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Screening extracts of *Euphorbia garuana* N.E.Br. for *in-vitro* cytotoxicity

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Summary

Two biological assays utilising *in-vitro* uptake of ^{3}H-thymidine into TLX/5 lymphoma and rat basophilic leukaemia cells have been used for rapid detection and subsequent isolation of active compounds from the latex of *Euphorbia garuana* (Euphorbiaceae). Two potent esters were characterized from the latex of *E. garuana* reportedly used in traditional medicine for the treatment of certain forms of cancer.

Résumé

Deux essais biologiques employant l'absorbtion *in vitro* de ^{3}H-thymidine dans TLX/5 lymphone et dans les cellules leucémiques de rat basophilique sont utilisés pour la détection rapide et l'isolation subséquent des corps composés actifs extraits du latex d'*Euphorbia garuana* (Eiphorbiaceae). Deux esters puissants ont été extraits du latex de *E. garuana* que la médicine traditionelle emploie pour le traitement de certaines formes de cancer.

Introduction

The genus *Euphorbia*, the largest member of the family Euphorbiaceae, has about 2000 species [1]. A number of *Euphorbia* species are used in traditional medicine [2–5].

Euphorbia species also have a long folklore history of use for the treatment of cancers and warts. Watt and Breyer-Brandwijk [6] listed three species, whilst Hartwell [7], in a comprehensive survey of plants showing anti-cancer activity, listed 80 *Euphorbia* species as being potentially useful.

The succulent *Euphorbia* species are known to produce toxic diterpenes of the tigliane, daphnane and ingenane types exhibiting inflammatory or tumour-promoting activities [8,9].

Although a series of diterpenes isolated from the genus *Euphorbia* have been cited as being active against various experimental tumour systems, activity has in most cases been attributed to the inflammatory esters [10,11] at relatively high doses.

This paper describes two rapid and sensitive *in-vitro* biological assays that have been used for the detection of the cytotoxic compounds from *Euphorbia garuana* N.E.Br. extracts.

Materials and methods

Phytochemical analysis

Euphorbia garuana latex was collected into methanol from plants growing near Vom in Nigeria. The plant was authenticated at the herbarium of the Department of Botany, Ahmadu Bello University, Zaria. The methanol-preserved latex was macerated with acetone for 7 days. Acetone was evaporated under reduced pressure and the dry residue dissolved in methanol : water (2 : 3). The lipids and steroids were removed by partitioning into n-hexane. The aqueous phase was re-extracted with aliquots of diethylether. The combined ether extract was washed with 0.25% sodium carbonate solution followed by saline. The n-hexane and ether extracts were tested against TLX/5 lymphoma and rat basophilic leukaemia cells. The active crude ether resin was subsequently fractionated on activated florosil by gradient elution column chromatography using mixtures of hexane, benzene and ethylacetate [12]. Similar column fractions, monitored by adsorption thin-layer chromatography (TLC), were bulked together to give fractions A–D. Fractions A–D

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were each tested for cytotoxic activity against TLX/5 lymphoma cells.

Active column fraction D was semi-purified by preparative partition TLC (20 × 20 cm plates, 0.5-mm thickness) on Kieselguhr G (Merck, Darmstadt, FRG) impregnated with 15% dipropylene glycol in acetone. The plates were developed in heptane : benzene (70 : 30) (system A). Four zones (D1–D4) were scraped off the plates and extracted with acetone. Acetone was evaporated under reduced pressure and the isolated bands recovered from glycol by partitioning between saline and carbon tetrachloride (2 : 1). Carbon tetrachloride was evaporated as before and the dry residue tested for biological activity (Fig. 2). The components of active band D4 were eventually purified by preparative partition TLC as described above and/or adsorption TLC on silica gel GF_{254} (0.5 mm thickness) developing in hexane : ether : benzene (1 : 2 : 1) (system B).

Biological assays

TLX/5 mice lymphoma cells were obtained from the Chester Beatty Cancer Research Institute, London, and propagated *in vivo* in female CBA/LAC mice. The rat basophilic leukaemia cells (RBL) were supplied by Dr D. Gordon (Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons, London, U.K.). The growth media consisted of Dulbecco's medium plus 10% foetal calf serum, containing 100μg streptomycin and 100 IU penicillin/ml (tissue culture medium). ^{3}H-Thymidine (24 Ci/mmol aqueous solution) was from the Radiochemical Centre (Amersham, U.K.), whilst TC 199 and horse serum were obtained from Wellcome Research Laboratories (Beckenham, U.K.).

