Antitumor Activity and Preliminary Phytochemical Examination of *Tagetes minuta* (Compositae)

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Abstract □ A random collection antitumor screening program revealed that the whole flowering plants of *Tagetes minuta* were significantly active against the Lewis lung carcinoma *in vivo*. In the present phytochemical examination of this plant, the flavone glycosides quercetagetrin and patuletrin were isolated in addition to the monomethyl ester of fumaric acid and syringic acid.

Keyphrases □ *Tagetes minuta* (Compositae)—phytochemical study, antitumor activity □ Antitumor activity—isolation and testing of constituents of *Tagetes minuta* □ Medicinal plants—phytochemical study of *Tagetes minuta*, antitumor activity

Extracts from *Tagetes minuta*[1] were found to elicit significant activity against the Lewis lung carcinoma *in vivo*. Results of a preliminary phytochemical investigation of this plant are reported here.

Prior phytochemical studies on *T. minuta* were limited to the isolation and characterization of insecticidally active compounds such as 5-(3-buten-1-ynyl)-2,2′-bithienyl, α-terthienyl (1), 5-(but-1-chloro-2-ol-3-ynyl)-2,2′-bithienyl, *cis*-5-(1-acetoxy-but-3-enyl)-2,2′-bithienyl, 5-(but-1-en-3-ynyl)-2,2′-bithienyl, 5-(4-chloro-3-hydroxy-but-1-ynyl)-2,2′-bithienyl, and 5-(but-1-yn-4-olyl)-2,2′-bithienyl (2), in addition to the terpenes ocimene, *d*-limonene, estragole, tagetone, and α-dimethyl-Δ^{α}-octen-ε-one (3, 4). A number of rather rare flavonoids, *i.e.*, patuletin, patuletrin, quercetagetin, quercetagetrin, tagetiin, and kaempferitrin (5–8), in addition to a number of carotenoids, *i.e.*, helinine, violaxanthin, rubichrome, rubixanthin epoxide, lutein, antheraxanthin, α-cryptoxanthin, phytofluene, α-carotene, β-carotene, and helenien (9–11), have been isolated from other tagetes species but not *T. minuta*.

EXPERIMENTAL[2]

Plant Material—Originally, the aerial parts of the plant[3] *T. minuta* L. (Compositae) were collected along an open sunny bank, below the Harar-Jijiga road, near the southeastern limits of Harar, Hararge Province, Ethiopia, in December 1964. The collection was made at latitude 9° 18′ N., longitude 42° 7′ E. at an elevation of 2134 m. (7000 ft.). A second collection of plant material (PR-15256), on which the current investigation is based, was collected along the Acachi River in Shoa Province, Ethiopia, 17 km. south of Addis Ababa [elevation 2082 m. (6830 ft.), latitude 8° 52′ N., longitude 38° 50′ E.] in January 1968. This material corresponded in every detail with the original collection.

Extraction and Initial Fractionation—A 6.0-kg. sample of milled aerial parts of *T. minuta* was extracted with hexane[4] in a Lloyd extractor for 24 hr. The resulting extract was concentrated and removed from the extractor; fresh solvent was added and the extraction was continued for an additional 24 hr. This procedure was repeated two additional times, and the extracts were pooled and concentrated to a thick syrup (39.8 g., Fraction A).

The defatted plant material was then air dried, placed back into the extractor, and extracted with 95% ethanol in a similar manner as for the hexane extraction. After removing the ethanol from the extract, a fraction (B) weighing 372 g. was obtained. Fraction B was then partitioned between 2 l. each of chloroform and water, and the aqueous phase was separated and extracted with three additional 2-l. volumes of chloroform. All chloroform extracts were combined and taken to dryness *in vacuo*, and a third fraction (C) weighing 43.5 g. was obtained. Entrained chloroform was removed from the aqueous phase *in vacuo*, and the fraction was frozen and lyophilized to yield 329.5 g. of Fraction D.

Fraction D was dissolved in a convenient volume of distilled water, and a flavonoid fraction was prepared by several extractions with ethyl acetate. The ethyl acetate extraction was continued until a negative cyanidin reaction was obtained (12). In this manner, 59.3 g. of Fraction E was obtained. Further extraction of Fraction D with ethyl acetate–methanol (95:5 and 8:2) yielded only negligible quantities of residue. Finally, Fraction D was exhaustively extracted with *n*-butanol, which yielded 190 g. of Fraction F.

