	17.35	8″	1.68(t, J=7.5)	17.32	1.63 (t, J=7.5)
9	151.00	9	-	150.95	-
10	104.50	10	9.40(s)	104.33	9.40(s)
11	138.00	11	-	137.96	-
12	129.02	12	-	129.02	-
12′	12.02	12′	3.62(s)	12.01	3.62(s)
13	129.02	13	-	129.02	-
13′	***	13′	-	189.59	-
13″	64.80	13″	6.23(s)	64.77	6.23(s)
13‴	169.00	13‴	-	169.61	-
13⁗	53.00	13⁗	3.85(s)	52.81	3.85(s)
14	149.70	14	-	149.70	-
15	105.30	15	-	105.28	-
16	161.00	16	-	161.23	-
17	51.00	17	4.20(m)	51.21	4.18(m)
17′	31.30	17′	2.28(m)	31.12	2.28(m)
17″	29.50	17″	2.58(m)	29.92	2.59(m)
17‴	***	17‴	-	173.34	-

18	50.00	18	4.44(dq, J=7.3,2.0)	50.17	4.43(dq, J=7.3,2.0)
18′	23.09	18′	1.80(d,J=7.3)	23.08	1.80(d, J=7.3)
19	172.40	19	-	172.15	-
20	93.00	20	8.53(s)	93.09	8.52(s)

3.3 Cytotoxicity screening

3.3.1. Effects of the positive control, hydrogen peroxide, and Compound 1 on cell viability and morphology

Hydrogen peroxide was used in this study as a positive control to establish the susceptibility of the cells to a cytotoxic agent, as hydrogen peroxide is a well-established oxidant used as a cytotoxic agent (Hencart, 2012; Fatokun et al., 2006). As revealed by both AB and CTG assays, hydrogen peroxide (up to 1 mM) following 48 h treatment reduced the viability of both the H460 and the Caco-2 cells in a concentration-dependent manner, although it was more potent against the H460 cell line than the Caco-2 cell line, with 50 µM and 200 µM as the least concentrations that induced significant cytotoxicity, respectively (Fig. 3 for the AB assay; similar results were obtained for the CTG assay). The IC_{50} values for the cytotoxic effects of hydrogen peroxide on H460 and Caco-2, respectively, were 45.8 µM and 213.2 µM for the AB assay, and 36.3 µM and 226.9 µM for the CTG assay. The reduction in viability induced by peroxide was associated with morphological damage, manifested in the loss of confluency (H460) or disrupted confluency (Caco-2) and the rounding up of the remaining cells (H460), compared to negative control cells which are generally in contact with one another in a sheet-like, confluent, epithelial monolayer (Fig. 4). The toxic effects of hydrogen peroxide on the viability and morphology of the

MC3T3-E1 cell line have been previously demonstrated by us, in which 200 µM hydrogen peroxide or higher, applied for between 1 and 6 h, reduced viability significantly (Fatokun *et al*., 2006).

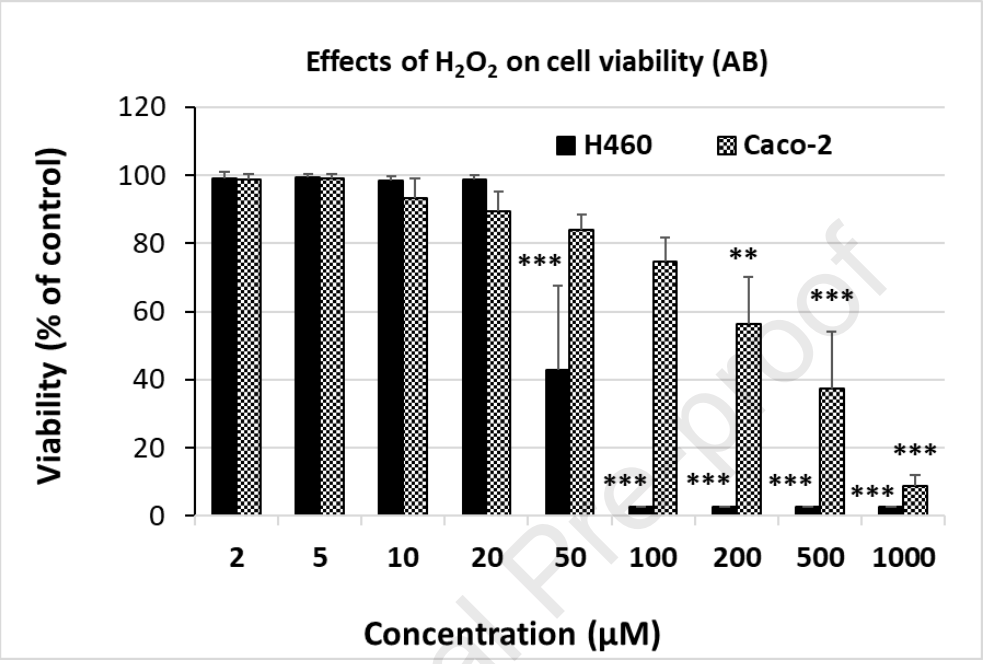


Fig. 3. Hydrogen peroxide-induced reduction (indicative of cytotoxicity) in the viability of H460 and Caco-2 cells, quantified using a fluorescence-based alamar blue (AB) assay. Treatment duration was 48 h and the experiment was run three independent times (n=3). **P<0.01 and ***P<0.001, compared to the respective negative control. Similar results were obtained using the CTG assay.

