

## Evaluation of the Antiplasmodial Properties of *Andrographis paniculata* (Burm.f.) and *Peperomia pellucida* (L.) Kunth

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**Abstract:** Plasmodium species are the infectious agents that are responsible for malaria, a disease that claims the lives of approximately 400,000 people annually. The fact that drug resistance against malaria is on the rise suggests that new antimalarial compounds need to be discovered. It is well known that medicinal plants present the best opportunity for the identification of novel antimalaria chemicals. Both the *Andrographis paniculata* (Burm.f.) and *Peperomia pellucida* (L. Kunth) species have been tested for their antiplasmodial ability against the *Plasmodium falciparum* strain. The *P. pellucida* (L. Kunth) species has also been subjected to in vitro and in vivo biological research. *P. pellucida* was used to isolate the steroid known as 3-hydroxy-24-ethyl-5,22-cholestadiene (1) and the triterpenoid known as 3-hydroxy-9-lanosta-7,24E-dien-26-oic acid (2). Both compounds were then tested for their activity in vitro. In the mice model, triterpenoid 2 had a substantial chemo-suppressive impact.

**Keywords:** *A. paniculata* (Burm.f.); *P. pellucida* L. Kunth; *Plasmodium falciparum*; inhibition; 3 $\beta$ -hydroxy-9-lanosta-7,24E-dien-26-oic acid

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### ■ INTRODUCTION

Malaria is a significant threat to global public health, as it affects individuals in every region of the world. The disease is brought on by the parasite *Plasmodium*, which is transmitted from female *Anopheles* mosquitoes to human hosts [1]. Since there are not enough preventative measures in place, and because the malaria parasite is becoming more resistant to many antimalarial drugs that are already on the market, such as quinine, chloroquine, and piperazine; this disease continues to be a concern for the health of people all over the world [2-4]. In spite of the devoted research that has been conducted over the

course of several decades, there is still no malaria vaccine that can be bought in stores at this moment. As a result, chemotherapies that make use of combination medications appear to be the best alternative, and novel antimalarial chemicals are an absolute necessity. Regular mining is done on plants that are used for therapeutic purposes with the purpose of determining whether they have the potential to provide novel active chemicals [5]. For instance, Tu [6] created Artemisinin in China in the early 1970s. This medication was derived from *Artemisia annua* L. and was utilized in the treatment of malaria. It is widely known that tropical plants contain a rich reservoir of bioactive compounds. Because of this, there

is the possibility that these plants could serve as sources for the development of novel antimalarial drugs [7].

One of the most common plant families that are farmed in tropical and subtropical regions is Acanthaceae family [8]. Traditional uses for this herb include the treatment of acute diarrhea, coughing, the common cold, inflammation, boils, skin eruptions, and seasonal fever [9]. Other ailments that have been alleviated by using this herb include boils, skin eruptions, and seasonal fever. The investigation of phytochemicals has resulted in the identification of a wide range of secondary metabolites that have significant pharmacological effects. The *Andrographis paniculate* species, more often known as "Sambiloto," which could be found all over Indonesia, is regularly exploited as an element of traditional medical practices [10]. Traditional cultures held the belief that combining this plant with *Peperomia pellucida* could boost the efficacy of antimalarial treatment [11-13]. In spite of the fact that this plant possesses anti-plasmodial activity against many strains of *Plasmodium falciparum*, this is still the case. We investigated the antiplasmodial activity of the *A. paniculata*, and *P. pellucida* isolates against a malarial parasite strain in order to give data in favor of this theory. In addition, we tested the efficacy of these compounds on infected mice and isolated the active compound(s) in an effort to gain a deeper comprehension of the biological role that these compounds play. On the other hand, the investigations regarding the isolated compounds of PP have not been documented in detail. Only one of the studies that we looked at in the literature had isolated chemicals that came from the *P. pellucida* plant.

## ■ EXPERIMENTAL SECTION

### Materials

In June 2016, fresh leaves and stems of *A. paniculata* and *P. pellucida* were harvested in north Gorontalo, Gorontalo Province, Indonesia. The plant was identified at the Herbarium Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo, and voucher specimens (No. 130/H47.B4.Bio.Lab Bio/LL/2016; for *P. pellucida* and No.

131/H48.B4.Bio.Lab Bio/LL/2016; for *A. paniculata*) were deposited to the Herbarium.

### Instrumentation

The instruments used in the experiment for molecular determination were Japan Electron Optics Laboratory (JEOL) 125 MHz  $^1\text{H-NMR}$  and JEOL 500 MHz  $^{13}\text{C-NMR}$ , for the photoelectron analysis the instrument used was JEOL UV-Vis Spectrophotometer, the cell culture was done in ESCO Scientific Laminar Cabinet, and the infrared instrument used was ThermoFisher Scientific Infrared Spectrometer.

### Procedure

#### *Plant extraction and isolation*

Dried leaves and stems of *P. pellucida* (PP) (2 kg) and *A. paniculata* (AP) (2 kg) were macerated with methanol (12 L) at room temperature for 2 d. After filtrating, the filtrate was evaporated under reduced pressure to yield the PP extract (211 g) and AP extract (278 g). The PP extract was suspended with water (500 mL) and partitioned successively with *n*-hexane and ethyl acetate. Evaporation gave the crude *n*-hexane (15.3 g), ethyl acetate (13.2 g) and aqueous (18.5 g) extracts. The isolation procedures are shown in detail in Scheme S1. The ethyl acetate extract was purified by column chromatography using silica gel G60 (132 g), eluted by *n*-hexane/EtOAc (200 mL) with a linear gradient from 10% to 100% of EtOAc to yield eleven fractions (A1–A11). Fraction A6 (0.27 g) was subjected to silica column chromatography using the isocratic elution mixture of *n*-hexane/EtOAc (90:10-0:100) to yield 40 fractions (B1–B40). Fractions B9 were recrystallized using *n*-hexane and chloroform to afford compound **1** (18 mg) as a needle-shaped crystal. Fraction A7 (0.97 g) was subjected to silica column chromatography (10 g of silica G60) using an isocratic system, eluted by *n*-hexane/EtOAc/MeOH (7:2:1) to yield 25 fractions (B1–B25). Fractions C13–C15 (90 mg) were combined and purified by silica column chromatography on Octa Decyl Silane (ODS), eluted by MeOH/H<sub>2</sub>O (4:1) to yield compound **2** (12 mg) as a white powder.

### **Synchronization and maintenance of parasite strain**

The University of Tokyo's chloroquine-sensitive malaria parasite Pf D-10. Parasite Pf D-10 was maintained in fresh O+ human erythrocytes with a hematocrit of 4% in RPMI 1640 culture medium (Gibco) supplemented with 10% (v/v) human serum, 25 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, Sigma Aldrich), 45 µg/mL hypoxanthine, 32 mM NaHCO<sub>3</sub>, and 50 µg/mL gentamicin, incubated at 37 °C under a gas mixture of 5% O<sub>2</sub> and 5% CO. After 96 h, parasites were microscopically synchronized with 5% sorbitol.

### **In vivo antiplasmodial activity**

The Animal Experimental Development Unit at Universitas Gadjah Mada, Yogyakarta, Indonesia, donated 30 male BALB/c albino mice aged 6–8 weeks weighing 25–28 g for this investigation. At the Universitas Airlangga Institute of Tropical Disease Animal Laboratory, the mice were fed and watered ad libitum. Eijkman Institute for Molecular Biology in Jakarta provided the parasite Pb ANKA strain. At the Institute of Tropical Disease, Universitas Airlangga, male BALB/c mice and cryogenic storage maintained the parasite.

As described by Peter [14], with slight modifications, the sample extract AP/PP (20/80 mg) and isolated chemical (10 mg) were tested in mice infected with Pb ANKA for antiplasmodial activity. Thirty BALB/c albino mice were intraperitoneally infected with Pb ANKA (106 parasitized erythrocytes). The volume of Inoculum used was 200 µL. Five six-mouse groups were formed (three experimental and two control groups). Three groups received the sample at 1, 10, and 100 mg/kg/body in 0.5% CMC-Na (200 µL). A negative control group received 0.5% CMC-Na while a positive control group received chloroquine diphosphate (25 mg/kg/body) intraperitoneally once a day for 4 d. After 4 d, the animals ceased treatment. On the 6<sup>th</sup> day, thin mouse tail blood smears were made on slides. The slide was stained with 15% Giemsa-dyed solution for 10 min [15-16]. Rinsing and drying at room temperature completed the slide. Giemsa-stained blood smears and microscopy determined parasitemia and growth inhibition. Day 21 post-infection survival was monitored.

