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3-O-ANGELOYLINGENOL, THE TOXIC AND SKIN IRRITANT FACTOR FROM LATEX OF *EUPHORBIA ANITIQUORUM* L. (EUPHORBIACEAE) AND FROM A DERIVED THAI PURGATIVE AND ANTHELIMINTIC (VERMIFUGE) DRUG

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Abstract

From latex of Euphorbia antiquorum *L. as well as from the purgative and anthelmintic (vermifuge) Thai drug yang Sa-Lad-Dai (dried, powdered latex) the highly skin irritant and toxic Euphorbia factor* An_1 *was isolated by combination of countercurrent distributions and chromatography. Spectroscopic and chemical characterisation of* An_1 *established its structure as 3-O-angeloylingenol (I). Because of the acute toxicity of factor* An_1 *and of the possible risk of co-carcinogenesis by tumor promotion, utilization of drugs made up from dried or fresh latex as practiced in Thailand in purgatives and vermifuges should be abandoned.*

Introduction

Euphorbia antiquorum L. is a branched succulant, between 2 and 4 m high and native to tropical India and Cylon[1]. It is widespread also in neighbouring regions such as Iran, Burma and Thailand (e.g.ref. 2). The plant is used in parks and gardens for ornamentals and also for protective hedges, due to the fragility of its branches together with the well known skin irritancy of its latex. While young shoots of the plant are used as a vegetable (Morton J.F, private communication), the woods and the latex—the latter dried to yield a resin—have been and still are used as aphrodisiacs[3] and as drug in folk medicine (in Sanscrit : Vajrakantaka) to treat various diseases[1]. In parts of Thailand in which infestation of people with worms is widespread a drug ("Yang Sa-Lad-Dai") made up of the powdered resin is commercially available on markets and used as purgative and vermifuge. Also, in the form of a decoction a preparation known in Thai as "Ya Nam Radom Pol" containing the fresh whole drug (latex) with some other ingredients is used as a purgative and available in drugstores without prescription.

Many species of the plant family Euphorbiaceae are known to contain highly irritant, toxic and frequently co-carcinogenic, especially tumor promoting diterpene esters of the ingenane, tigliane and daphnane type[4,5]. So far, from *E. antiquorum*, using powdered stem bark and latex, triterpenes, inactive as irritants, were isolated [6-8]. Therefore, extracts of the latex of *E. antiquorum* and of the Thai drug Yang Sa-Lad-Dai were investigated to clarify the chemical nature of their irritant and perhaps tumor promoting constituents.

Materials and Methods

Biological assays. Irritant doses 50 (ID_{50}^{24}) were determined on the mouse ear 24 hours after application of materials or compounds according to the standard procedure[9,10]. In case of factor An_1 the ID_{50} was also determined two hours after application (ID_{50}^{2}). The tumor promoting activity was determined using 100 nmole 7,12-dimethylbenz(a)anthracene (DMBA) as initiator for groups of 28 female NMRI-mice in the standard assay on the back skin[10]

Spectra. Mass spectra (MS) were measured with a Varian MAT 711 spectrometer, uv-spectra (UV) with a Beckman DK 2a far uv-spectrometer, ir-spectra (IR) with a Perkin Elmer spectral photometer 521 and ^{1}H-nmr-spectra (NMR) with a Burker HX 90 spectrometer. Chemical shifts refer to tetramethylsilane (δ = 0.00 ppm) as internal standard and $CDCl_3$ as solvent.

Separation methods. For machinery and methods of multiplicative distributions see[9]. For analytical thin layer chromatography (TLC) precoated plates, 0.25 mm (Merck) were used and for preparative TLC (PTLC) either precoated plates, 0.5 mm, or Merck silica gel PF_{254} were used. Spots were detected under uv-light (254 nm) and/or by spraying with vanillin/sulfuric acid[9].

Plant materials. 1 l of latex of *Euphorbia antiquorum* L., preserved under methanol, was obtained by Dr. E.F. Steinmetz Co., Amsterdam. Of the Thai drug Yang Sa-Lad-Dai 50 g were purchased in 1976 from a local drugstore of Thai ancient medicine in Bangkok, Thailand.

Extraction and fractionation procedures (Table 1).