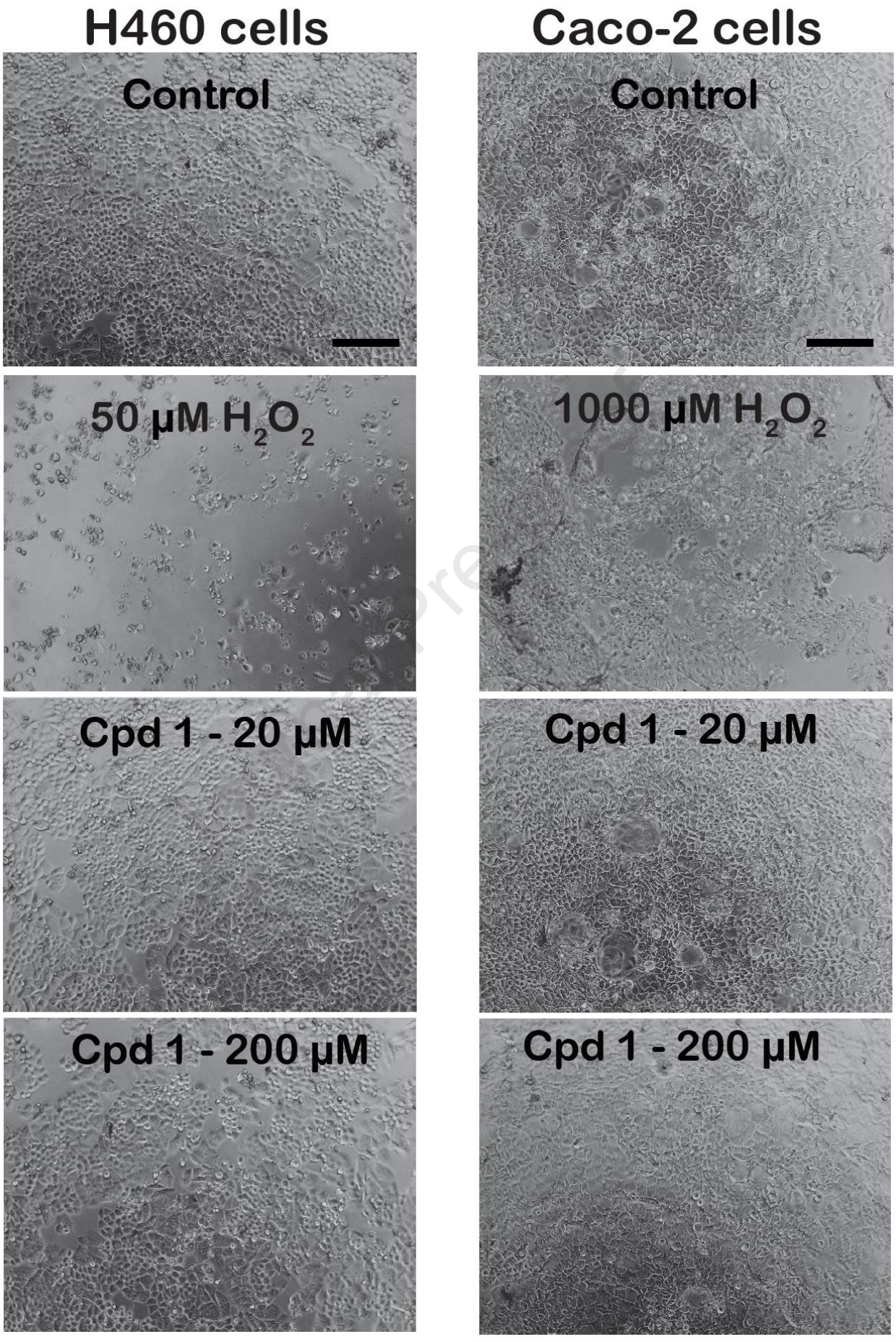

H460 cells
Caco-2 cells
Control
Control
50 µM H_2O_2
1000 µM H_2O_2
Cpd 1 - 20 µM
Cpd 1 - 20 µM
Cpd 1 - 200 µM
Cpd 1 - 200 µM

Fig. 4. Photomicrographs showing the toxic effects of hydrogen peroxide and lack of effect of compound 1 on the morphology of H460 and Caco-2 cells. Images were acquired, following 48 h treatments, on a Nikon Eclipse TS100 inverted microscope (x10 magnification) fitted with a camera. Scale bar = 20 µm.