### **Antimalarial assay in vitro**

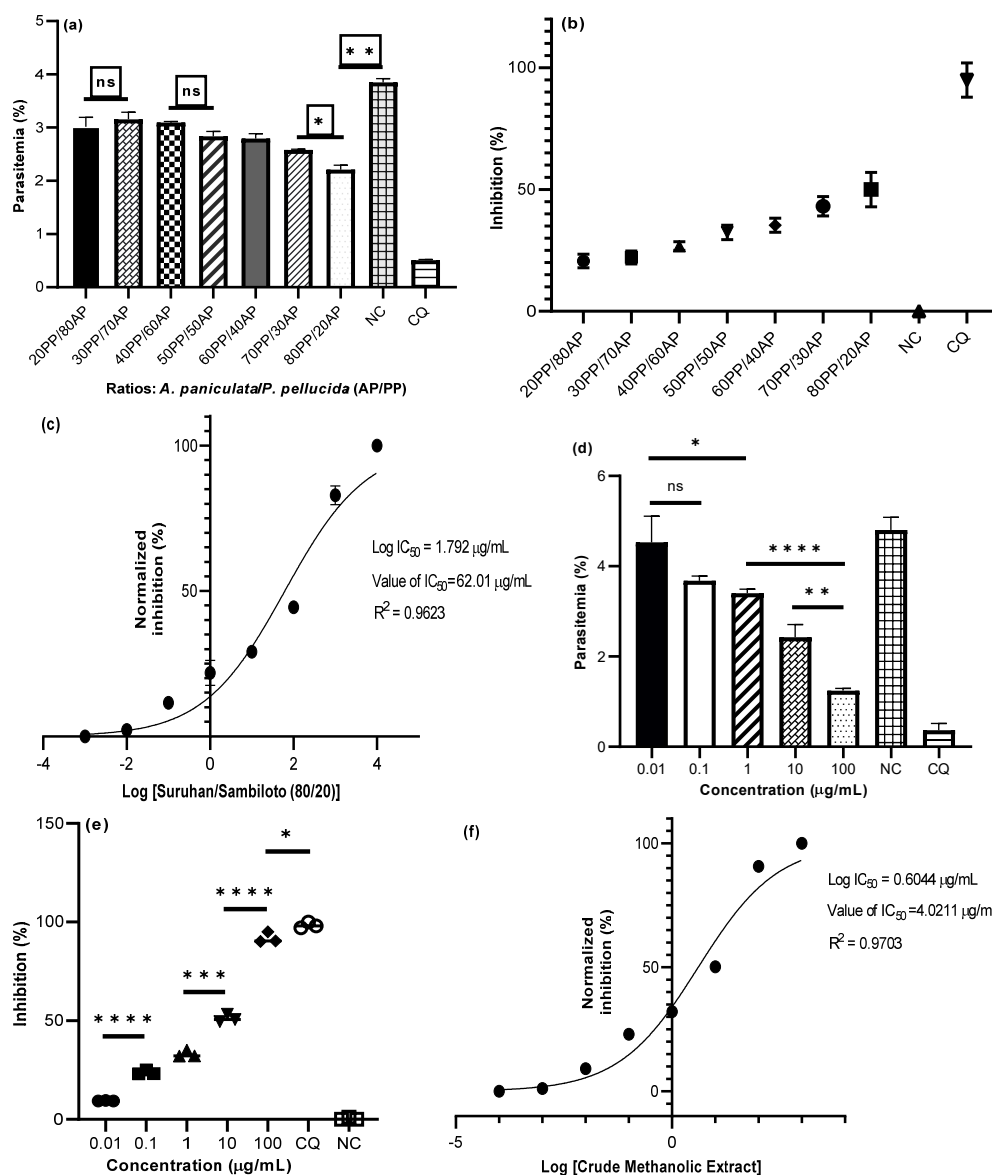
The antimalarial activity of the compounds was tested using the Desjardins method [17]. The test was performed using *P. falciparum* 3D7 strain. The antimalarial examination used 96 wells, each well filled by parasitemia culture 1%. The RPHS media was replaced by a sample containing RPHS media with different sample concentrations. All wells were then stored in the Laminar Airflow cabinets until all solvents were evaporated. Further, we added 50 µL of parasitemia red blood cells into the well and then incubated at 37 °C. After 48 h, slide preparation was stained using Giemsa 20%. The parasite was then swapped with Giemsa-colored blood. The parasitemia percentage was calculated by comparing the number of infected erythrocytes to 500 erythrocytes.

### **Statistical analysis**

The experiments were performed independently in triplicate, and the average data are presented. Statistical analysis was performed by unpaired two-tailed *t*-test using GraphPad Prism. The statistics were significant when \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001.

## **RESULTS AND DISCUSSION**

Fresh leaves and stems of species AP and PP were extracted with crude methanolic extracts. A thin blood smear stained with Giemsa was obtained after incubating synchronized *P. falciparum* D-10 strain (Pf D-10) with the AP-PP extract at varied ratios (80:20 to 20:80) in dimethyl sulfoxide (DMSO), chloroquine (CQ) (1 M), and 0.2% DMSO. Microscopically, parasitemia, parasite growth, and inhibition rates were assessed. The chemoprevention of parasitemia against Pf D-10 was investigated. The activity of the AP-PP extract was limited (2.8 to 3.0%) at a ratio of 80:20 to 40:60. However, when the PP extract ratio was increased, the parasitemia rate reduced considerably (Fig. 1(a)). Low suppressions of Pf D-10 were seen at ratios ranging from 80:20 to 40:60, whereas a ratio of 20:80 indicated a 50% inhibitory effect (*p* 0.05). The AP-PP extract's antiplasmodial activity was boosted in a ratio-dependent way (Fig. 1(b)). Furthermore, the IC<sub>50</sub> value of AP-PP



**Fig 1.** The AP-PP extract was tested *in vitro* against Pf D-10 at a variety of ratios, ranging from 80:20 to 20:80, as well as the PP extract on its own. (a) The level of parasitemia, and (b) the percentage of parasites that were inhibited by treatment with the AP-PP extract, (c) A result of the  $IC_{50}$  of the AP-PP extract when it was mixed in a ratio of 20:80. (d) The prevalence of parasitemia, and (e) the percentage of parasites that were inhibited by the PP extract. (f) A value for the  $IC_{50}$  of the PP extract. With the use of the GraphPad Prism program, an  $IC_{50}$  value was determined. As a negative control, we used parasites that had been treated with 0.2% DMSO, and as a positive control, we employed CQ at a dose of 1  $\mu$ M. Every experiment was carried out three times, so the total number of participants was three. The standard deviation is represented by the error bars. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , not significant (ns)

extract at a 20:80 ratio was investigated (Fig. 1(c)). The extract was tested at concentrations of 0.01, 0.1, 1, 10, and 100 g/mL and the inhibition rates were determined after 48 h of incubation. The  $IC_{50}$  value was calculated through an examination of inhibition-response curves. Based on

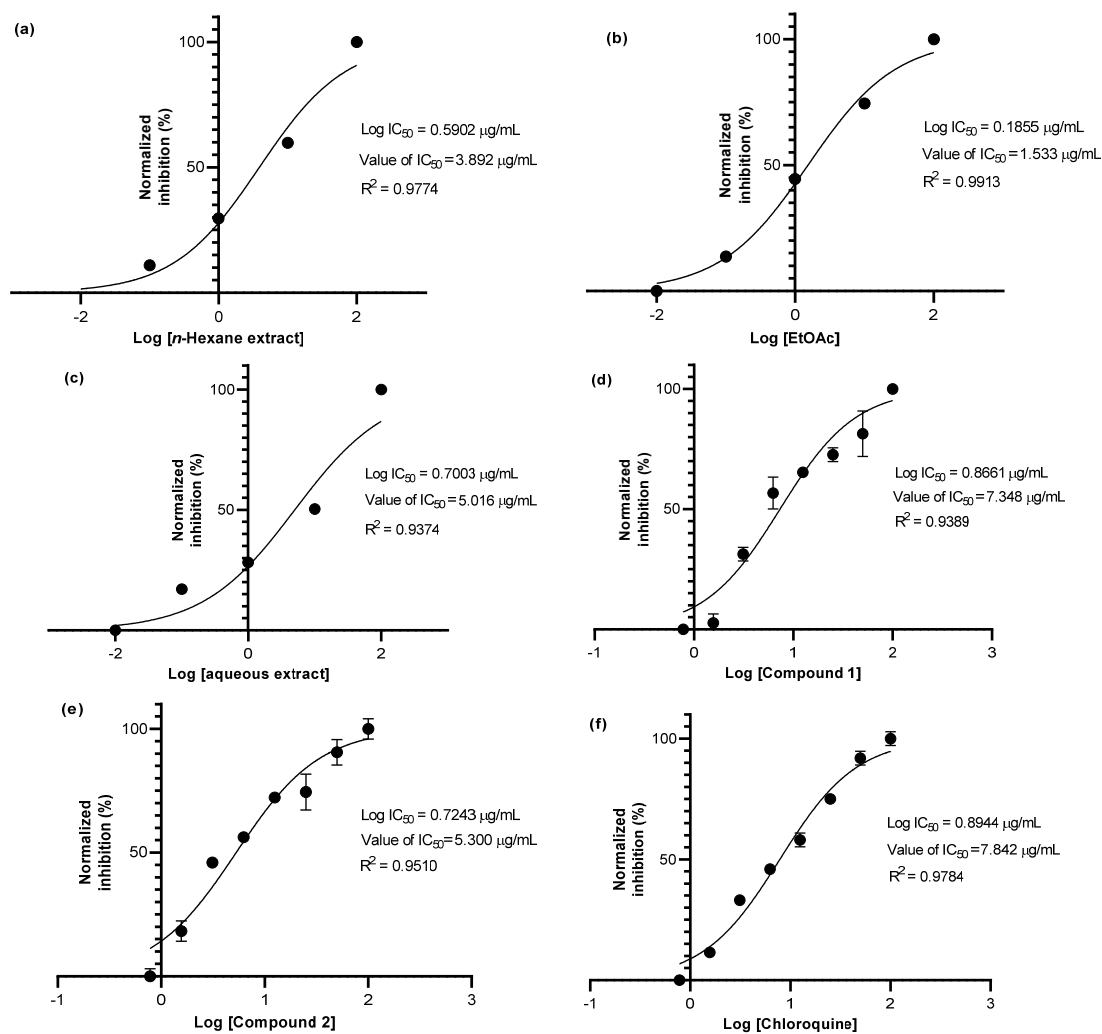
an *in vitro* study with an  $IC_{50}$  value of 62.01 g/mL, the AP-PP extract at a 20:80 ratio was rated mildly potent (Table S1) [17].

The PP extract alone must be assessed because its larger ratio had a stronger antiparasitoid effect than the

AP extract. *In vitro* concentrations were 0.01 to 100 g/mL. Fig. 1(d) shows that PP extract at 0.1 to 100 g/mL had antiplasmodial potential. The greatest dose of *P. pellucida* extract reduced parasitemia by 1.2% ( $p$  0.01). PP extract was investigated for parasite PfD-10 inhibition (Fig. 1(e)). At 10 and 100 g/mL, 51% ( $p$  0.01) and 92% ( $p$  0.001) inhibition were observed. The PP extract demonstrated promising antiplasmodial activity with an  $IC_{50}$  of 4.0 g/mL (Table S1). According to Kwansa-Bentum et al. [17], PP L. Kunth crude methanolic extract had higher antiplasmodial activity than AP (Burm.f.) Nees ( $IC_{50}$ , 7.2 g/mL). These results indicate that the PP extract ratio increased antiplasmodial activity.

In addition, we tested the PP extract's efficacy in BALB/c albino mice infected with *Plasmodium berghei* ANKA (Pb ANKA) (Fig. S1). The extract had antiplasmodial action, with a 50% effective dose ( $ED_{50}$ ) of 12.86 mg/kg/body weight (Fig. S1(a)). A strong chemosuppression of parasitemia (Fig. S1(b)), inhibitory effect (Fig. S1(d)), and chemosuppression of parasitemia from day 0 to day 4 were clearly demonstrated after the fourth day of treatment (Fig. S1(c)). The treatment of the *P. pellucida* extracts intraperitoneally improved the survival of infected mice (Fig. S1(e)).