A. Latex preparation: 1 l of the latex of *Euphorbia antiquorum* was exhaustively extracted with acetone to yield besides 100 g of an inso luble residue 125 g of an acetone extract, ID_{50}^{24} : 4.2 µg/ear. O'Keeffe distribution of 115 g of the acetone extract, in the solvent sytem petroleum ether/methanol/water = 15/10/0.5 afforded 76 g of a hydrophobic fraction (ID_{50}^{24} : > 100 µg/ear) and 38 g of a hydrophilic fraction, ID_{50}^{24} : 1.5 µg/ear. 25 g of the hydrophilic fraction were subfected to a Craig distribution in the same solvent sytem (n= 6000 transfers, z = 500 elements, V = 10 ml/10 ml, T =20 °C, single withdra-

wal procedure). Fractions were combined to sections according to TLC. All withdrawn sections and most of the sections in the battery amounting to 20.0 g of material were inactive as irritants : ID_{50}^{24} : 50 µg/ear. Two irritant active sections were obtained: r = 300-360 (1.9 g; ID_{50}^{24} : 0.2 µg/ear) and r = 361-430 (0.8 g; ID_{50}^{24} : 0.2-0.4 µg/ear).

B. Resinous drug : Extraction of 40 g of the resinous drug of *E. antiquorum* yielded 27 g of acetone extract (ID_{50}^{24} : 100 ug/ear). O'Keeffe-distribution afforded 22.05 g of an inactive hydrophobic fraction (ID_{50}^{24} : 100 ug/ear) and 2.9 g of a hydrophilic fraction, ID_{50}^{24} : 15 µg/ear. After Craig-distribution of the hydrophilic fraction (n = 700 transfers, z = 1000 elements; V = 10 ml/10 ml; all solvent systems as used for the latex) 1.2 g of inactive sections r = 101-700, ID_{50}^{24} : 100 µg/ear) and 1.3 g of an active section (r = 0-100, ID_{50}^{24} : 6.5 µg/ear) were obtained.

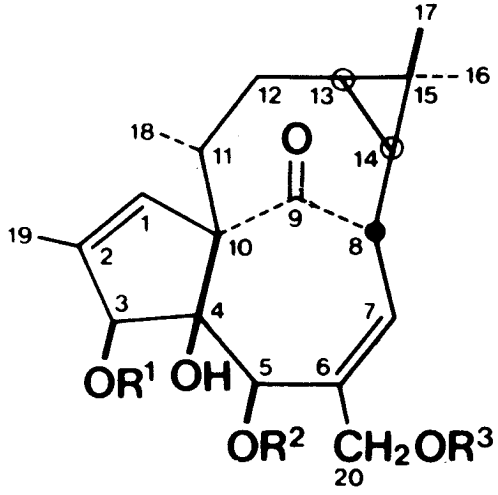
Isolation of Euphorbia factor An_1.

A. Active section of the latex : By PTLC of 200 mg of section r = 300-360 in the system petroleum ether/ethyl acetate = 1/1 105 mg of Euphorbia factor An_1 (I) was obtained, R_f-value : 0.28 (system above); for ID_{50}-values see Table 1 . MS ; parent ion m/e 430. IR (CH_2Cl_2) : 3500 (OH), 1718 (CO), 1642 cm^{-1} (C = C).
UV (methanol) : λ (ε) : 194 nm (18500), λ_{max} (ε) : 211 (16700), 296 nm (370). NMR: 1-H, 7-H: 6.05 (m), 3-H: 5.53 (s); 20-H_2: 4.14 (s); 5-H: 4.05 (s,br.) ; 8-H: 4.0-4.2 (superimposed with 5-H); 19-H_3 : 1.80 (d, J = 1.5 Hz); 16-H_3, 17-H_3 : 1.06 and 1.09; 18-H_3 : 0.96 ppm (d, J = 7 Hz); OH (exchangeable with D_2O) : 3.52 ppm; acid moiety : 1 olefinic H : 6.15 (m) ; 2 vinylic CH_3 : 1.9-2.1 ppm (m).
Factor An_1 was also detected by analytical TLC in the active section r = 361-430.

B. Active section of the resinous drug : 200 mg of section r = 0-100 were separated by PTLC in the system ether/petroleum ether = 4/1 to yield 7.0 mg of an active fraction (ID_{50}^{24} : 0.4 µg/ear) which still exhibited several peaks as demonstrated by HPLC (reversed phase, ODS, methanol/water = 70/30). 3.5 mg of this fraction were further purified by PTLC in the system chloroform/ethanol = 95/1 to afford 0.7 mg of an irritant factor An_1 ; MS : m/e 430, NMR : multiplicity and chemical shifts of all peaks correspond to those in the spectrum of factor An_1.

Chemical characterization of Euphorbia factor An_1.

