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Preliminary Phytochemical Investigation of *Euphorbia millii*

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Abstract □ *Euphorbia millii* (Des Moulins) was investigated. β-Sitosterol, cycloartenol, and β-amyrin acetate were isolated from the petroleum ether extract. The presence of lupeol and euphol as minor constituents was demonstrated by GC, and the existence of flavonoids in the methanol extract was shown.

Keyphrases □ *Euphorbia millii*—phytochemical investigation, isolation and identification of constituents □ Medicinal plants—preliminary phytochemical investigation of *Euphorbia millii* □ GC—isolation and identification of *Euphorbia millii* constituents

Euphorbia millii (Des Moulins) is a native shrub of the Island of Madagascar and is widely cultivated as an ornamental plant in the South Florida area. It has been reported (1) to contain toxic substances in its latex.

The genus *Euphorbia* has been very prolific in the production of substances with biological properties. Antitumor activity has been reported in extracts of *Euphorbia drumondii* (2), antimicrobial alkaloids have been found in *Euphorbia thymifolia* (3), and extracts from *Euphorbia ingens* (4) have produced epidermal hyperplasia and tumors in mice.

In addition, a variety of chemical compounds have been isolated from *Euphorbia* species. Dumkow (5) isolated kaempferol 3-α-monorhamnopyranoside from *Euphorbia myrsinites*, Wingnes and Andrew (6) reported the presence of D-glucaric acid in the latex of *Euphorbia canariensis*, and Morales Mendez (7) identified triterpenoids in *Euphorbia caracasana*.

Interest in this investigation arose out of the limited phytochemical reports on *E. millii* and the many medicinal applications of other species from the genus *Euphorbia*.

EXPERIMENTAL

Plant Material—The plants[1] used in this investigation were air dried for 1 month and then ground in a mill (Fitzpatrick), yielding 2.4 kg. of fine, powdered material.

[1] Obtained from Melrose Nursery, Miami, Fla., during the spring of 1969.

Table I—Chromatography on Silicic Acid of the Petroleum Ether Extract

	Fraction Number	Fraction Weight, g.	Eluent	L.B. Test[a]
A	(1–22)	18.4	Heptane–benzene (1:1)	–
B	(23–28)	5.2	Heptane–benzene (1:1)	+
C	(29–39)	18.6	Benzene	+
D	(40–43)	3.4	Benzene	+
E	(44–55)	14.0	10% Ethyl acetate in benzene	+
F	(56–63)	8.4	10% Ethyl acetate in benzene	+
G	(64–79)	13.9	20% Ethyl acetate in benzene	–

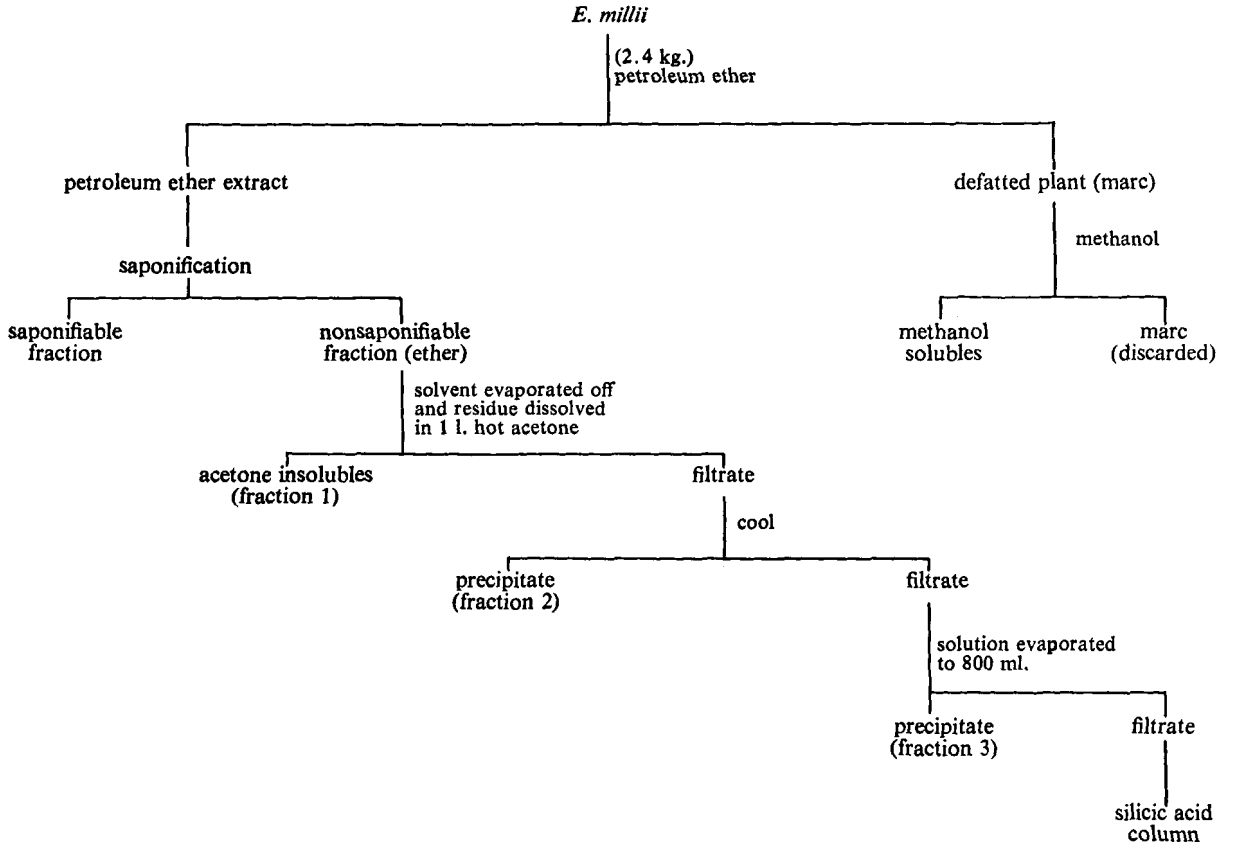
[a] Liebermann–Burchard test.

