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Isolation of an anti-tumour terpenoid from stem bark of *Spondianthus preussii var. preussii* Engl.

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Summary

We report a biologically monitored phytochemical separation of stem bark of *Spondianthus preussii* var. *preussii* against a panel of human cancer cell lines *in vitro* and the P-388 murine lymphocytic leukemia cells in culture. An ethylacetate extract of the stem bark exhibited selective cytotoxicity against human melanoma (ED_{50} = 10.0ug/ml). Further activity-guided fractionation of the ethylacetate extract by flash chromatography and subsequent purification on preparative thin layer chromatography led to the identification of a lupane-type triterpene, 3β-hydroxy-20(29) – lupenoic acid, by spectroscopic methods. This is the first report of the occurrence of this compound in *S. preussii* var. *preussii*. It is also the first time this triterpene is being shown to exhibit *in vitro* anti-tumor activity against human melanoma (ED_{50} = 2.4ug/ml). This compound could be a promising bioactive natural product since it has been previously reported to exhibit a range of biological activities including *in vivo* and *in vitro* antiplasmodial activity and it is not toxic.

Keywords: *Spondianthus preussii, Euphorbiaceae, Anti-tumour activity, Hydroxylupenoic acid.*

Résumé

Biologiquement dirigée, nous rapportons une séparation phytochimique du tronc et la tige de Spondianthus preussii var. preussii contre un panneau de cancer humain, lignes cellulaires in vitro et les P-388 cellules de la leucémie du lymphocyte du murine dans la culture. Un extrait de l'ethylacetate du tronc et la tige a exposé le cytotoxicité sélectif contre la mélanome humain (ED50 = 10.0ug/ml).En plus, les fractionnaires de l'ethylacetate extrait par l'éclat de chromatographie et la purification subséquente sur la chromatographie de la couche minces ont mené à l'identification d'un triterpene du lupane-type, 3 -hydroxy-20(29) - acide du lupenoique, par les méthodes du spectroscopique. C'est le premier rapport de l'événement de ce composé dans S. preussii var. preussii. C'est aussi la première fois que ce triterpene est montré pour exposer dans l' activité de l'anti-tumeur du vitro contre mélanome humain (ED50 = 2.4ug/ml). Ce composé pourrait être un bioactive prometteur produit naturel depuis qu'il a été rapporté d'exposer une gamme d'activités biologiques qui incluent in vivo et in vivo dans l'activité 'anti-plasmode du vitro et ce n'est pas toxique.

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Introduction

There are two African varieties of *Spondianthus preussii* Engl. (Euphorbiaceae) namely *S. preussii* var. *preussii* and *S. preussii* var. *glaber [1]*. Both varieties are commonly found in swampy areas of the rain forest. *S. preussii* var. *preussii* is known in Yoruba as "Obo ekute" [1] and it is locally recognised as a poisonous plant. However, it is sometimes used for the treatment of toothache [2] and as a rat poison [1].

Some species of the family Euphorbiaceae have recently yielded interesting terpenoids which have become of great interest to the National Cancer Institute (NCI), Bethesda, Maryland, USA. Several of them exhibit very good *in vivo* anticancer activity [3].

In the course of our search for anticancer agents of plant origin, our attention turned to *S. preussii* var. *preussii* after an ethylacetate extract of the stem bark was found to exhibit significant anti-tumour activity when tested against a panel of human cancer cell lines and the P-388 murine lymphocytic leukemia system in cell culture. We now report for the first time the isolation and identification of 3β-hydroxy-20 (29) – lupenoic acid as the major anti-tumour constituent of the stem bark. We are not aware of any previous biological or phytochemical report on this variety of *S. preussii*.

Experimental

Plant material: Stem bark materials of *Spondianthus preussii* Engl. var *preussii* were collected in Aponmu area of Ondo State in April 1991 and authenticated at the Forestry Research Institute of Nigeria, Ibadan where a herbarium specimen (voucher number FHI 20213) is deposited. Stem bark samples were dried at 45°C for 10 days and powdered for analysis.

Extraction: 1kg of the powdered sample was percolated with methanol (2Lt) at room temperature overnight. The process was repeated daily for 5 days. The pooled methanol extract was concentrated *in vacuo* and suspended in methanol/water (2:1). This was partitioned into equal volume of n-hexane (1 vol.). The hexane extract was discarded. Methanol was completely evaporated and 500ml water added. This aqueous suspension was then partitioned with aliquots (2 x 300ml) of ethylacetate. The pooled ethylacetate extract was dried over anhydrous sodium sulphate, filtered and the solvent evaporated *in vacuo* to give the ethylacetate extract (yield = 4.5gm). Accurately weighed quantities of the dried ethylacetate and aqueous extracts were submitted for bioassay.

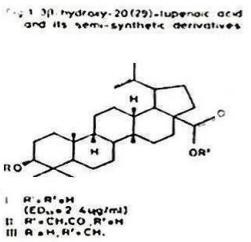

Fig. 1: 3β-hydroxy-20(29)-lupenoic acid and its' semi-synthetic derivatives

Fractionation of active ethylacetate extract: 4.0gm of the active ethylacetate extract (ED_{50} = 10.0ug/ml, Melanoma) was fractionated by flash chromatography over florisil (Fischer Scientific Co., Itasca, IL.) eluting with gradient mixtures of hexane, toluene and ethylacetate. Four fractions (A – D; pooled on the basis of similar TLC profiles) were obtained. Fraction B (1.4gm) eluted with mixtures of toluene / ethylacetate (1: 1, 1:2, 2Lt) yielded a cytotoxic residue which was finally purified on preparative thin layer chromatography on Kieseigel 60: F_{254} (0.5mm, E. Merck, Darmstadt) developing in hexane / ethylacetate/ toluene (1:1:1). This yielded compound I (figure 1).