Having established that the three different cell lines are susceptible to chemically induced toxicity, the potential effects of Compound **1** on the cells were examined. The AB assay revealed that the compound was not toxic to the cells up to 200 µM (Fig. 5), while the CTG assay showed that the compound was not cytotoxic below 200 µM, but at 200 µM induced mild-to-moderate but statistically significant decreases in the viability of the cells ($P<0.05$ for MC3T3-E1, $P<0.01$ for H460 and Caco-2), with values still above 80% of the negative control in each case (Fig. 6). As further evidence that Compound 1 is generally non-toxic, photomicrographs of cultures treated with it retained a phenotype that is indistinguishable from the control phenotype (Fig. 4).

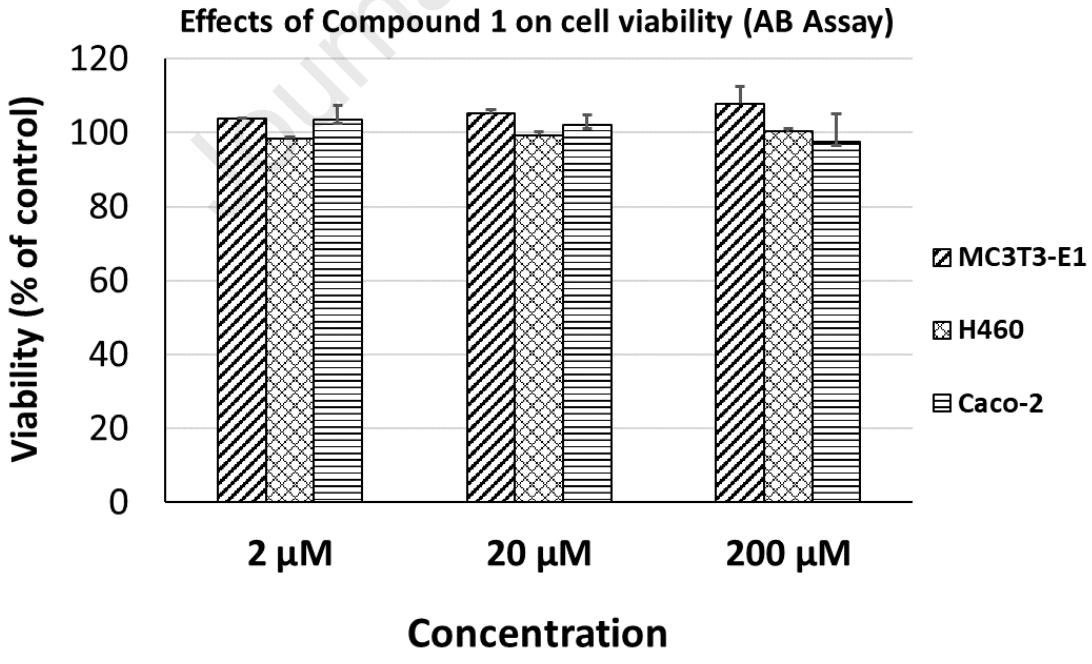


Fig. 5. Evidence of no toxic effect of Compound **1** on the viability of MC3T3-E1, H460 and Caco-2 cell cultures exposed to it for 48 h. Viability was quantified using a fluorescence-based Alamar blue (AB) assay. Each treatment was conducted in triplicate and the experiment was run three independent times (n=3) for the MC3T3-E1 cell line and four independent times (n=4) for the other two cell lines.