The ascites tumour cells were removed from the intraperitoneal cavity of two mice 6–7 days after implantation, and added to lysing solution. The centrifuged tumour cells were resuspended in tissue culture mediun (TC 199: horse serum = 60 : 40). An aliquot of the suspension, diluted with 1 N saline solution, was counted either in a Coulter counter or by microscopy using a haemocytometer after staining with trypan blue dye. A suspension of the cells was then made in fresh tissue culture medium to give a final test concentration of not less than 2 × 10^6 cells/ml of test suspension. For the purpose of screening all the column fractions and TLC bands, a single dose (15 μg/ml) of each extract dissolved in 0.1 ml dimethylsulphoxide (DMSO) was added to aliquots (15 ml) of the cell suspension. The TLX/5 lymphoma cells plus 0.1 ml DMSO without the test extract served as a control. The test suspensions and control were gassed with 10% CO_2 in air and incubated, with shaking, at 37°C for 90 min. ^{3}H-Thymidine (1 ml, 1.3 μCi/ml) was added to each test suspension and to the control, and further incubated for 40 min. Aliquots (1 ml) were removed from each test and control suspension at intervals of 10 min, then were added to sterile test-tubes containing ice-cold 1 N saline (5 ml) and placed in ice to stop metabolism. Five replicates were withdrawn at each time interval from each test suspension. The cells from each tube were filtered separately on glass-fibre filters previously soaked in 1 N saline. The filters were successively washed with 1 N saline, trichloro-acetic and distilled water. The filters were placed into scintillation vials, dried at about 60°C for 1 h, and phosphor solution (10 ml) added. ^{3}H-Thymidine incorporated into the cells was recorded on a Packard TriCarb Liquid scintillation spectrophotometer as counts per minute (CPM).

The pure compounds were assayed for cytotoxic potency *in vitro* using cultured rat basophilic leukaemia cells (RBL). The cells were centrifuged and suspended in tissue culture medium. A suspension of cells was then prepared to give a concentraton of about 2 × 10^6 cells/ml of test suspension.

Aliquots (180 μl) of the RBL cell suspension were added into the wells (200 μl) of microtitre plates. Ten serial dilutions of a test compound were prepared from a 1 mg/ml solution of ingol esters (Fig. 3) in redistilled acetone to give final test doses ranging from 0.003 to 30 μg/ml.

Test doses of a test compound were added at random into the wells of one plate (one plate per compound and five replicates per dose). Wells containing only RBL cells and tissue in culture medium served as control. All plates were incubated at 37°C in an atmosphere of 5% CO_2 in moist air for 24 h. All assays were performed under sterile conditions.

^{3}H-Thymidine (1 μCi/ml) was added to each test suspension and to the control, and incubated for a further 24 h.

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The contents of each well were harvested on the third day using a standard automatic radioactive sampler onto a Mash 11 glass-fibre filter paper (Grade 934 AH). The filter papers were continuously washed with distilled water, oven-dried at 60°C and placed in scintillation vials. Toluene scintillation fluid (1 ml : from 10-ml Zip pipette) was added to each vial and counted as above in a liquid scintillation spectrophotometer. The uptake of precursor into RBL cells were recorded as counts per minute and converted to percentage inhibition. The ED_{50} values were computed from their corresponding dose–response curves.

Results and discussion

Compounds 1 and 2 (Fig. 3), which were the major components of band D4, were characterized as 7-benzoyl-ingol-3,8,12-triacetate (ED_{50}, RBL, 0.8 μg/ml and ingol-3,7,8,12-tetra acetate (ED_{50}, RBL, 1 μg/ml), respectively. The significant mass and Proton NMR spectra data of compound 1 (Rf 0.40, system B) are given below:

MS: (Electron impact, 70eV, 170°C): m/z 596 ($M^{+\cdot}$, 4%), 536 (6%), 491 (1.5), 476 (10), 474 (13), 472 (2), 416 (8), 414 (6), 354 (30), 330 (35), 312 (26) 294 (62), 122 (45), 105 (100), 83 (92), 43 (70).