Fraction E, following TLC on polyamide plates using methanol as the developing solvent and 25% aqueous lead subacetate solution as the spray reagent, showed the presence of three yellow-orange spots at R_f 0.90, 0.41, and 0.59 when the chromatograms were viewed under UV light.

Isolation and Identification of Patuletrin—An aqueous slurry of polyamide (370 g.) was prepared and poured into a glass chromatographic column (4 × 7 cm.). A charge of 18.05 g. of Fraction E was absorbed on 10 g. of polyamide, and this was placed on top of the prepared column as a dry powder. Elution was initiated with water, then with water containing increasing amounts of methanol, and finally with methanol. Fifty-milliliter fractions were collected from the column, and these fractions were monitored on cellulose TLC plates, using *n*-butanol–acetic acid–water (4:1:5) as the developing solvent. Fractions were grouped and pooled on the basis of TLC patterns after spraying the plates as previously described.

Fractions 50–69, which were eluted from the column with water–methanol (1:1), were pooled and taken to dryness to yield 1.01 g. of residue. The residue was dissolved in 500 ml. of hot methanol and this was reduced *in vacuo* to 50 ml. and filtered; then the filtrate was allowed to evaporate spontaneously at room temperature. In this manner, three crops of amorphous material were harvested, which were shown to be identical by TLC. The material consisted of two major components, giving flavonoid reactions, at R_f 0.66 (major) and 0.77 (minor). The precipitate was crystallized from methanol to give 20 mg. of crystals. However, the crystals still contained a small amount of the R_f 0.77 substance, which was separated from the major component by TLC on cellulose plates. Elution of the mixture was with *n*-butanol–acetic acid–water (4:1:5). The zone at R_f 0.66 was removed by scraping, dissolved in methanol, and crystallized in the usual manner to yield microcrystals having, after drying *in vacuo* at 100° for 24 hr., m.p. 254–256°. An IR spectrum of the isolate showed absorption maxima at 3380 (s), 2940 (sh), 1660 (m), 1595 (m), 1485 (s), 1375 (m), 1200 (m), 1070 (s), 990 (s), and 700 (s) $cm.^{-1}$.

Direct comparison of the aglycone of the isolate by UV and IR analyses and by TLC analysis in three different systems showed it to be identical with patuletin. The glycone, analyzed after acid hydrolysis, was shown to be D-glycose. Attachment of the D-glucose at the 7-position was determined by UV analysis when no bathochromic shift in the UV spectrum from 259 nm. (observed) for the intact glycoside was observed after treatment with sodium acetate

[1] Collected in Ethiopia as part of the anticancer screening program of the U. S. Department of Agriculture and the National Cancer Institute.

[2] Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were obtained using a Beckman model DK-2 spectrometer, and the IR spectra were determined in KBr pellets using a Beckman model IR-8 instrument.

[3] A voucher specimen is in the Herbarium of the U. S. National Arboretum and in Kew Herbarium. The original specimen was determined at Kew.

[4] Skellysolve B.

(14). It was thus concluded that the isolated flavone was 3,5,3′,4′-tetrahydroxy-6-methoxyflavone-7B-D-glucopyronoside (patuletrin).

Isolation and Identification of Quercetagetrin—TLC on cellulose plates of combined fractions 96–115 from the column, which were eluted with water–methanol (1:3), showed the presence of one major flavonoid at R_f 0.16 and five minor flavonoids. The fraction (0.74 g.) was dissolved in a minimum volume of methanol and was applied to several thick-layer cellulose plates (0.5 mm.); it was separated, using *n*-butanol–acetic acid–water (4:1:5), by triple development of the plates. The major component (R_f 0.16) was removed and eluted from the cellulose matrix with methanol to give 410 mg. of one-spot material as evidenced by TLC. After drying *in vacuo* at 100° for 24 hr., the isolate exhibited a melting point of 237–238°. An IR spectrum (KBr) of the isolate showed absorption bands at 3400 (s), 2940 (w), 1655 (s), 1605 (s), 1485 (s), 1385 (m), 1250 (w), 1070 (s), 990 (sh), 855 (w), and 825 (w) cm.$^{-1}$. The isolate exhibited an unusual dull black color under UV light on paper and thin-layer chromatograms, which is an unusual characteristic for flavonoids but which has been described previously for two rather rare flavonoids, *i.e.*, quercetagetrin and its aglycone (16). This isolate was also shown to be a flavonol as evidenced by the typical UV absorption at 363 nm. (methanol), and the *o*-dihydroxy grouping was demonstrated to be present as a result of a hypsochromic shift of 56 nm. in aluminum chloride when compared with the absorption in aluminum chloride–hydrochloric acid.