Transesterification and acetylation of Euphorbia factor An_1 : 66 mg of Euphorbia factor An_1 were treated with 30 ml of 0.1 M sodium methoxide in methanol for 4 hours. After neutralisation with excess buffer, pH 6.8 and usual work-up, 46 mg of the parent alcohol II were obtained, R_f : 0.43 (CH_2Cl_2/methanol = 10/1), MS : parent ion m/e 348. Reaction of the transesterification product with acetic acid anhydride/pyridine afforded



I : R^1 = $COC(CH_3) \overset{Z}{\equiv} CH(CH_3)$, R^2 = R^3 = H
II : R^1 = R^2 = R^3 = H
III : R^1 = R^2 = R^3 = $COCH_3$
IV : R^1 = $COC(CH_3) \overset{Z}{\equiv} CH(CH_3)$ R^2 , R^3 = isopropylidene

Figure Euphorbia Factor An_1(3-O-angeloylingenol,I) isolated from latex and Thai drug of *Euphorbia antiquorum* L. and of its derivatives prepared : ingenol (II), ingenol-3, 5, 20-triacetate (III) and the 5,20-isopropylidene-derivative IV of Factor An_1.

TABLE 1 : YIELDS AND IRRITANT DOSES 50 (ID_{50}^{24}) OF EXTRACTS AND VARIOUS FRACTIONS AND EUPHORBIA FACTOR An_1 AS OBTAINED BY THE SEPARATION PROCEDURES FOR THE ACETONE EXTRACTS FROM LATEX AND DRUG OF *EUPHORBIA ANTIQUORUM* L.

Standard : 12-O-tetradecanoylphorbol-13-acetate (TPA), ID_{50}: 0.01 µg/ear or 0.016 nmoles/ear [9,10].

Fraction/Factor	Latex		Drug	
	Yield[a](%)	ID_{50}^{24} (µg/ear)	Yield[a](%)	ID_{50}^{24} (µg/ear)
Acetone Extract	--	4.2	67.5	100
Hydrophilic Fraction[b]	33	1.5	10.7	15
Active Section (s)[c]	3.5	0.2-0.4	4.8	6.5
Factor An_1 [d]	1.8	0.06[e]	0.017	0.06[e]

[a] Yields of hydrophilic fractions, active sections and factor An_1 refer to the acetone extracts of latex and drug (= 100 %).

[b] after O'Keeffe-Distribution;

[c] after Craig-distribution;

[d] after PTLC of active sections;

[e] or 0.14 nmoles/ear; ID_{50}^{2} : 0.003 µg/ear or 0.007 nmoles/ear.

after PTLC in ether/petroleum ether = 1/1 38 mg of the acetylation product III which was crystallized from ether/petroleum ether : M.p. 195-197 °C; IR (KBr) : 3430, 1743, 1733, 1710, 1640 cm^{-1}.

Isopropylidene-derivative of Euphorbia factor An_1 : 55 mg of Euphorbia factor An_1 were dissolved in 10 ml of acetone and treated with catalytic amounts of p-toluene sulfonic acid hydrate. After 40 min. the reaction was stopped by adding excess buffer. PTLC of the reaction product in ether/petroleum ether = 1/1 afforded 35 mg of IV, R_f : 0.4; MS : parent ion m/e 470; UV (methanol) : λ (ε) : 194 (19000); λ_{max} (ε) : 209 (16800), 298 nm (400). NMR : 1-H: 6.05 (m); 7-H: 5.75 (m); 3-H: 5.63 (s); 20-H_2 : 4.18 (s); 8-H : 4.0-4.2 (partially superimposed); 5-H : 4.0 (s, br.); 19-H_3 : 1.8 (d, j = 1.5 Hz); 16-H_3, 17-H_3 : 1.04 and 1.09; 18-H_3 : 0.97 (d,j = 8 Hz); OH (exchangeable with D_2O) : 3.18 ppm; acid moiety : 1 olefinic H : 6.0-6.1 (m), 2 vinylic CH_3: 1.9-2.1 ppm; isopropylidene : 1.42 (s) and 1.46 ppm (s).

TABLE 2 : COCARCINOGENIC ACTIVITY IN THE STANDARD INITIATION/PROMOTION EXPERIMENT MEASURED AS AVERAGE TUMOR RATES AND AVERAGE TUMOR YIELDS AFTER 12 AND 18 WEEKS[a] FOR THE ACETONE EXTRACT (AC.-EXTR.) FROM LATEX OF *EUPHORBIA ANTIQUORUM* L. AND FOR EUPHORBIA FACTOR An_1

Standard : 12-O-tetradecanoylphorbol-13-acetate (TPA).

Extract/Factor	Duration of experiment (weeks)	Promoting dose (p)	Tumor rate[b] 12 weeks	Tumor rate[b] 18 weeks	Tumor yield[c] 12 weeks	Tumor yield[c] 18 weeks	Survival rate after 18 weeks (%)
Ac.-Extr.	30	2 mg[d]	6/27	8/24	9/27	10/24	86[e]
Factor An_1	18	43 µg (100 nmoles)	0/26[f]	0/10[f]	0/26[f]	0/10[f]	36
TPA	48	6.16 µg (10 nmoles)	7/28	21/27	16/28	83/27	96

[a] Initiation : i = 0.1 mmole of DMBA (7,12-dimethylbenz (a) anthracene); Promotion twice weekly doses p of the promoter.