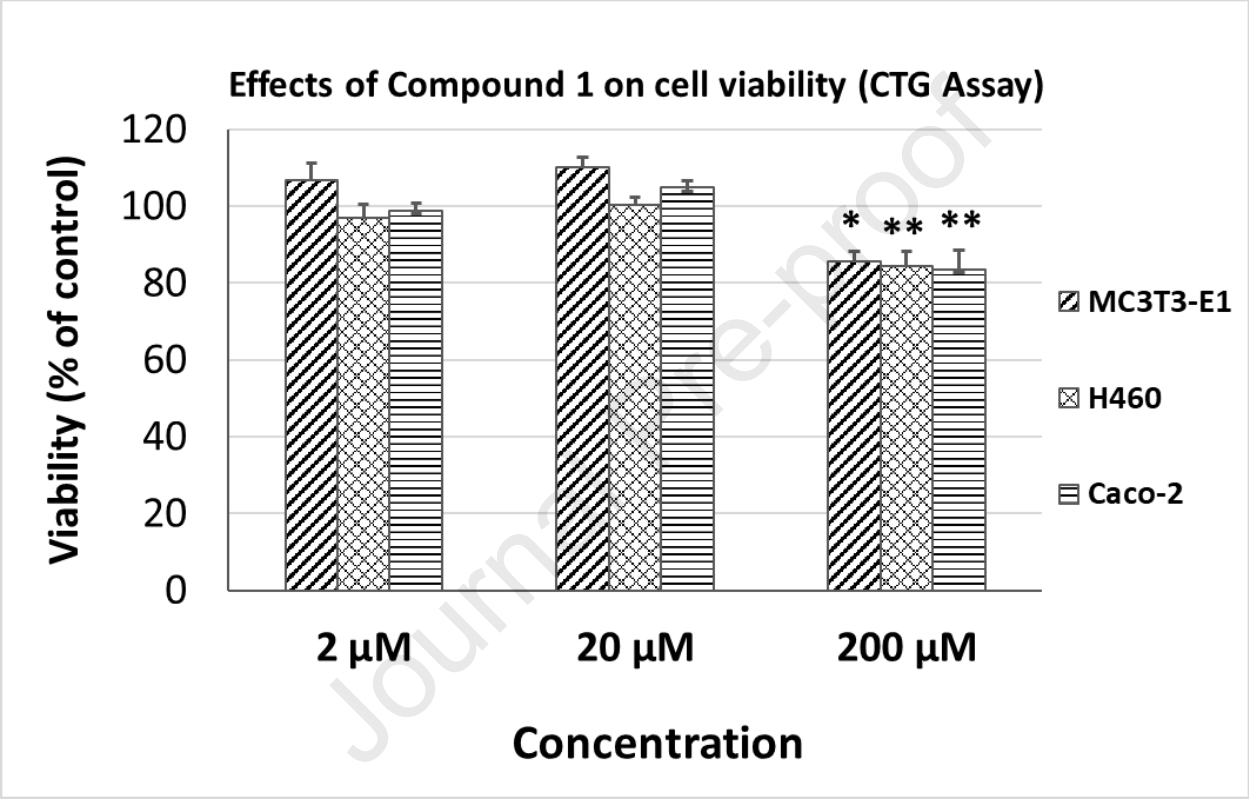


Fig. 6. The effect of Compound **1** on the viability of MC3T3-E1, H460 and Caco-2 cell cultures exposed to it for 48 h. Viability was quantified using a luminescence-based CellTiterGlo (CTG) assay. Each treatment was conducted in triplicate and the experiment was run three independent times (n=3). *P<0.05 and **P<0.01, compared to the negative control.

4. Discussion

This work describes the preparation, from *S. mombin* leaf extract, of three different extracts which were screened for their potential anthelmintic activity against *H. placei*. The acetone extract elicited the most potent anthelmintic activity, suggesting that the potently active principle(s) was/were of medium polarity by nature. The active principle in the acetone extract underlying activity was subsequently isolated and identified as phaeophorbide-a, a compound that was found to be relatively non-toxic to mammalian cells. To our knowledge, this is the first time it is being reported in *S. mombin* and its observed anthelmintic activity is novel.

Spectroscopic techniques were employed to identify the compound, which was isolated from an active fraction of the acetone leaf extract as a blackish green powder. It was identified as **1** by comparing its ^{1}H & ^{13}C NMR and ESIMS data with data from the literature. The compound was isolated in a dim room, shielded from light, and stored under strict conditions to prevent photodegradation.

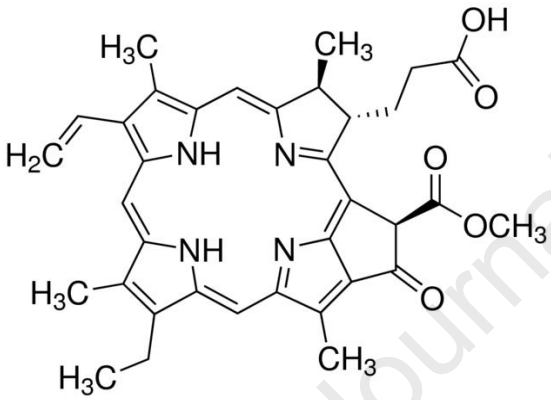
The ESIMS in +ve mode showed an $[M+1]^+$ peak at m/z 593($C_{35}H_{36}N_4O_5$) as expected for **1** as reported by Silva *et al*. (2006). A $[M-59]^+$ peak indicated the loss of the methyl ester from C-13″. Furthermore, the ^{1}H & ^{13}C NMR spectra of Compound **1** were similar to those reported for **1** in the literature (Sakata *et al*., 1990; Ohshima *et al*., 1994), with the slight differences in the δ values likely due to the effect of concentration on the chemical shifts, which is a characteristic of chlorins (Abraham and Rowan, 1991). Two δC carbonyl signals were not visible on the ^{13}C spectrum because they are usually low-intensity signals and the small sample size of the compound analysed was too small to reveal the two low-intensity

δC signals downfield; however, taken together, the experimental and published spectral data confirmed that **1** is phaeophorbide-a.