The crude methanolic extract of PP L. Kunth demonstrated promising antiplasmodial action (Fig. 1(f)),



**Fig 2.** The  $IC_{50}$  values for each of the extracts compounds 1-2, and chloroquine, which served as a positive control. To determine the  $IC_{50}$  values of the separated compounds, concentrations ranging from 0.78 to 100 g/mL were used in the experiment

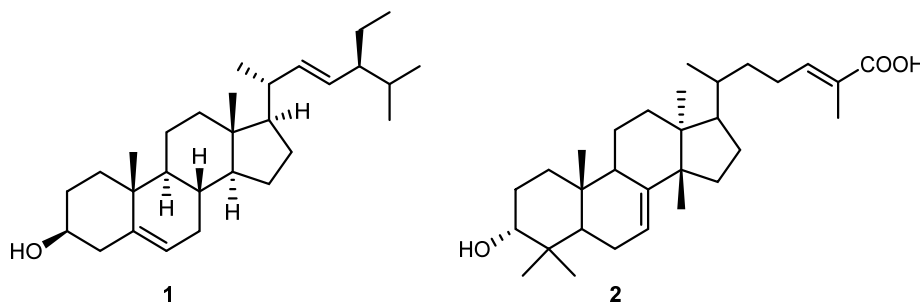
indicating the existence of chemicals responsible for this activity [17]. To further understand the mechanism of antiplasmodial activity of the PP extract, the compound(s) must be isolated and identified to assist in finding their biological function and mode of action. The antiplasmodial activity of crude *n*-hexane (E1), ethyl acetate (E2), and aqueous (E3) extracts were first determined. The antiplasmodial activity test shows the EtOAc (Fig. 2(b)) fraction has the most potent antiplasmodial activity due to having the lowest IC<sub>50</sub> value of 1.533 µg/mL among the other fraction of *n*-hexane (Fig. 2(a)) with IC<sub>50</sub> of 3.892 µg/mL and aqueous extract (Fig. 2(c)) with IC<sub>50</sub> value of 5.016 µg/mL.

Because E2 was the most active extract in our investigation, we isolated and identified the compound(s) from it. The E2 was treated to standard, reverse-phase silica column chromatography and recrystallizations, yielding compounds 1–2 (Fig. 3).

The IR spectrum of compound 1 revealed the existence of O-H stretching, C-O bond, C-C, CH<sub>2</sub> vibrations, hydroxyl, and cycloalkane groups at 3373.6, 1247.0, 1641.0, 1457.3, 1381.6, 1038.0, and 881.6 cm<sup>-1</sup>, respectively. Two methyl singlets, three methyl doublets, and a methyl signal as a triplet was observed in the proton NMR of 1. Characteristic signals were also detected at 4.91, 5.18, and 5.31 ppm, indicating the presence of trisubstituted and disubstituted olefinic groups. The <sup>13</sup>C-NMR spectrum exhibited the presence of six *sp*<sup>3</sup> methyl carbons resonated at δ<sub>C</sub> 12.4 (C-29), 18.4 (C-28), 19.3 (C-27), 20.4 (C-26), 12.9 (C-24) and 40.5 (C-18) and four *sp*<sup>2</sup> carbons resonated at δ<sub>C</sub> 140.5, 121.9, 138.7 and 129.4 ppm indicated to a steroid compound related with stigmasterol

[18]. An oxygenated carbon peak at δ<sub>C</sub> 71.9 ppm suggested the presence of a hydroxyl group and was usually located at C-3. Furthermore, the correlation of proton and carbon signals of 1 was confirmed by <sup>1</sup>H-<sup>1</sup>H COSY and <sup>1</sup>H-<sup>13</sup>C HMBC experiments. Therefore, isolated compound 1 was identified as a 3β-Hydroxy-24-ethyl-5,22-cholestadiene.

Compound 2 was obtained as a white crystal (12 mg), and the IR spectrum displayed absorption peaks for hydroxyl, carboxyl, carbonyl and olefinic groups. The <sup>1</sup>H-NMR spectrum of 2 showed the presence of seven signals of methyl groups as a singlet, resonating at δ<sub>H</sub> 0.89–1.70 ppm. An oxymethine protons resonated at δ<sub>H</sub> 3.49 (1H, dd, *J* = 4.0 and 12.0 Hz, H-3) indicated as a β isomer [19]. Additional functionalities included the signals of two olefinic protons resonated at δ<sub>H</sub> 5.26 (1H, dd, *J* = 6.5 and 3.0 Hz (H-7) and 5.98 (1H, t, *J* = 7.8 Hz, H-24). A total of 30 carbons resonances were observed in the <sup>13</sup>C-NMR and DEPT 135° spectra. Four *sp*<sup>2</sup> carbons include two methine carbons and two quaternary carbons resonated at δ<sub>C</sub> 119.6 (C-7), 144.3 (C-24), 147.4 (C-8), and 128.6 (C-25), respectively. Seven *sp*<sup>3</sup> methyl carbons resonated at δ<sub>C</sub> 24.7 (C-18), 17.1 (C-19), 18.4 (C-21), 12.6 (C-27), 29.5 (C-28), 23.7 (C-29), and 30.8 (C-30). Nine methylene carbons resonated at δ<sub>C</sub> 35.5 (C-1), 28.1 (C-2), 23.1 (C-6), 22.8 (C-11), 33.4 (C-12), 34.7 (C-15), 28.5 (C-16), 35.0 (C-22), and 25.5 (C-23). Five *sp*<sup>3</sup> methines resonated at δ<sub>C</sub> 77.1 (C-3), 48.5 (C-5), 48.4 (C-9), 53.5 (C-17), and 35.9 (C-20). Five *sp*<sup>3</sup> quaternary carbon signals resonated at δ<sub>C</sub> 38.8 (C-4), 35.8 (C-10), 43.5 (C-13), and 52.7 (C-14) while one carbonyl group resonated at δ<sub>C</sub> 171.8 (C-26)



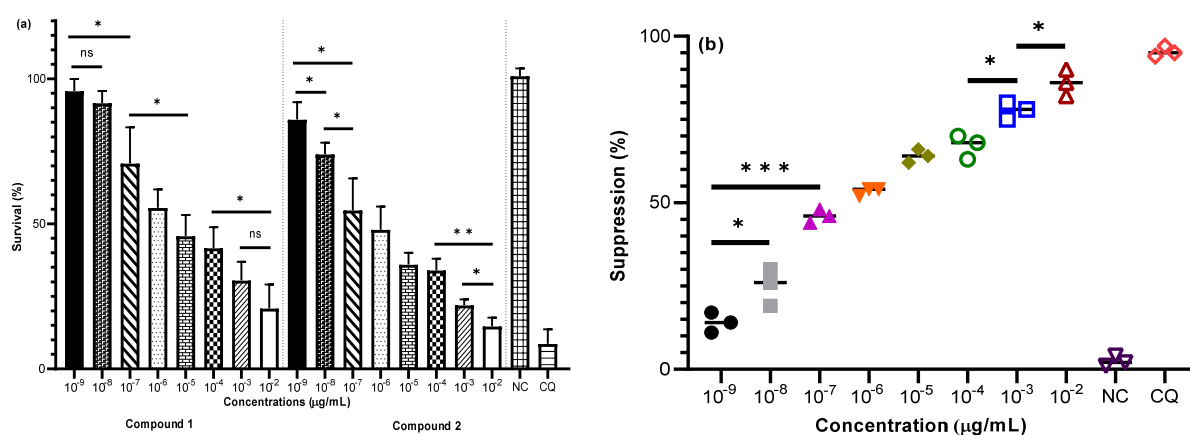
**Fig 3.** Chemical structure of 3β-hydroxy-24-ethyl-5,22-cholestadiene (1) and 3β-hydroxy-9-lanosta-7,24*E*-dien-26-oic acid (2)

was assigned as a carboxylic acid group. The presence of seven  $sp^3$  methyls, one carboxylic acid group, and nine  $sp^3$  methylene carbons were assigned as a triterpenoid tetracyclic similar to a lanostane derivative skeleton [20-21]. The position of the tetracyclic systems (A, B, C and D) were assigned by the correlation between proton and carbon using HMBC and  $^1\text{H}$ - $^1\text{H}$  COSY spectra. The HMBC correlations between six  $sp^3$  methyl carbons (C-18, C-19, C-21, C-27, C-28, C-29, and C-30) and six  $sp^3$  quaternary carbon C-13, C-10, C-20, C-4, C-4, and C-14, respectively, confirmed the tetracyclic systems. The position of the hydroxy group at C-3 was confirmed by the correlation between  $sp^3$  oxygenated methine carbon at  $\delta_c$  77.1 and  $sp^3$  methyl carbons at  $\delta_c$  29.5 (C-28) and 23.7 (C-29). The position of an olefin group at C-7/C-8 was confirmed by HMBC correlations between H-5, H-6, H-9 to C-7 and H-6, H-9, H-11 to C-8. Additional correlation between H-24 to C-25 and C-27; H-27 to C-24, C-25, and C-26 ( $\delta_c$  171.8), suggested that a carboxylic acid group is not located in the tetracyclic systems. Another olefin group was confirmed at C-24/C-25 by HMBC correlations between H-23 to C-24 and H-27 to C-25. The side chain of **2** was further confirmed by a continuous sequence from C-15 to C-24 as deduced from HMBC and  $^1\text{H}$ - $^1\text{H}$  COSY and spectra. In addition, the HMBC correlation of H-21 to C-20 indicated the side chain is connected to C-20. Therefore, isolated compound **2** was

identified as a  $3\beta$ -hydroxy-9-lanosta-7,24*E*-dien-26-oic acid. The isolated compound **2** was first reported from this plant. All compounds were identified, as reported previously [22-23].