[b] Averge tumor rate : number of tumor bearing animals/ number of survivors of the group.

[c] Average tumor yield : number of tumors/number of survivors of the group.

[d] Due to the toxic effect, the dose was reduced to 0.5 mg/application following the 4^{th} application.

[e] Survival rate after end of experiment : 32 %.

[f] At 8 and 13 weeks : two tumor bearing animals with 3 tumors; tumors are lost after 11 and 15 weeks, respectively, due to the necrotic lesions on the back skin of the mice.

In trials to acetylate the isopropylidene-derivative IV with acetic acid anhydride/ pyridine only starting material was recovered.

Results and Discussion

The acetone extract of the latex of *Euphorbia antiquorum* L. shows considerable irritant activity of the mouse ear (Table 1). In the assay for tumor promoting activity on the back skin of mice the acetone extract exhibits only weak activity as expressed by the average tumor rates and yields after 12 or 18 weeks of promoting treatment (Table 2). However, it proved to be highly toxic on the mouse back skin, causing severe ulcerations and necrosis in a dose of 2 mg/application. Because of its acute toxicity the promoting dose p of the acetone extract, used initially (p = 2.0 mg), had to be reduced after the fourth application to p = 0.5 mg (Table 2). While the survival rate was satisfactory but lower still than that of TPA up to 18 weeks, after 30 weeks the experiment was terminated because only 32% of the animals had survived.

A separation procedure, monitored by the quantitative assay for irritant, was developed for the acetone extract of the latex to isolate irritant principle(s). After sequential countercurrent distributions (O'Keeffe and Craig distributions) and separation of an irritant section by preparative TLC, an irritant factor An_1 was isolated (Table 1) and characterized by spectroscopic and chemical means as 3-O-angeloylingenol (I, see Fig.). I readily formed the 5,20-acetonide IV upon treatment with acidic acetone. IV could not be acetylated with acetic acid anhydride/pyridine proving 3-position of the acyl residue. Transesterification of factor An_1 and reacylation of the diterpene moiety with acetic acid anhydride/pyridine afforded ingenol - 3,5,20 - triacetate (III) yielding identical spec - troscopic data and melting point as an authentical sample[11]. 3-O-Angeloylingenol (I) was also isolated from *Euphorbia helioscopia* (Gotta, H, Adolf, W., Opferkuch, H.J. and Hecker, E, in preparation) and in minor amounts from latex of *Euphorbia paralias*[12].

From the Thai purgative drug yang Sa-Lad-Dai an acetone extract was prepared yielding 67.5% of acetone solubles (Table 1). It exhibited very weak irritant activity (Table 1) as compared to the extract of the latex. After O'Keeffe and Craig distributions of the acetone soluble fraction and further purification by preparative TLC as in case of the acetone extract of the latex, factor An_1 was isolated too, although in a much lower yield.

In the assay for irritant activity on the mouse ear factor An_1 is highly active : read 2 hours after application it is about twice as irritant as TPA (Table 1). However, if read 24 hours after application it is by about a factor of 10 less active than TPA. The irritant dose 50 of TPA is not time dependent within this interval[13]. In the assay for tumor promotion factor An_1 proves to be very toxic in a dose of 100 nmoles/application causing similar effects as observed with the acetone extract. In case of An_1 after 18 weeks only

36%of the animals had survived and the experiment was terminated. The tumor response is low, only in the 8th and 13th week two tumor bearing animals are noticed (Table 2). Most probably the low response is due to the toxicity of An_1 in the doses p used as may be seen by the comparatively good response elicited by the acetone extract : High local toxicity may eliminate cells prone to form tumors i.e. "initiated cells" or "potential tumor cells".

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บทคัดย่อ

ได้แยกสาร An_1 ซึ่งมีฤทธิ์ระคายผิวและเป็นพิษจากยางของ *Euphorbia antiquorum* L. และจากยาระบายและยาถ่ายพยาธิ "ยางสลัดได" (ผงยางแห้งจากพืชนี้) โดยใช้ countercurrent distributions และ โครมาโตกราฟี ได้พิสูจน์ว่าสารนี้คือ 3 - O - angeloylingenol (I) โดยวิธีทางสเปกโตรสโคปีและทางเคมี เนื่องจากสาร An_1 นี้มีความเป็นพิษสูง และเนื่องจากจะมีโอกาสเป็นตัวร่วมก่อให้เกิดมะเร็ง ควรเลิกใช้เป็นยาระบายหรือยาถ่ายพยาธิ ไม่ว่าจะมาจากยางสด หรือแห้งก็ตาม