Extraction of Powdered Plant—The fine, powdered material was extracted in a 3-l. soxhlet apparatus with petroleum ether (b.p. 30–60°) for 72 hr. At the end of this period, the petroleum ether was removed and the marc was extracted with methanol for an additional 72 hr.

The petroleum ether extract was evaporated to dryness with the aid of a continuous circulating-type evaporator and a rotavapor (Bachi). The gummy, greenish residue obtained (196 g.) was then subjected to saponification.

Saponification of Petroleum Ether Extract—Before saponification of the petroleum ether extract was undertaken, tests for the presence of sterols were performed on the extract. Samples were subjected to the Liebermann–Burchard and Salkowski tests (8, 9), giving positive reactions characteristic of steroids or triterpenoid-type compounds.

The gummy residue obtained from the petroleum ether extraction was dissolved in 1.5 l. of ethanol and saponified by refluxing with 30 g. of potassium hydroxide for 1.5 hr. The ethanol was then removed with the help of the rotavapor, and the residue was mixed with 1.5 l. of water. The water suspension was placed in a 2-l. separator and extracted with six 500-ml. portions of ether. The ether fraction was tested for the presence of sterols with the Liebermann–Burchard reagent and gave a positive test. The solvent was then evaporated and the residue was dissolved in 1 l. of hot acetone. Thirteen grams of acetone-insoluble material was removed (Frac-



Scheme I—Extraction and Fractionation of E. millii

tion 1) and the filtrate was allowed to cool to room temperature. A greenish amorphous material (4.5 g.) precipitated upon cooling and was filtered (Fraction 2). The acetone solution was then evaporated to approximately 800 ml. and cooled, yielding a precipitate (3.0 g.) of brown amorphous material (Fraction 3).

Fractions 1, 2, and 3 gave negative results when subjected to the Liebermann–Burchard test and were set aside. The mother liquor remaining from the acetone solution was evaporated to dryness and the material was subjected to column chromatography (Scheme I).

Chromatography on Silicic Acid of Petroleum Ether Extract—The acetone solution, upon evaporation, yielded 105 g. of a gummy residue. This material was added to the top of a column (8.5 × 105 cm.) packed with a slurry of 2 kg. silicic acid[2] in heptane–benzene (1:1) and eluted initially with the same solvent mixture, followed by benzene, 10% ethyl acetate in benzene, and finally 20% ethyl acetate in benzene; 250-ml. fractions were collected.

The fractions collected were monitored by means of TLC on silica gel plates. Plates 20 × 20 cm. in area and 250 μ in thickness and a solvent system of heptane–benzene–ethanol (50:50:2) were employed. The spots were detected by spraying with 20% antimony trichloride in chloroform. Liebermann–Burchard tests were performed on each fraction. Fractions were pooled on the basis of changes in TLC patterns and their behavior to the Liebermann–Burchard reaction (Table I).