Compounds **1-2** were evaluated for their ability to suppress parasitemia and inhibit the parasite growth. The *in vitro* antiplasmodial activity of **1-2** and chloroquine as a positive control are reported in Fig. 2(d-e). Steroid **1** exhibited antiplasmodial activity against Pf D-10 with an  $\text{IC}_{50}$  value of  $7.35 \mu\text{g/mL}$ . Interestingly, triterpenoid **2** displayed a more pronounced antiplasmodial activity ( $\text{IC}_{50}$ ,  $5.30 \mu\text{g/mL}$ ) than chloroquine ( $\text{IC}_{50}$ ,  $7.84 \mu\text{g/mL}$ ). The  $\text{IC}_{50}$  value of chloroquine is in good agreement with Perumal et al. [23].

Next, we take a look at the parasitemia as well as the inhibition rates of **1-2**. In the same way, as was the case with the PP extract, the antiplasmodial activity of compounds **1** and **2** increased in a manner that was dependent on the concentration. For **1**, the prevalence of parasitemia ranged from roughly 4.6 to 1.1%, and for **2**, the prevalence ranged from approximately 3.7 to 0.7% within a range of 0.78 to  $100 \mu\text{g/mL}$ , respectively (Fig. 4(a)). Fig. 4(b) demonstrates that the growth of the parasite Pf D-10 was stifled as a result of exposure to **1-2**. Under the same conditions, steroid **1** showed a moderate suppression of Pf D-10 at a concentration of

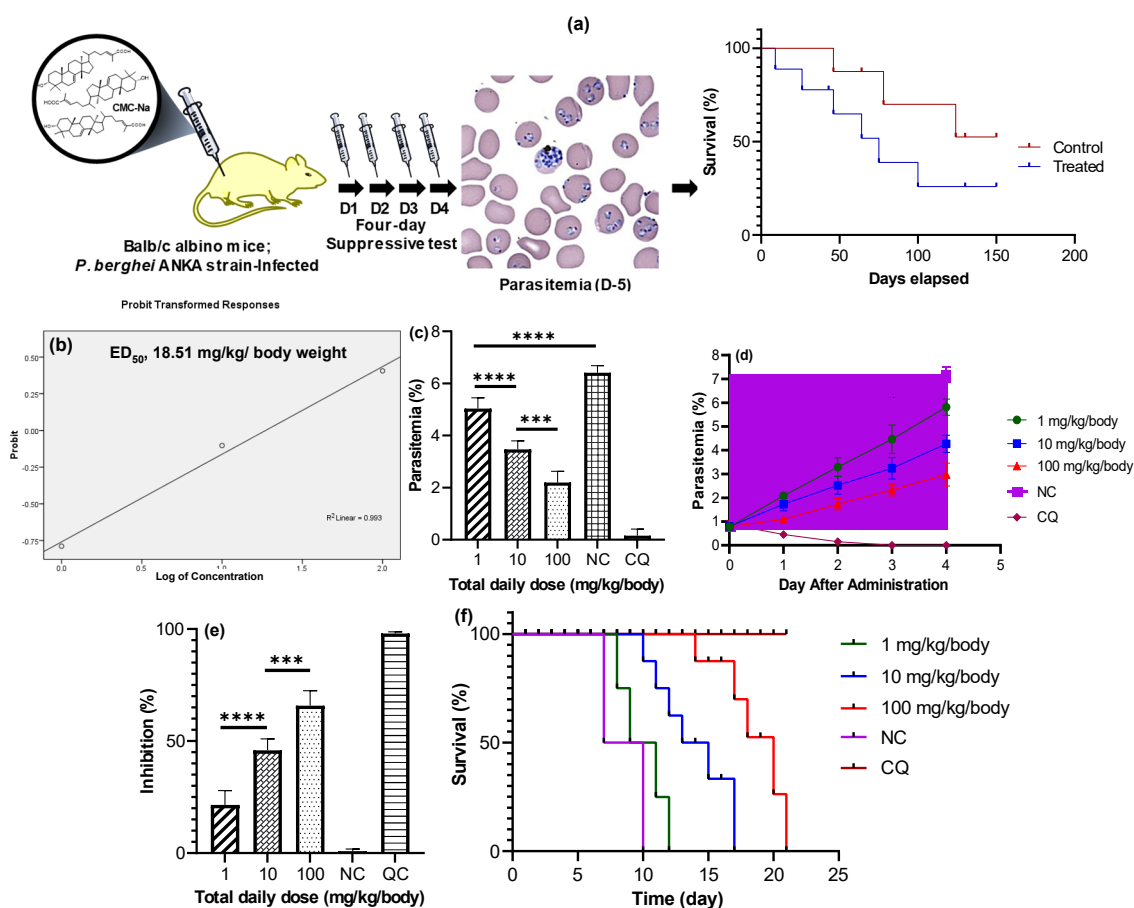


**Fig 4.** An *in vitro* antiplasmodial activity of steroid **1** and triterpenoid **2**. (a) Parasitemia and (b) inhibition rates of untreated parasites (0.2% DMSO) and parasites treated with compounds **1-2** at a concentration of 0.78 to  $100 \mu\text{g/mL}$  and chloroquine ( $1 \mu\text{M}$ ) are shown. All experiments were performed in triplicate ( $n = 3$ ). The standard Deviation (SD) is indicated by the error bars. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and not significant (ns)

0.78 g/mL (1.7%) and 1.56 g/mL (4.0%), but triterpenoid **2** showed a considerable suppression impact of 14% and 25% ( $p < 0.05$ ). In addition, the ability of compounds **1** and **2** to preserve the life of parasitized red blood cells was investigated. Steroid **1** had a moderate effect (about 96%) when tested at a diluted quantity, whereas triterpenoid **2** demonstrated superior parasite killing at a rate of 86% when tested at the same doses. The survival rates dropped in a manner that was dependent on the concentration of the organisms (Fig. S2).

The substantial response of triterpenoid **2** against Pf D-10 was mediated by a carboxyl functional group, which promoted carboxyl-carboxylate association for the

purpose of providing a stabilizing force in protein interaction [24]. Contrary to compound **2**, a low response of **1** is most likely caused by the compound's cytotoxicity rather than particular action against the parasite itself or being negatively influenced by poor pharmacokinetics [25-27]. This is because compound **1** has poor pharmacokinetics. The antiplasmodial activity of compounds **1-2** supported the hypothesis that the species *Pf L. Kunth* could serve as a possible source of novel antimalarial metabolites. This allowed for the identification of potential antiplasmodial leads derived from *Pf L. Kunth*, which could lead to the discovery of a new anti-malarial candidate.



**Fig 5.** *In vivo* test of triterpenoid **2** in BALB/c albino mice infected with *Pb* ANKA. (a) *In vivo* four-day suppressive test. Infected mice were treated with 0.5% CMC-Na as a negative control (NC), CQ as a positive control (25 mg/kg/body) and triterpenoid **2** at a daily dose of 1, 10 and 100 mg/kg/body. Mice received treatment from day 1 to day 4. On the 5<sup>th</sup> day, Giemsa-stained blood smears were prepared and analyzed microscopically. (b) ED<sub>50</sub> value of triterpenoid **2** in mice. (c) Parasitemia after the fourth day of treatment. (d) Chemo suppression of parasitemia from day 0 to day 4. (e) Suppression rates after the fourth day of treatment. (f) Survival rates of *Pb* ANKA in BALB/c albino mice. SD is indicated by the error bars. \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$



Triterpenoid **2** proved to be the most powerful antimalarial agent in this investigation, so the next thing we did was test it on mice infected with the malaria parasite Pb ANKA. The method described by Peters et al. [26] was implemented for the purpose of analyzing the *in vivo* test of **2**. Mice of the BALB/c albino strain were given Pb ANKA by the intraperitoneal route. After 4 d of treatment with 0.5% CMC-Na as a negative control, chloroquine diphosphate as a positive control, and triterpenoid **2** at daily doses of 1, 10, and 100 mg/kg/body, thin blood smears stained with Giemsa were obtained for each mouse on the fifth day. This was followed by 4 d of treatment with chloroquine diphosphate as a positive control. On a microscopic level, parasitemia and the reduction of parasite development were observed and measured (Fig. 5(a)). Up until day 21, the survival of mice was carefully monitored and tracked. It was believed that triterpenoid **2** had some biological activity, with an ED<sub>50</sub> value of 18.51 mg/kg/body weight (Fig. 5(b)). The drug quinine, which is available for clinical use, has an elimination half-life (ED<sub>50</sub>) of 34 mg/kg/day and a slow clearance [28]. Triterpenoid **2** was successful in inhibiting the development of parasitemia in mice infected with Pb ANKA, as shown in Fig. 5(c). Even at the lowest dose (1 mg/kg/body), intraperitoneal administration of triterpenoid **2** resulted in a 78% reduction in parasitemia. To be more specific, **2** was successful in killing 22% of the parasites. The incidence of parasitemia fell to lower levels as higher doses of **2** were administered. The rates of parasitemia were reduced to approximately 54 and 34%, respectively when triterpenoid **2** was administered at doses of 10 and 100 mg/kg/body. In addition, the effectiveness of chemotherapy in reducing parasitemia was evaluated from day 0 to day 4 of treatment (Fig. 5(d)). The levels of parasitemia in mice that had not been given any treatment rose consistently after infection, but the number **2** therapy had a significant chemo-suppressive effect that was dose-dependent. Fig. 5(e) shows the suppression of parasite growth that was caused by triterpenoid **2**. This compound had a substantial inhibitory effect against Pb ANKA in mice. Triterpenoid **2** strongly suppressed Pb ANKA, as can be seen in Fig. 5(e), which suggests that it also inhibited the proliferation of parasites

in mice. The suppression rates varied from 66 (at 100 mg/kg/body) to 46 (10 mg/kg/body) to 22% (1 mg/kg/body). These findings made it abundantly evident that factor **2** was responsible for eliminating the parasites. The treatment of **2** also increased the number of ill mice that survived longer. Based on the *in vivo* experiment (Fig. 5(f)) mice that were not given any treatment succumbed to the Pb ANKA infection after 10 d, whereas mice that were given 1, 10, or 100 mg/kg/body of triterpenoid **2** lived until 12, 17, and 21 d, respectively. Positive control groups were given chloroquine diphosphate at a daily dose of 25 mg/kg/body for as long as 21 d, which led researchers to conclude that the Pb ANKA infection was completely eradicated in the mice who participated in the study. These findings are in agreement with those found by Fang et al. [29].