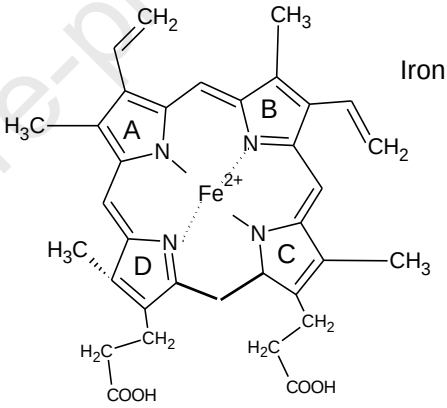
Phaeophorbide-a may have been formed from the degradation of chlorophyll a by the enzymes Mg-dechelatase and chlorophyllase (Silva *et al*., 2010) in the leaf extract of *S. mombin*. However, the possibility also exists that it might not be a degradation compound but an actual secondary metabolite of *S. mombin*, and while this would be of great interest to researchers (Teles *et al*., 2015), it is outside the scope of this study.

The structural elucidation of phaeophorbide-a revealed it to be a porphyrin belonging to the class of compounds called chlorins. The mechanism of action of this compound is proposed to be due to biological antagonism, because the compound is structurally similar to haem (Fig. 7). Parasitic worms cannot synthesize haem because they lack most of the enzymes necessary for haem biosynthesis (Sah *et al*., 2002; Panek *et al*., 2002). These include several medically relevant helminths as well as the *Haemonchus* spp. (Rao *et al*., 2005). Rao *et al*. (2005) further proved this by demonstrating that biosynthetic enzyme activities were undetectable in the many parasitic nematodes of different host specificities (that they tested), thereby showing that these helminths lacked the necessary enzymes for haem biosynthesis. Haem is essential to the survival of parasitic helminths; therefore, in order to compensate for their inherent deficiencies in haem biosynthesis, these parasites have evolved highly efficient haem uptake channels (Rao *et al*., 2005; Halton, 1997). These haem uptake channels are so efficient that some studies have shown that non-iron porphyrins can easily gain entrance into the parasites through these haem transport systems, thereby leading to toxicity, because without the central iron metal chelate, oxidation-reduction reactions cannot occur (Stojiljkovic *et al*., 1999). Phaeophorbide-a is structurally similar to haem; therefore, it could be exerting its observed anthelmintic effect by biological antagonism (competing with haem and thus eventually starving helminths of the needed haem). Interestingly, another parasitic

organism, the *Leishmania* spp., which is also deficient in some of the genes that encode for haem biosynthesis, was found in one study to be highly susceptible to phaeophorbide-a (Hortensteiner *et al*., 1998). Pharmacological targeting of haem transport pathways in parasitic worms could become an important control measure for helminthic infections (Rao *et al*., 2005; Heinemann *et al*., 2008; Chitwood *et al*., 2003). Phaeophorbide-a has already been isolated from several plants, including *Ficus platypoda* (Miq.) leaves (Afifi *et al*., 2014); *Scutellaria barbata* plant (Chan *et al*., 2006); *Psychotria acuminate* plant (Glinski *et al*., 1995) and the leaves of *Arrabidaea chica* (Miranda *et al*., 2017).



Phaeophorbide-a **(1)**



Iron protoporphyrin IX

Iron protoporphyrin or haem

Fig. 7. Structural similarities between phaeophorbide-a **(1)** and haem

The cytotoxic/cytostatic effects of chlorins are well known and are already being exploited in the form of photodynamic therapy where their ability to generate reactive oxygen species have been applied in killing cancer cells (Pandey *et al*., 1992). However, the cytotoxic effect of the compound tested in this study was

not pronounced (the mild-to-moderate effect occurred at a high concentration of 200 µM and this was only revealed by an assay that depends on changes to ATP levels, i.e., the CTG assay, which is based on measuring ATP levels in living cells (Hannah et al., 2001)). This apparent lack of cytotoxic effect observed could perhaps have been due to the care taken in handling the compound by shielding it as much as possible from light to prevent photodegradation. This reasoning is based on the fact that the effects of photoactivation of chlorins have been shown in a study by Yao *et al*. (2008), where some chlorins assessed for cytotoxicity in the dark were found to be mostly non-cytotoxic when not exposed to light, whereas phaeophorbide-a has been shown in the literature to possess cytotoxic effects when photoactivated (Wongsinkongman *et al*., 2002; Lee *et al*., 2004). It is therefore exciting to note the prospect of employing photoactivation as a switch to target the therapeutic application of chlorins to disease areas where death of cells is desired (e.g., cancer), on the one hand, and to those in which achievement of a therapeutic effect without cytotoxicity (e.g., to the host cells) is the goal, on the other hand, for example, anthelmintic treatments. Interestingly, apart from their application in Photodynamic Therapy (Roeder, 1990) and the observed anthelmintic activity reported in this paper, chlorins are biologically active compounds with other observed biological activities. For example, reports in the literature have shown that they also possess anti-malarial activity (Afifi *et al*., 2014), potent HIV-1 replication inhibition activity (Zhang et *al*., 2003), and *in vitro* activity against influenza virus proliferation (Yasuda *et al*., 2010).