Examination of Fractions B, C, D, and E—TLC of Fractions B, C, D, and E (Tubes 23–55) on silica gel plates, utilizing the same solvent system already mentioned, showed a minimum of five spots, ranging in R_f values from 0.20 to 0.42. These fractions were recombined and subjected to column chromatography.

Isolation of β-Sitosterol—Fraction F (Tubes 56–63) was examined by TLC on silica gel; it showed one large spot at R_f 0.20 and traces of other material at R_f 0.32 and 0.38. Approximately 2.5 g. of material from Fraction F was dissolved in hot methanol, and the resulting solution was allowed to cool slowly. After 24 hr., a crystalline material (I) was removed which, upon harvesting and drying in a vacuum desiccator overnight, weighed 120 mg. (m.p. 136–137°).

Chromatography on Alumina of Fractions B, C, D, and E—The solution resulting from the combined fractions was evaporated to dryness, yielding 40 g. of an orange gelatinous material. This was dissolved in 200 ml. of benzene and added to the top of a column (4.5 × 90 cm.) packed with a slurry of 1.8 kg. of alumina[3] in benzene. The column was eluted with benzene, followed by 10% ethyl acetate in benzene and finally 20% ethyl acetate in benzene. Then 250-ml. fractions were collected, monitored, and combined after examination on TLC and by the Liebermann–Burchard reaction. Table II shows the results of the color tests, the weight of each combined fraction, and the elution solvent.

Isolation of β-Amyrin Acetate—Fraction I (Tubes 7–14) contained 7.5 g. of material which, when subjected to TLC on silica gel using a solvent system of heptane–benzene–ethanol (50:50:2), showed three spots, with one major spot at R_f 0.38. Attempts were made to purify this material by preparative TLC. The plates were prepared by a slightly different method. Thirty grams of silica gel[4] were shaken with 75 ml. distilled water until a homogeneous mass was obtained. The suspension was immediately placed on a 20 × 20-cm. dry, clean plate, and the plate was vibrated by hand to allow the material to spread to all corners. The plate was allowed to air dry for 12 hr. and was then dried in an oven at 110° for 2 hr.

Three hundred milligrams of material from Fraction I was placed on a preparative plate with the aid of a micropipet and developed in a solvent system of heptane–benzene–ethanol (50:50:2). One major band at approximately R_f 0.38 was scraped off the plate and extracted with 10% methanol in chloroform. Upon evaporation of the solvent, 200 mg. of a gelatinous yellow material was obtained; this material would not crystallize from methanol, acetone, or chloroform.

[2] 100 mesh, Mallinckrodt analytical reagent.

[3] Woelm, neutral, activity grade 1.

[4] $HF_{254+366}$ according to E. Stahl, Merck A. G., Darmstadt, Germany.

Table II—Chromatography on Alumina of the Combined Fractions (B, C, D, and E)

	Fraction Number	Fraction Weight, g.	Eluent	L.B. Test[a]
H	(1–6)	6.8	Benzene	–
I	(7–14)	7.5	Benzene	+
J	(15–27)	8.1	10% Ethyl acetate in benzene	+
K	(28–41)	2.8	10% Ethyl acetate in benzene	+
L	(42–57)	2.0	20% Ethyl acetate in benzene	–
M	(58–67)	1.5	20% Ethyl acetate in benzene	–

[a] Liebermann–Burchard Test.

Acetylation of this material was undertaken by dissolving it in 2 ml. of dry pyridine and reacting it with 10 ml. of acetic anhydride. The solution was allowed to stand at room temperature overnight. Fifty milliliters of distilled water was added and the crude acetate precipitated. TLC of the sample, as previously described, using a solvent system of heptane–benzene (1:1), showed it to have three spots, with one major spot at R_f 0.45. Then 180 mg. of this material was subjected to preparative TLC on silica gel and treated in the same manner. The band at R_f 0.45 was scraped off the plate and extracted with 10% methanol in chloroform. Evaporation of the solvent yielded 150 mg. of a white, powdery material. Crystallization of this substance from acetone–methanol (1:1) yielded crystals (II), m.p. 235–236°.