## ■ CONCLUSION

In this study, the PP extract displayed a more pronounced antiplasmodial activity than the AP extract. Steroid **1** and triterpenoid **2** exhibited promising antiplasmodial activity. This is the first report of 3 $\beta$ -hydroxy-9-lanosta-7,24*E*-dien-26-oic acid (**2**) as an antiplasmodial agent, and we have shown its promising efficacy in an *in vivo* mouse model. Our results suggest a novel triterpenoid that has the potential for use as an antimalarial agent. Although 3 $\beta$ -hydroxy-9-lanosta-7,24*E*-dien-26-oic acid may not yet be an ideal drug candidate for treating malaria disease in humans, it may provide a useful lead structure to produce more effective antiplasmodial agents. We plan to do an *in vivo* test with the combination of triterpenoid **2** and antimalarial drug artesunate to improve the efficacy of drug action [30].

## ■ ACKNOWLEDGMENTS

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Ghosh at the Max Planck Institute for their helpful discussions.

## ■ AUTHOR CONTRIBUTIONS

The experiment was conducted by NB with the analysis being conducted by MAM and YS. The NMR and *in vivo* analysis were done by WM and AW. The manuscript was arranged and revised by AMI, BS, and JS. All the authors mentioned have agreed to the final version of this manuscript.

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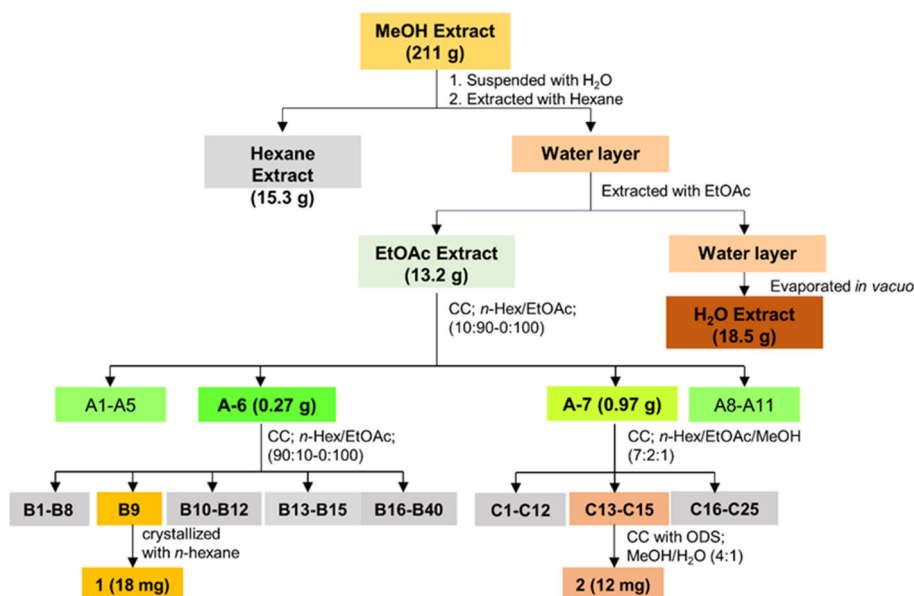
## Supplementary Data

This supplementary data is a part of a paper entitled “Evaluation of the Antiplasmodial Properties of *Andrographis paniculata* (Burm.f.) and *Peperomia pellucida* (L.) Kunth”.

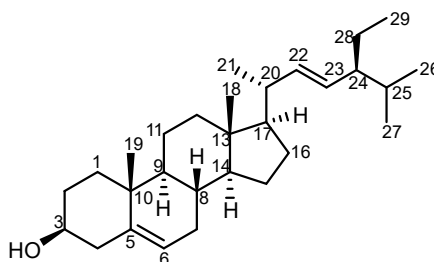
### General information

Commercial grade solvents were used unless stated otherwise. Solvents for chromatography were distilled prior to use. Analytical thin layer chromatography (TLC) was performed on Kieselgel 60 F254 glass plates and Octa Desyl Silane (ODS) RP-18 (Merck). Spots were visualized by 10% sulfuric acid in ethanol. Silica column chromatography was performed on silica gel G60 (Merck) (230-400 mesh) and ODS RP-18.

Optical rotations were recorded with an ATAGO AP-300 polarimeter. InfraRed (IR) spectra were measured by using a PerkinElmer spectrum-100 FT-IR spectrometer in KBR.  $^1\text{H}$ ,  $^{13}\text{C}$  and two-dimensional NMR spectra were measured with a JEOL JNM A-500 (500 MHz) spectrometer at 296 K. Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ( $\text{CD}_3\text{OD}$ :  $\delta$  4.78 in  $^1\text{H}$ -NMR). Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ( $\text{CDCl}_3$ :  $\delta$  7.26 in  $^1\text{H}$  and 77.20 in  $^{13}\text{C}$ -NMR;  $\text{CD}_3\text{OD}$ :  $\delta$  4.78 in  $^1\text{H}$ -NMR and 49.20 in  $^{13}\text{C}$ -NMR). The following abbreviations are used to indicate peak multiplicities: s singlet; d doublet; dd doublet of doublets; t triplet; m multiplet. Coupling constants ( $J$ ) are reported in Hertz (Hz). Mass spectra were recorded using a Waters Xevo QTOF mass spectrometer.

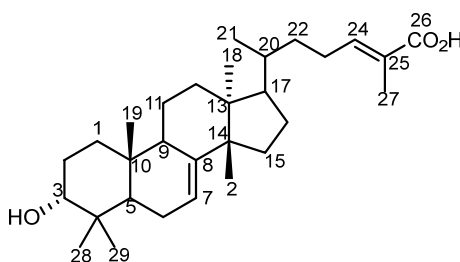


Scheme S1. Isolation procedures of compound 1-2



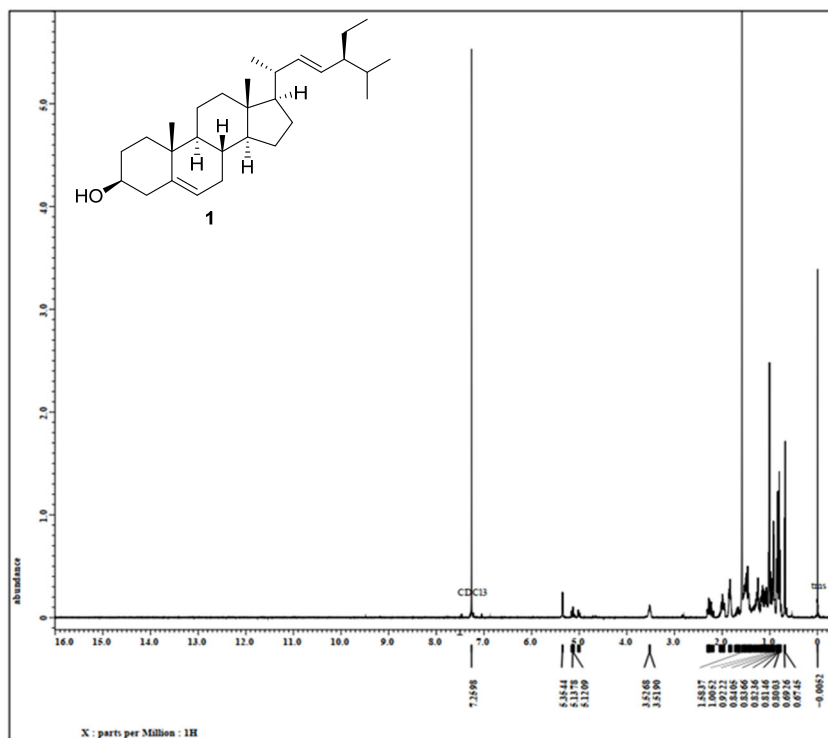
**3 $\beta$ -Hydroxy-24-ethyl-5,22-cholestadiene (1)**

A white crystal, m.p 150–152 °C;  $[\alpha]_D$  -38.6 (c 0.3, CHCl<sub>3</sub>); IR (KBr) $V_{\max}$  3401, 2861 1457 and 1039 cm<sup>-1</sup>; <sup>1</sup>H-NMR (500 MHz, Chloroform-d).  $\delta$  1.08 (m, 1H, H-1), 1.84 (m, 1H, H-1'), 1.49 (m, 1H, H-2), 1.81 (m, 1H, H-2'), 3.52 (m, 1H, H-3), 2.28 dd,  $J = 2.0, 5.2$  Hz, 1H, H-4), 2.30 (dd,  $J = 2.0, 5.2$  Hz, 1H, H-4'), 5.35 (d,  $J = 5.2$  Hz, 1H, H-6), 1.54 (m, 1H, H-7), 1.96 (m, 1H, H-7'), 1.46 (m, 1H, H-8), 0.94 (m, 1H, H-9), 1.46 (m, 1H, H-11), 1.49 (m, 1H, H-11'), 1.15 (m, 1H, H-12), 1.95 (m, 1H, H-12'), 1.03 (s, 1H, H-14), 1.07 (m, 1H, H-15), 1.56 (m, 1H, H-15'), 1.26 (m, 1H, H-16), 1.67 (m, 1H, H-16'), 1.13 (m, 1H, H-17), 0.67 (s, 3H, H-18), 1.00 (s, 3H, H-19), 2.02 (m, 1H, H-20), 0.92 (d,  $J = 9.5$  Hz, 1H, H-21), 5.16 (dd,  $J = 8.5, 15.0$  Hz, 1H, H-22), 5.00 (dd,  $J = 8.5, 15.0$  Hz, 1H, H-23), 1.53 (m, 1H, H-24), 1.45 (m, 1H, H-25), 0.84 (d,  $J = 6.4$  Hz, 3H, H-26), 0.82 (d,  $J = 6.1$  Hz, 3H, H-27), 1.15 (t,  $J = 3.2$  Hz, 1H, H-28), 0.80 (t,  $J = 6.0$  Hz, 1H, H-29); <sup>13</sup>C-NMR (125 MHz, Chloroform-d).  $\delta$  37.4 (C-1), 31.8 (C-2), 72.0 (C-3), 42.5 (C-4), 140.9 (C-5), 121.9 (C-6), 32.1 (C-7), 21.3 (C-8), 50.3 (C-9), 36.7 (C-10), 21.3 (C-11), 39.9 (C-12), 42.5 (C-13), 56.9 (C-14), 24.5 (C-15), 28.4 (C-16), 56.1 (C-17), 12.1 (C-18), 19.5 (C-19), 40.7 (C-20), 21.2 (C-21), 138.5 (C-22), 129.5 (C-23), 51.4 (C-24), 31.8 (C-25), 21.3 (C-26), 19.1 (C-27), 25.6 (C-28), 12.2 (C-29); HR-TOFMS  $m/z$  413.3748 [M+H]<sup>+</sup>, (calculated for C<sub>29</sub>H<sub>48</sub>O,  $m/z$  412.3704). In agreement with published data [1].