'HNMR: (250 MHz, $CDCl_3$, TMS = 0.000 p.p.m.): 8.064 – 7.456 (m, 5H, aromatic), 5.576 (s, H-5), 5.244 (d., J = 8.46 Hz, H-3), 5.227 (d, J = 1.84 Hz, H-7) 4.884 (dd, J = 11.03 Hz, 4.04 Hz, H-12), 4.603 (dd, J = 10.30 Hz, 1.84 Hz, H-8), 2.950 (q, J = 4.04 Hz, H-13), 2.789 (dd, J = 15.08 Hz, 9.10 Hz, H-1) 2.126–2.079 (acetyls), 2.015 (s, 3H – 17), 1.693 (d, J = 15.08 Hz, H-1), 1.108, 0.86 (6H, 3H–18, 3H–19), 1.077 (3H, d, J = 6.99 Hz, 3H–20), 0.925 (3H, d, J = 7.35 Hz, 3H–16) p.p.m.

The structure of compound 2 (Rf 0.53, system A) has previously been published [12]. Confirmation of the structure of compounds 1 and 2 was also obtained by co-chromatography on TLC with authentic samples. Compound 1 is a new ingol ester from *Euphorbia garuana*. The result shown in Fig. 1 indicate that fraction D is most potent of all the column fractions and that cytotoxic activity was closely associated with TLC bands D3 and D4 (Fig. 2). The two compounds are tetra-esters of ingol (compound 3, Fig. 3) and both esters exhibited significant *in-vitro* activity against the test systems. In con-

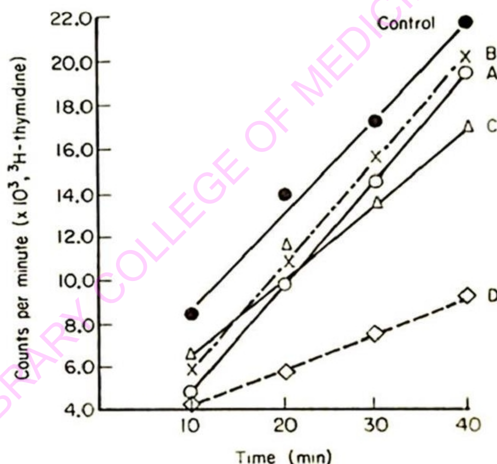

Fig. 1. Effect of column fractions A–D at 15 μg/ml on the uptake of ^{3}H-thymidine into TLX/5 lymphoma cells.

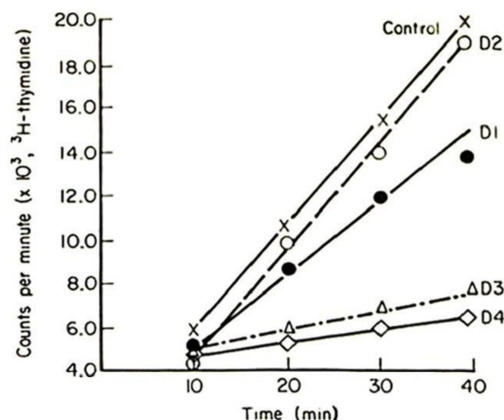

Fig. 2. Inhibition of uptake of ^{3}H-thymidine into TLX/5 lymphoma cells by TLC bands D1–D4 at 15 μg/ml.

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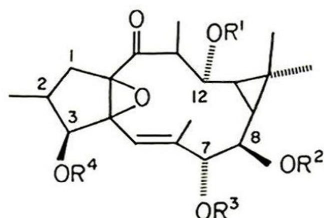

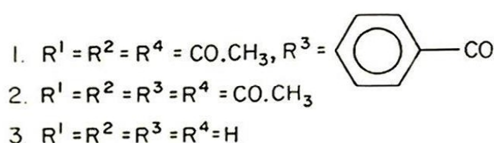

Fig. 3. Ingol macrolides from fraction D4.

clusion, the folklore reports concerning the antitumour activity of *Euphorbia* species could be explained by the action of the non-irritant macrocyclic diterpenes rather than the inflammatory diterpenes.

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