TLC of the acetylated isolate (II), using silica gel and a solvent system of heptane–benzene–ethanol (50:50:2), showed it to be a homogeneous material with an R_f value of 0.38.

Isolation of Cycloartenol from Fraction J—Fraction J (Tubes 15–27) yielded, upon evaporation of the solvent, 8.1 g. of a dark-orange, gummy residue. This material was dissolved in hot chloroform, boiled for several minutes with decolorizing charcoal, and filtered while hot. The chloroform was then removed *in vacuo* and the residue was crystallized from a small volume of ethanol, yielding crystals (III), m.p. 84–85°. Recrystallization of this substance from acetone yielded white needles, m.p. 94–95°.

Fraction K (Tubes 28–41) yielded another 250 mg. of β-sitosterol after recrystallization from methanol.

Detection of Euphol and Lupeol as Minor Constituents by GC—A sample of Fractions B, C, D, and E from the silicic acid column (Table I) was analyzed by GC[5]. Three different liquid phases were used: 3% OV-17, 1% OV-1, and 1.6% SE-30, all on Chromosorb W solid phase. The operating conditions and retention times are given in Table III. The sample was demonstrated to be composed of euphol and lupeol in addition to the other three compounds already isolated.

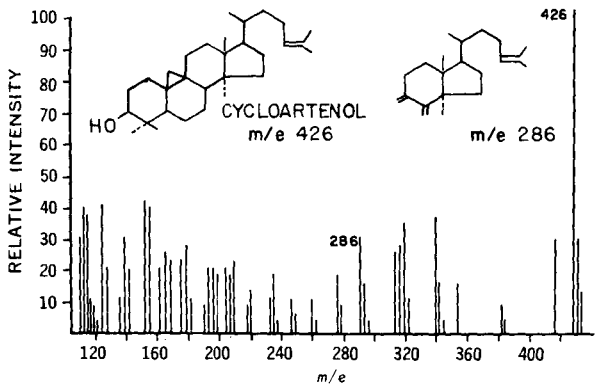


Figure 1—*Mass spectrum of cycloartenol.*

[5] Varian 2100 gas chromatograph.

Table III—GC[a] of Fractions B–E[b]

Products	Retention Time, min. 3% OV-17	1% OV-1	1.6% SE-30
Euphol	11.8	10.7	11.0
Lupeol	14.3	10.0	10.3
β-Sitosterol	11.1	10.2	10.8
β-Amyrin	12.0	11.2	11.8
Cycloartenol	16.6	14.2	15.4

[a] Flow rate, 50 ml./min.; oven temperature, 250° for OV-17 and 200° for OV-1 and SE-30. [b] Fractions B through E were combined, the solvent was evaporated off, the residue was dissolved in acetone, and 25 μl. was injected.

Detection of Flavonoids from Methanol Extract—The methanol extract was evaporated to approximately 900 ml. At this point, most of the methanol had evaporated and the residual water solution was extracted with six 500-ml. portions of chloroform. The solvent was evaporated off, and a 5-g. sample of the residue was dissolved in 30 ml. of chloroform and added to the top of a column (3 × 60 cm.) packed with a slurry of 100 g. silicic acid in chloroform. The column was then eluted with chloroform, 40% ethyl acetate in chloroform, and ethyl acetate. Fifty-milliliter fractions were collected, which were monitored and recombined on the basis of their behavior on TLC. All of the combined fractions were subjected to the cyanidin reaction (10). The combined Fractions 7–12 gave a positive cyanidin test. TLC of this material on polyamide plates[6], using methanol as the developing solvent and followed by exposure to ammonia vapors, revealed the presence of several yellow spots suggestive of the presence of flavonoids (11). Work is now underway to purify this material further.

RESULTS AND DISCUSSION

The carbon–hydrogen analyses for Compounds I, II, III, and their derivatives are reported in Table IV.

Mass spectrometric analysis of Compound I showed a molecular ion peak at *m/e* 414 that was identical to an analysis of a reference standard of β-sitosterol. IR, NMR, and TLC analyses of I compared to β-sitosterol proved I to be β-sitosterol, m.p. 136–137° [lit. (12) m.p. 137–138°]. Acetylation of I yielded an acetate which was identical to β-sitosterol acetate, m.p. 123–124° [lit. (13) m.p. 125–127°].