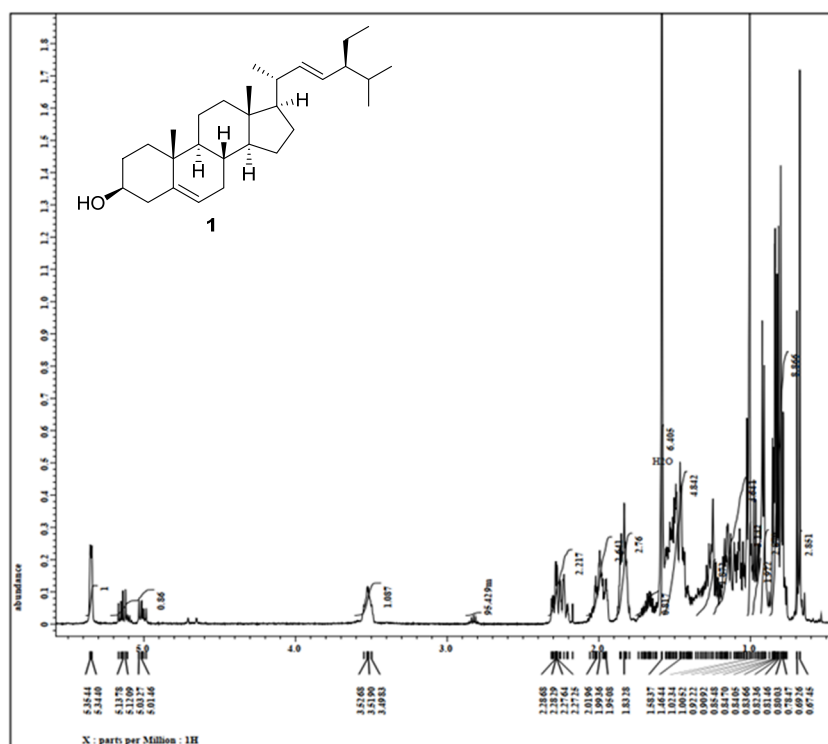
**3 $\beta$ -hydroxy-9-lanosta-7,24E-dien-26-oic acid (2)**

A white crystal, m.p 162–165 °C;  $[\alpha]_D$  +15.6 (c 0.3, EtOH); IR (KBr) $V_{\max}$  3241, 1701 1633 and 1120 cm<sup>-1</sup>; <sup>1</sup>H-NMR (500 MHz, Methanol-d).  $\delta$  1.54 (m, 2H, H-1), 1.59 (m, 2H, H-2), 3.49 (dd,  $J = 4.0, 12.0$  Hz, 1H, H-3), 1.45 (m, 1H, H-5), 1.85 (m, 2H, H-6), 5.26 (dd,  $J = 3.0, 6.5$  Hz, 1H, H-7), 2.18 (m, 1H, H-9), 1.40 (m, 2H, H-11), 1.32 (m, 2H, H-12), 1.40 (m, 2H, H-15), 1.96 (m, 2H, H-16), 1.47 (m, 1H, H-17), 0.96 (s, 3H, H-18), 0.89 (s, 3H, H-19), 1.61 (s, 1H, H-20), 0.85 (d,  $J = 6.5$  Hz, 3H, H-21), 1.51 (m, 2H, H-22), 2.02 (m, 2H, H-23), 5.98 (t,  $J = 7.8$  Hz, 3H, H-24), 2.48 (s, 3H, H-27), 1.08 (s, 3H, H-28), 0.73 (s, 3H, H-29), 1.70 (s, 3H, H-30); <sup>13</sup>C-NMR (125 MHz, Methanol-d).  $\delta$  35.5 (C-1), 28.1 (C-2), 77.1 (C-3), 38.8 (C-4), 48.5 (C-5), 23.1 (C-6), 119.6 (C-7), 147.4 (C-8), 48.4 (C-9), 35.8 (C-10), 22.8 (C-11), 33.4 (C-12), 43.5 (C-13), 52.7 (C-14), 34.7 (C-15), 28.5 (C-16), 53.3 (C-17), 24.7 (C-18), 17.1 (C-19), 35.9 (C-20), 18.4 (C-21), 35.0 (C-22), 25.5 (C-23), 144.3 (C-24), 128.6 (C-25), 171.8 (C-26), 12.6 (C-27), 29.5 (C-28), 23.7 (C-29), 30.8 (C-30); HR-TOFMS  $m/z$  455.3571 [M-H]<sup>-</sup>, (calculated for C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>,  $m/z$  456.3503). In agreement with published data [2].

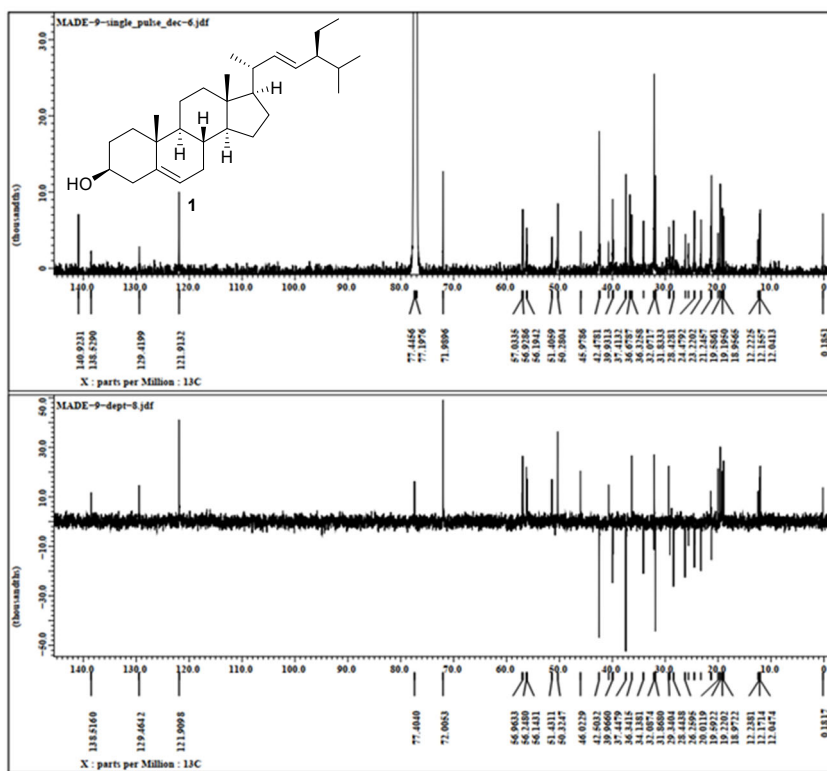
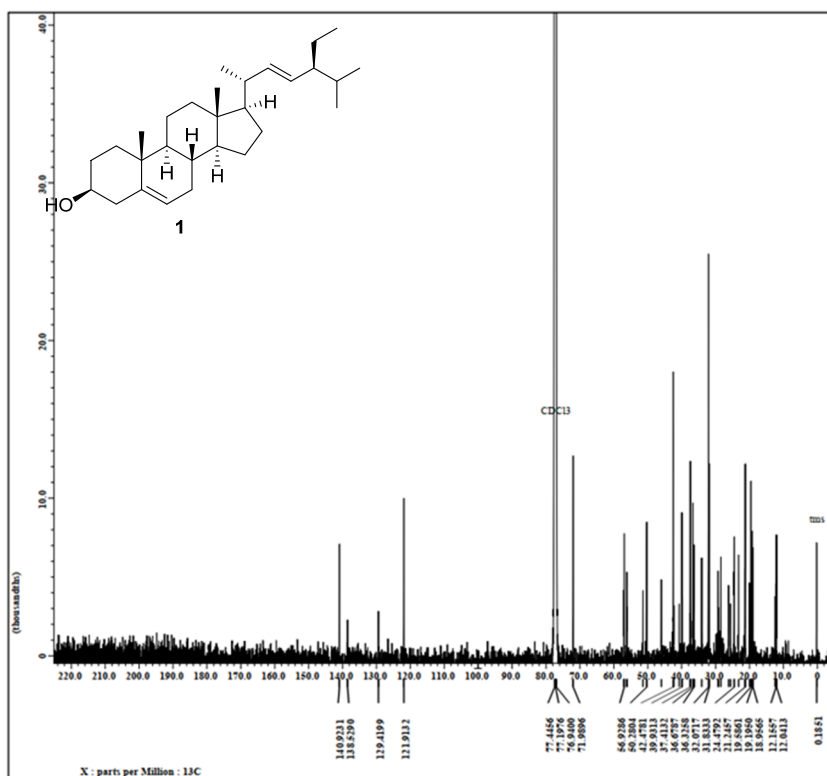
Nuclear Magnetic Resonance (NMR) of Isolated Compound 1-2

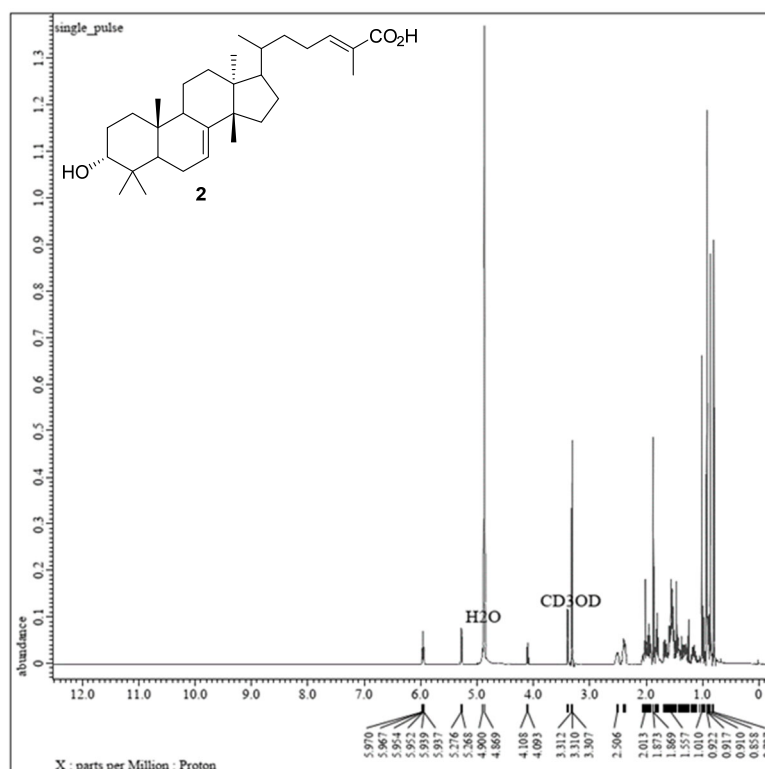


<sup>1</sup>H-NMR (CDCl<sub>3</sub>, 500 MHz)