Acetylation of compound I: 200mg of I was treated with acetic anhydride / pyridine (1:4) overnight and worked up as previously described [2]. This yielded compound II (160mg, figure 1) which co-chromatographed with an authentic sample supplied by Dr. O. Ekabo of the University of Illinois. Its spectral data (NMR and MS) were identical with previously published data [2].

Preparation of methyl ester of compound I: 120mg of I was dissolved in tetrahydrofuran and diazomethane in diethyl ether added. This was kept at 0°C overnight. The reaction mixture was evaporated *in vacuo* and purified on preparative thin layer chromatography as described above. This yielded compound III (60mg, M. pt 224-225° C, Rf = 0.72). Its spectral data were in agreement with those of published data [2,4,5].

Anti-tumour activity: Anti-tumour activity was assessed under the auspices of the Bioassay Research Facility (BRF) of the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago in accordance with established protocols [6,7] of National Cancer Institute, National Institute of Health, Maryland, USA. A series of accurately weighed doses of the dried aqueous and ethylacetate extracts/isolates were prepared by serial dilution and tested against the following cancer cell lines *in vitro* : human sarcoma, lung cancer, human melanoma, colon cancer, squamous cell cancer, breast cancer, P-388 murine lymphocytic leukemia and prostate cancer with the cooperation of Professor J.M. Pezzuto (Director of BRF). ED_{50} values were obtained by probit analysis using a VAX computer program. By convention, plant extracts/ isolates with ED_{50} values < 20ug/ml in the human cell line and < 5ug/ml in the P-388 system are considered significantly active [6,7].

Spectroscopy: UV spectra was obtained with Beckman DB-G spectro-photometer, IR spectra were recorded on Beckman model 118-A instrument with polysterene calibration at 1601cm^{-1}. ^{1}H-NMR Spectra were recorded in pyridine-D_5 with a Varian XL-300 spectrophotometer at 300MHz with a Nicolet TT-7 Fourier Transform attachment. TMS was internal standard. High resolution MS was obtained using AEI-MS 902 double focussing instrument operating at 70 eV.

Results and discussion

Cancer in its many forms is without doubt, a most insiduous disease and is responsible for an estimated 500,000 deaths /year in the U.S.A. alone [8]. The search for plant ant-cancer agents is very intense.

Although plants of the family Euphorbiaceae are best known for their toxic actions [9-11], they have a folklore history of use for the treatment of cancers and warts and references to their use have appeared in the literature of many countries [12]. Indeed a number of anti-tumour

terpenoids have been reported in some species of the family Euphorbiaceae [13-18].

The result of the anti-tumour assays against the nine cancer cell lines showed that the ethylacetate extract of the stem bark of *S. preussii* var. *preussii* exhibited significant selective cytotoxicity against human melanoma (ED_{50} = 10.0ug/ml. The aqueous extract was inactive (ED_{50} > 20ug / ml). The ethylacetate extract was fractionated using same cell line for monitoring this process in which activity had originally been observed. The same cytotoxicity was observed during fractionation implying the active constituents were stable to chromatography. Purification of fraction B (1.4gm) produced a major cytotoxic compound which was identified as 3β –hydroxy-20 (29)-lupenoic acid (compound I, figure I, $C_{30}H_{48}O_3$, yield = 100mg, Rf = 0.69) by spectroscopic methods (UV, IR, NMR, MS).

Compound 1 exhibited major absorbance maxima in the 1R spectrum (KBr) at Vmax 3400, 1040, 950 and 715 cm^{-1}. Its mass spectrum (EIMS, 250°C, 70eV) showed significant fragment ions at m/z 456 (M^+, 20%, $C_{30}H_{48}O_3$), 438 (5, $M^+ - H_2O$), 423(45, $M^+ - CH_2 - H_2O$), 411 (20) 395 (24), 378 (59), 302 (15) and 189(62).

In the [13] CNMR spectrum ($CDCI_3$, 50MHz) principal peaks were observed at 178.7 (11%, C-28), 150.1 (55, C – 20), 109.1 (61, C – 30) 78.1 (71, C – 3), 49.7 (55, C – 19), 47.1 (70, C – 18) 39.2 (47, C – 4), 37.4 (68, C – 10), 28.2 (42, C – 23) and 19.4 (27, C – 29) ppm.

The spectral data of 1 and its semi-synthetic derivatives (compounds 11 and 111, figure 1) exhibited exact correlation with data previously reported [2, 4, 19, 20]. They also co-chromatographed with authentic samples giving unambiguous identification. Compound 1 exhibited anti-tumour activity against human melanoma *in vitro* (ED_{50} = 2.4μg / ml).

3β-hydroxy-20 (29)-lupenoic acid (betulinic acid) has been reported in other Euphorbiaceae plants [21]. It has been shown to possess a wide range of biological activities [22] including *in vivo* and *in vitro* anti-plasmodial activity [23]. Our present findings show that this is the first time this compound is being reported to exihibit potent *in vitro* anti-tumour activity in human solid tumour cells in culture. It is also the first time compound 1 is being isolated from the stem bark of *S. preussii* var *preussii*. Compound 1 could be a promising bioactive terpenoid since it has been reported to be non-toxic to experimental animals [2].

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