The discovery in our study of the anthelmintic activity of this compound and its minimal cytotoxicity makes it suitable for advancement to further studies to use it as a scaffold to generate new drug leads.

5. Conclusion

Phaeophorbide-a (**1**) isolated from the acetone extract of *S. mombin* leaf for the first time was shown to possess anthelmintic activity against *H. placei* worms. It was not toxic to the mammalian Caco-2, MC3T3-E1 and H460 cell lines below a relatively high concentration of 200 µM, suggesting that it is

reasonably safe for therapeutic use in mammalian organisms, including livestock. Phaeophorbide-a could, therefore, serve as a chemical lead for the development of new anthelmintic drugs.

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Conflicts of interest

The authors declare that there are no conflicts of interests.

Authors' contributions

ANOO collected plant material, performed experiments, analysed data and wrote the manuscript draft with contributions from other authors. IOA supervised the conduct of anthelmintic assays. CWW supervised isolation and structure elucidation of the active compound. SOI supervised chemistry experiments and the PhD work of ANOO, of which this study was part. AAF conducted and supervised cytotoxicity and imaging experiments, and also provided general supervision to ANOO during her visiting research. All authors read and approved the manuscript.

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FIGURE LEGENDS

Fig. 1. Concentration-effect plot of the anthelmintic activities of fraction 8, an impure sample of compound 1 and compound 1 against *H. placei* in an adult worm motility assay, with the table above the plot showing the LC_{50} parameters, including for the positive control levamisole hydrochloride (as reported in Ogedengbe et al., 2019).

Fig. 2 Structure of phaeophorbide-a (**1**)

Fig. 3. Hydrogen peroxide-induced reduction (indicative of cytotoxicity) in the viability of H460 and Caco-2 cells, quantified using a fluorescence-based alamar blue (AB) assay. Treatment duration was 48 h and the experiment was run three independent times (n=3). **P<0.01 and ***P<0.001, compared to the respective negative control. Similar results were obtained using the CTG assay.

Fig. 4. Photomicrographs showing the toxic effects of hydrogen peroxide and lack of effect of compound 1 on the morphology of H460 and Caco-2 cells. Images were acquired, following 48 h treatments, on a Nikon Eclipse TS100 inverted microscope (x10 magnification) fitted with a camera. Scale bar = 20 µm.

Fig. 5. Evidence of no toxic effect of Compound **1** on the viability of MC3T3-E1, H460 and Caco-2 cell cultures exposed to it for 48 h. Viability was quantified using a fluorescence-based Alamar blue (AB) assay. Each treatment was conducted in triplicate and the experiment was run three independent times (n=3) for the MC3T3-E1 cell line and four independent times (n=4) for the other two cell lines.

Fig. 6. The effect of Compound **1** on the viability of MC3T3-E1, H460 and Caco-2 cell cultures exposed to it for 48 h. Viability was quantified using a luminescence-based CellTiterGlo (CTG) assay. Each

treatment was conducted in triplicate and the experiment was run three independent times (n=3). *P<0.05 and **P<0.01, compared to the negative control.

Fig. 7. Structural similarities between phaeophorbide-a **(1)** and haem

TABLE LEGEND

Table 1. NMR spectral data of phaeophorbide-a compared with the literature. *Sakata *et al.* (1990). **Oshima *et al*. (1994). ***undetected signal due to small sample size