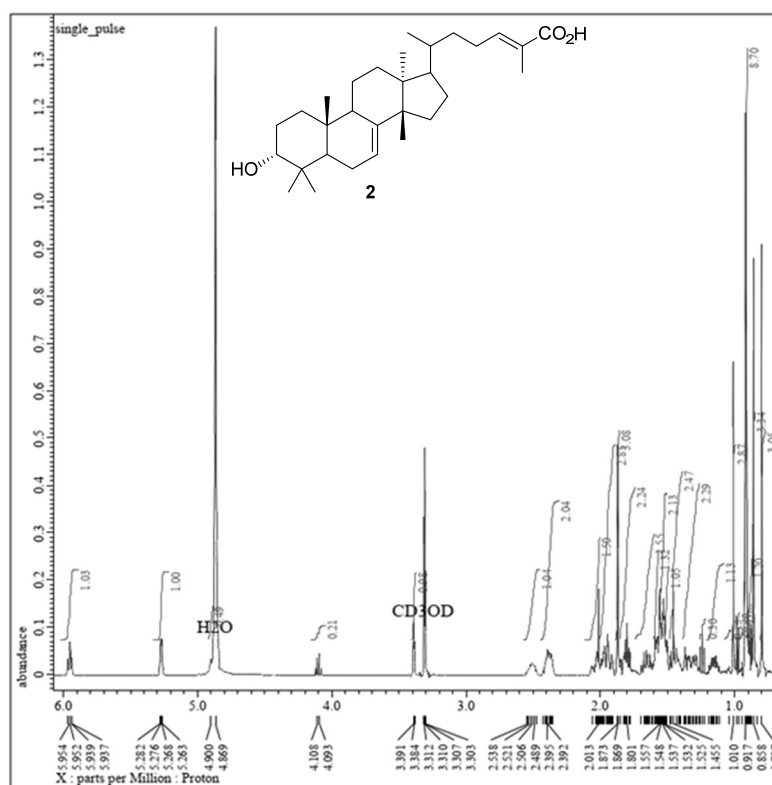


<sup>1</sup>H-NMR-Expansion (CDCl<sub>3</sub>, 500 MHz)



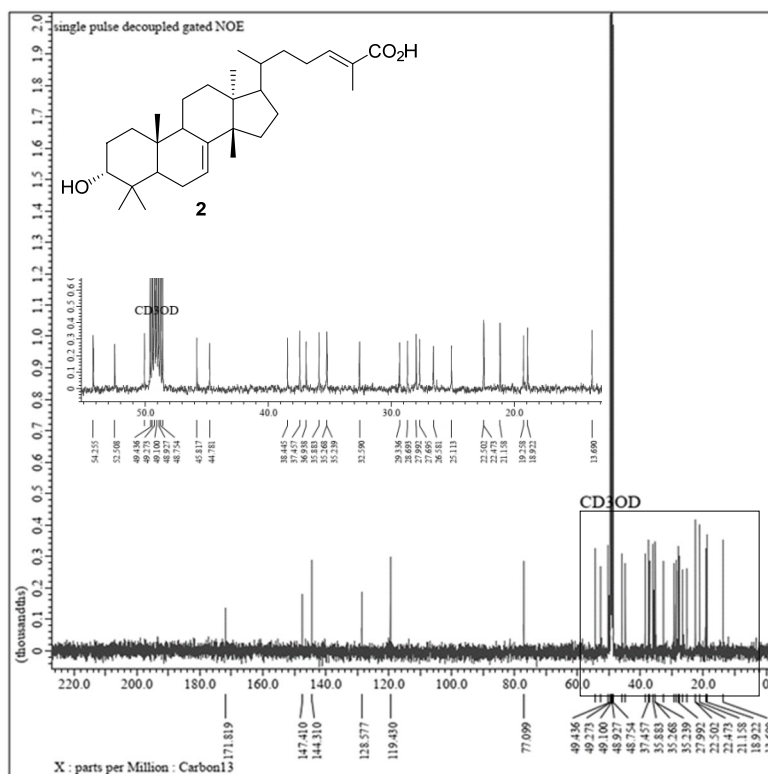
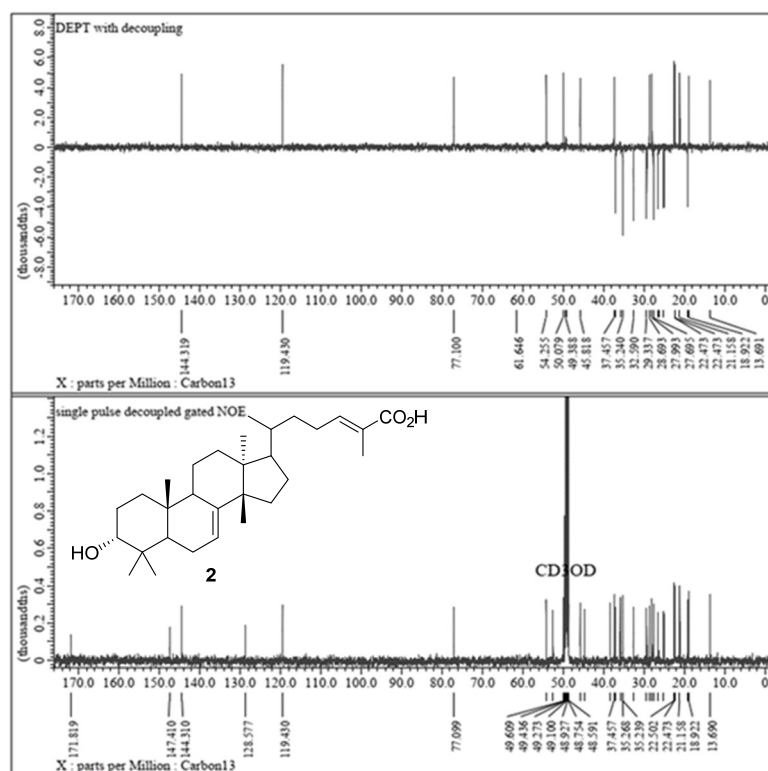


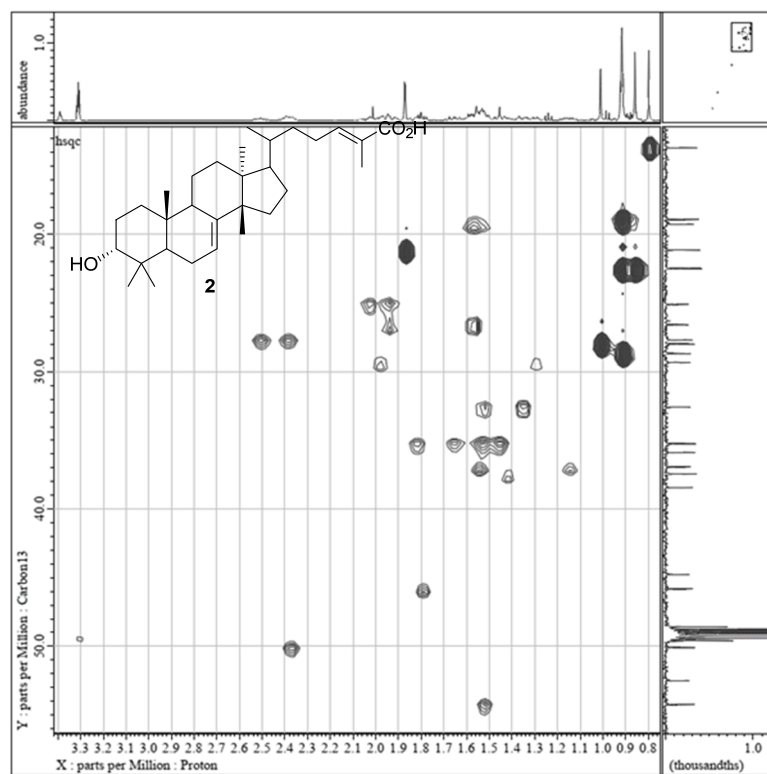
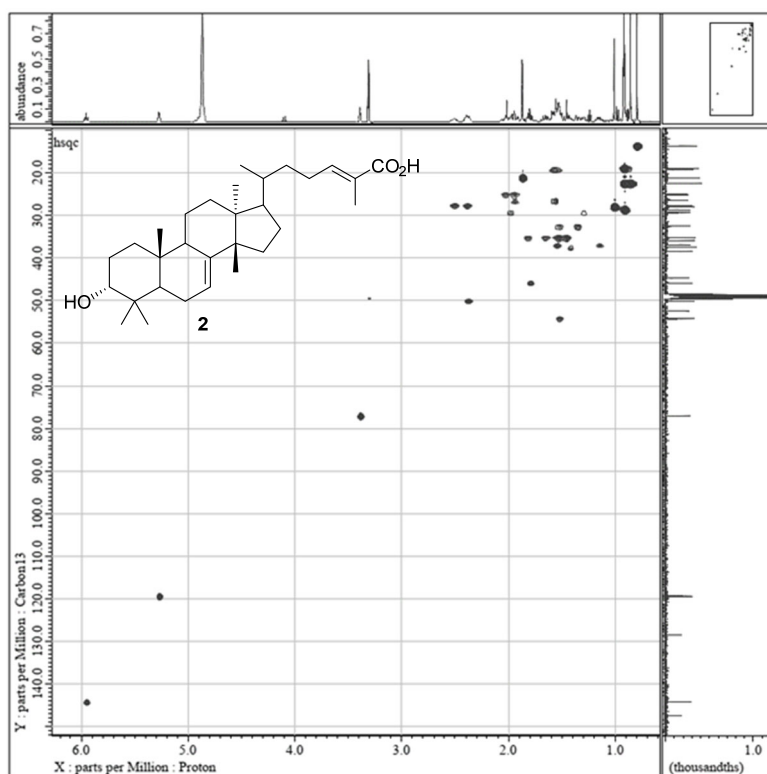
<sup>1</sup>H-NMR (CD<sub>3</sub>OD, 500 MHz)