Compound II exhibited a molecular ion peak at *m/e* 468 and a base peak at *m/e* 218 characteristic of pentacyclic triterpenes of the α- and β-amyrin series (14). IR and NMR analyses were identical to a reference standard of β-amyrin acetate. Compound II was hydrolyzed with potassium hydroxide, yielding the free alcohol which agreed with the melting point, NMR, IR, and TLC of a reference standard of β-amyrin, m.p. 196–197° [lit. (15) m.p. 197–198°]. Subsequent examination of a petroleum ether extract of *E. millii* that was not saponified indicated that β-amyrin also occurs in the plant as the acetate.

The mass spectra of Compound III (Fig. 1) showed a molecular ion peak at *m/e* 426 and a signal at *m/e* 286 characteristic of the mass spectrum of cycloartenol, which has been identified in certain other *Euphorbia* species (16, 17). The NMR and IR analyses were identical to those of a reference sample of cycloartenol. To confirm the identity of III further, it was acetylated with acetic anhydride in pyridine, giving an acetate that was identical in all respects to a reference standard of cycloartenol acetate, m.p. 118–119° [lit. (17) m.p. 120–121°].

The use of GLC, particularly when employing three different liquid phases, made it possible to screen accurately the different fractions and to identify euphol and lupeol in *E. milli.*

SUMMARY

The nonsaponifiable fraction from the petroleum ether extract of *E. millii* (Des Moulins) was investigated. Column chromatography

[6] MN-Polyamide, Macherey, Nagel and Co., Germany.

Table IV—Elemental Analyses of Compounds I–III and Their Derivatives

Compound	Formula	Analysis, %—Calc.	Found
I Free alcohol	$C_{29}H_{50}O$	C 83.99	84.26
		H 12.15	12.33
I Acetate	$C_{31}H_{52}O_2$	C 81.52	81.72
		H 11.48	11.55
II Free alcohol	$C_{30}H_{50}O$	C 84.40	84.92
		H 11.80	11.78
II Acetate	$C_{32}H_{52}O_2$	C 81.99	81.95
		H 11.18	11.26
III	$C_{30}H_{50}O \cdot H_2O$	C 81.02	81.26
		H 11.79	11.80

of this fraction on silicic acid yielded β-sitosterol. TLC on alumina of several fractions eluted from the silicic acid column yielded cycloartenol. Acetylation of a fraction from the alumina column yielded, after preparative TLC, β-amyrin acetate. Examination of a petroleum ether extract of the plant which was not subjected to harsh saponification methods indicated that β-amyrin also occurs as the acetate.

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Potential Antidiabetics XII: N^1-Phenylcarbamoyl-4-arylazo(3- and/or 5-substituted)pyrazoles and Their Biological Activities

H. G. GARG▲ and CHANDRA PRAKASH

Abstract □ The promising antidiabetic candidates N^1-phenylcarbamoyl-4-arylazo-3,5-dimethylpyrazoles, N^1-phenylcarbamoyl-4-arylazo-3-methyl-5-phenylpyrazoles, and N^1-phenylcarbamoyl-4-arylazo-3,5-diphenylpyrazoles were prepared in 55–70% yield by the cyclization of 2,3,4-pentanetrione-2-arylhydrazones, 1-phenyl-2-arylhydrazono-1,2,3-butanetriones, and 1,3-diphenyl-2-arylhydrazono-1,2,3-propanetriones, respectively, with 4-phenylsemicarbazide. No significant antidiabetic effects were observed in pharmacological testing of these compounds except for N^1-phenylcarbamoyl-4-(2,3-dimethylphenylazo)-3,5-dimethylpyrazole.

Keyphrases □ Antidiabetic agents, potential—synthesis and biological activity of N^1-phenylcarbamoyl-4-arylazo(3- and/or 5-substituted)pyrazoles □ Pyrazoles, N^1-phenylcarbamoyl-4-arylazo(3- and/or 5-substituted)—synthesis as potential antidiabetic agents, biological activity □ N^1-Phenylcarbamoyl-4-arylazo(3- and/or 5-substituted)pyrazoles—synthesis as potential antidiabetic agents, biological activity

The facts that N^1-phenylcarbamoyl-3,5-dimethylpyrazole possesses hypoglycemic activity 20–30 times that of tolbutamide (1) and that certain arylazohydroxyquinolines are diabetogenic (2) led to the synthesis of N^1-phenylcarbamoyl-4-arylazo-3,5-dimethylpyrazoles, N^1-phenylcarbamoyl-4-arylazo-3-methyl-5-phenylpy-