Supplementary material

Proton NMR of compound 1

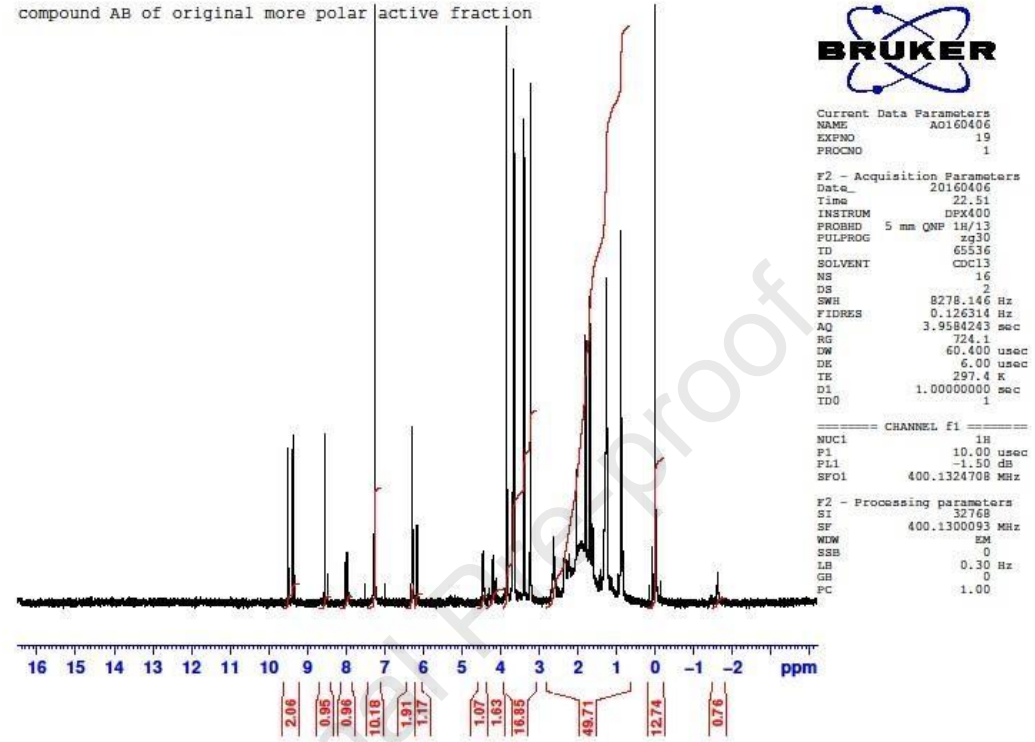

Fig. S1. ^{1}H NMR of compound 1 in $CDCl_3$ at 400MHz

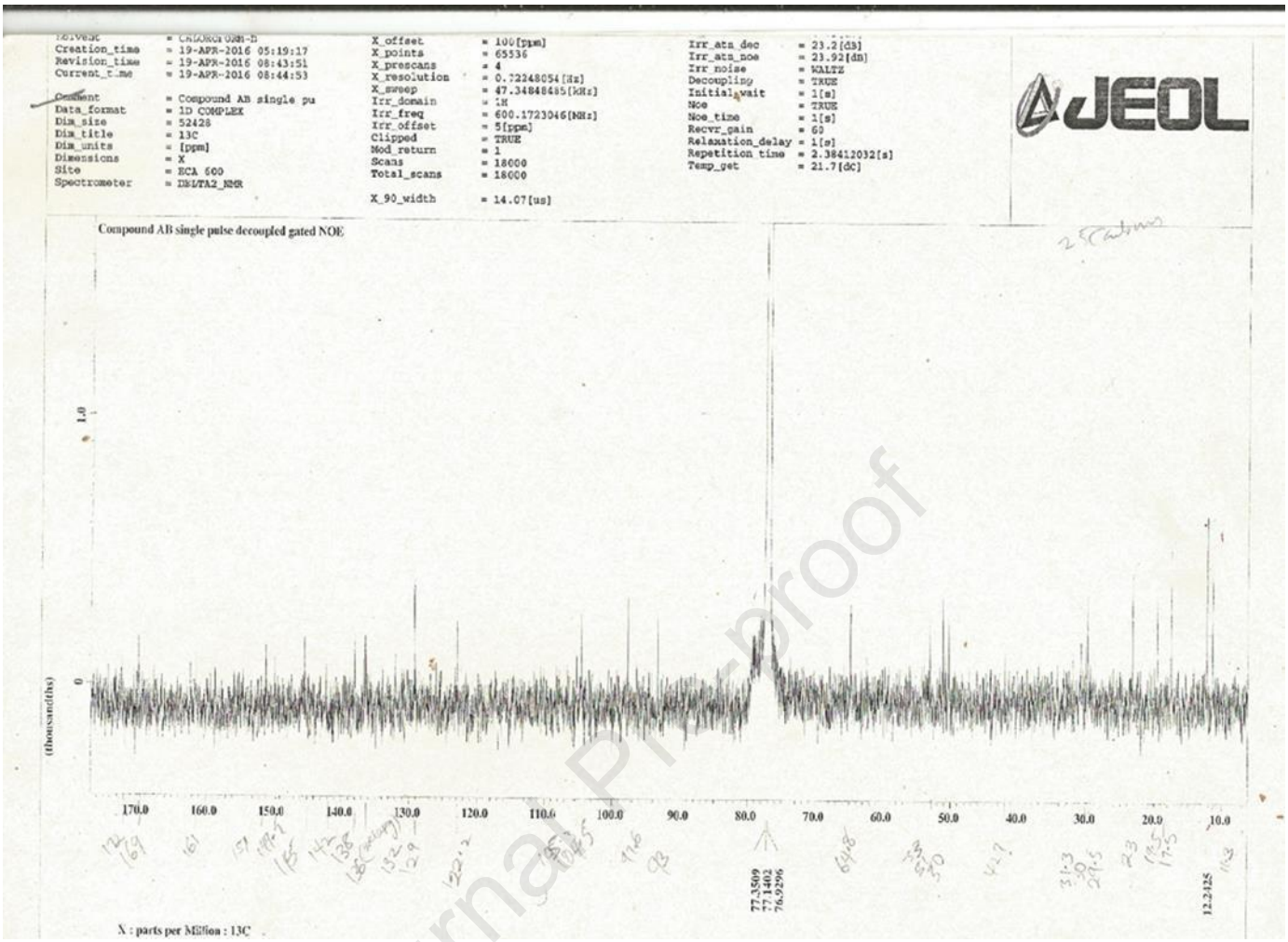

Fig. S2. ^{13}C spectrum of compound 1 in $CDCl_3$ at 600MHz

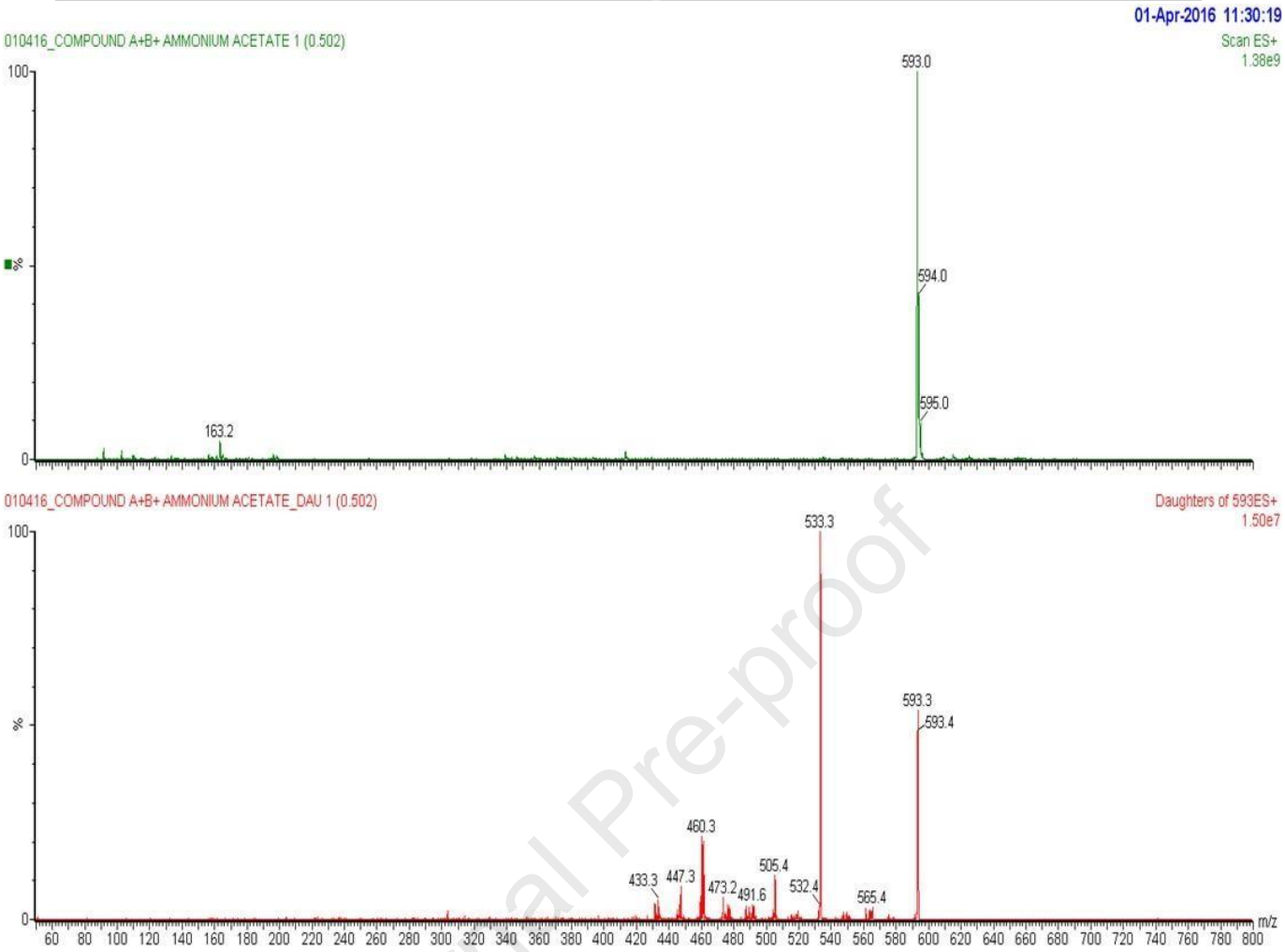

Fig. S3. ESIMS-LC of compound 1 (ammonium acetate added) and daughter ions in positive mode

HIGHLIGHTS:

- Phaeophorbide-a was isolated from acetone extract of *Spondias mombin* leaf.
- The compound exhibited anthelmintic activity, with an LC_{50} of 23.0 µg/mL (38.8 µM).
- It was not toxic to cultured HeLa, Caco-2 and MC3T3-E1 cells at concentrations below 200µM.
- Its chemical structure could serve as a pharmacophore for anthelmintic drug development.