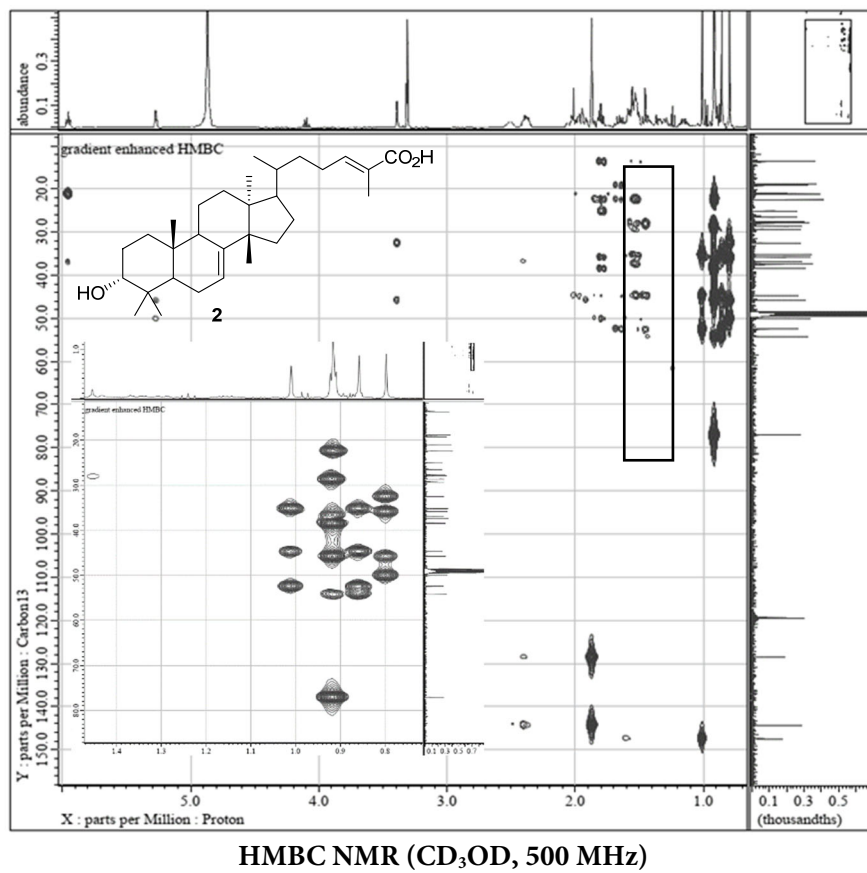


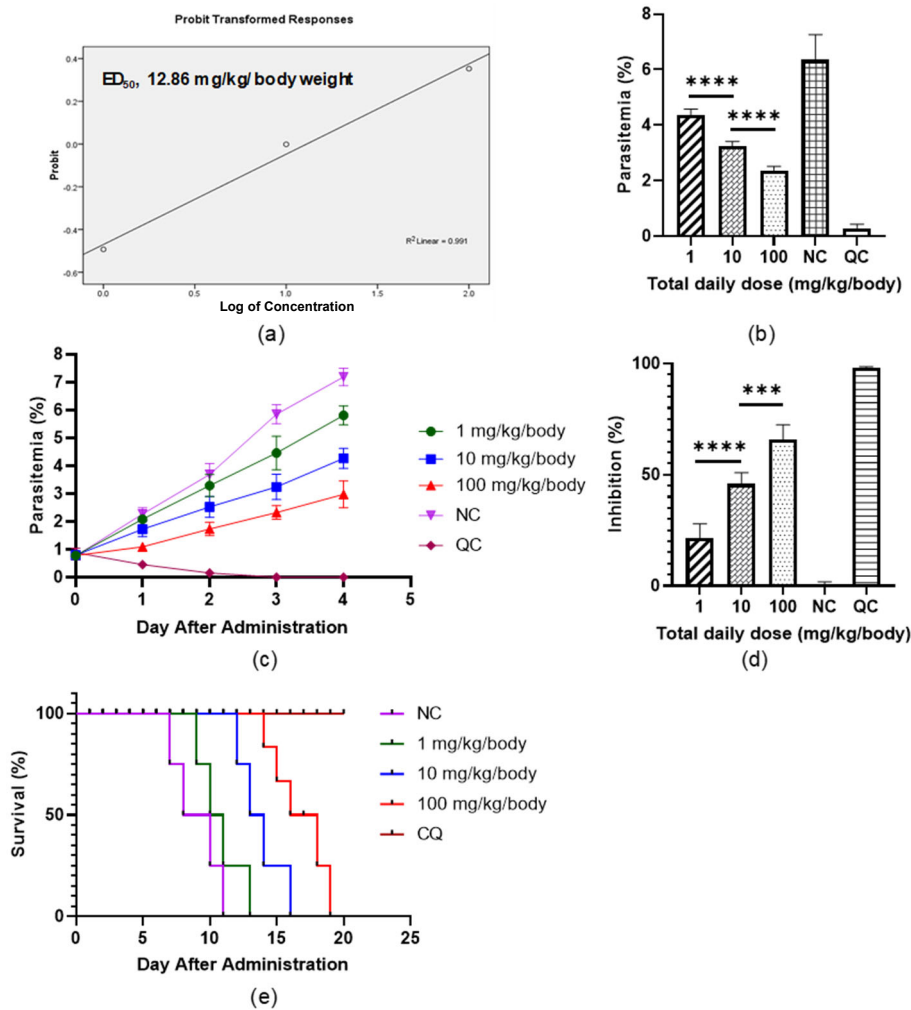
<sup>1</sup>H-NMR-Expansion (CD<sub>3</sub>OD, 500 MHz)



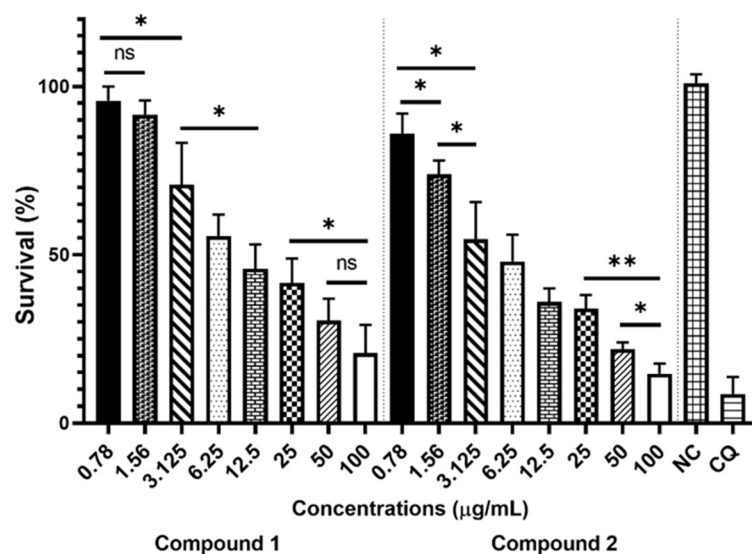
 $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ , 125 MHz)DEPT 135 and  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ , 125 MHz)







**Fig S1.** *In vivo* test of the PP extract against malarial parasite *Pb* ANKA strain in BALB/c albino mice. (a) ED<sub>50</sub> value of the PP extract in mice. (b) Parasitemia rates after the fourth day of treatment. (c) Chemosuppression of parasitemia from day 0 to day 4. (d) Inhibition rates after the fourth day of treatment. (e) Survival mice treated with 0.5% CMC-Na as a negative control, the PP extract at various doses (1, 10 and 100 mg/kg/body) and chloroquine as a positive control. Bars in Fig. S1(b) and S1(d) indicate the parasitemia and parasite growth inhibition rates treated with 0.5% CMC-Na (NC) (grid), CQ (horizontal) and the PP extract at a daily dose of 1 mg/kg/body (pencil striped), 10 mg/kg/body (herringbone) and 100 mg/kg/body (halftone), while in Fig. S1(c) and S1(e) indicate parasitemia reduction from day 0 to day 4 and survival of mice treated with 0.5% CMC-Na (pink), CQ (carmine) and the PP extract at a daily dose of 1 mg/kg/body (green), 10 mg/kg/body (blue) and 100 mg/kg/body (red). SD is indicated by the error bars. \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$



**Fig S2.** Survival rates of untreated parasites (0.2% DMSO) and parasites treated with compounds 1–2 at a concentration of 0.78 to 100 µg/mL and CQ (1 µM) are shown. Bars indicate parasite survival rates. Untreated parasites (NC - grid), CQ (horizontal) and compounds 1–2 at a concentration of 100 µg/mL (white), 50 µg/mL (small striped), 25 µg/mL (checkerboard), 12.5 µg/mL (brick), 6.25 µg/mL (halftone), 3.125 µg/mL (big striped), 1.56 µg/mL (hexagon) and 0.78 µg/mL (black). All experiments were performed in triplicate ( $n = 3$ ). Standard Deviation (SD) is indicated by the error bars. \* $p < 0.05$ , \*\* $p < 0.01$ , not significant (ns)

### Statistical analysis

Experiments were performed independently in triplicate and average are presented. Statistical analysis was performed by unpaired two-tailed  $t$ -test using GraphPad Prism. The statistics were significant when \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

**Table S1.** *In vitro* Antiplasmodial activity of plant extract against *Plasmodium falciparum* [3]

IC <sub>50</sub> value (µg/mL)	Category of activity
< 10	Promising
10–20	Moderate
20–40	Good
40–70	Marginally potent
> 70	Poor

### References

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- [2] Isaka, M., Chinthanom, P., Sappan, M., Supothina, S., Vichai, V., Danwisetkanjana, K., Boonpratuang, T., Hyde, K.D., and Choeyklin, R., 2017, Antitubercular activity of mycelium-associated *Ganoderma* lanostanoids, *J. Nat. Prod.*, 80 (5), 1361–1369.
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