A comparison of the UV, IR, and TLC data of the isolate with those of quercetagetrin proved the two compounds to be identical. Thus, the isolate was shown to be 3,5,6,3′,4′-pentahydroxyflavone-7B-D-glucopyranoside (quercetagetrin).

Isolation and Identification of Monomethyl Fumarate—An additional portion of Fraction E (41.25 g.) was dissolved in water containing only enough methanol to effect complete solution. This solution was then extracted several times with ether; the ether fractions were pooled, dried over anhydrous sodium sulfate, and taken to dryness to give 7.11 g. of residue. This residue was dissolved in chloroform and applied to a chloroform slurry-packed column of silicic acid (400 g.), and 20-ml. fractions were collected. TLC of each fraction from the column revealed that fractions 113–118 contained a single substance. The pooled fractions (113–118) were taken *in vacuo* to a syrupy consistency (510 mg.), and the addition of benzene to this syrup resulted in a copious formation of colorless needles. The needles were recovered by filtration, in addition to a second crop from the mother liquor. A total of 35 mg. of colorless needles was obtained which, after drying *in vacuo* for 24 hr., gave a melting point of 144.5°; IR (KBr): 3100 (s), 2975 (w), 2700 (w), 2530 (w), 1720 (s), 1685 (s), 1351 (s), 1285 (sh), 1275 (s), 1265 (s), 995 (s), 920 (m), 895 (m), 765 (m), and 650 (m) cm.$^{-1}$. The UV spectrum of the isolate, in ethanol, showed only end-absorption at 208 nm. (ϵ = 7333). A mass spectrum taken at 70 ev. showed the molecular weight of the isolate to be 130. Mass tables (17) were consulted for various combinations of C, H, and O having this molecular weight. Five formulas were found: $C_5H_6O_4$, $C_6H_{10}O_3$, $C_7H_{14}O_2$, $C_8H_2O_2$, and $C_8H_{16}O$. Since the IR spectrum indicated the presence of both an unsaturated acid and an unsaturated ester, all compounds with less than four O atoms were not considered further. This left only the formula $C_5H_6O_4$. The mass spectrum showed that the compound was a methyl ester, as evidenced by the large M − 31 ion at *m/e* 99. When the IR spectrum of the isolate was compared with that of a synthetic sample of the monomethyl ester of fumaric acid, they were found to be identical. The melting point of the isolate was 144.5°, which is in agreement with the literature value for the monomethyl ester of fumaric acid (18). A mixed melting point of the isolate and a synthetic sample of the monomethyl ester of fumaric acid showed no depression. These data confirmed the identification of this isolate as the monomethyl ester of fumaric acid.

Isolation and Identification of Syringic Acid—Fractions 161–204, obtained from the silicic acid column previously described, were combined and concentrated *in vacuo* to a thick syrup and placed into a vacuum oven to dry at 50°. After several hours, colorless needles were observed projecting from the syrup. These crystals were removed and dissolved in 10 ml. of methanol for recrystallization. Three milligrams of pure, colorless needles were obtained from this solution which, after drying for 72 hr. in a vacuum desiccator at 25°, exhibited a melting point of 197–199°.

An IR spectrum of the isolate (KBr) showed absorptions at 3300 (s), 2980 (w), 2930 (w), 1695 (s), 1610 (s), 1515 (s), 1455 (s), 1415 (s), 1365 (m), 1240 (m), 1200 (s), 1025 (w), 905 (m), 860 (m), 795 (w), 765 (s), and 680 (s) cm.$^{-1}$. The carbonyl absorption at 1695 cm.$^{-1}$ was suggestive of an unsaturated aromatic acid.

The UV spectrum, in methanol, showed bands at 267 (ϵ = 4812) and 217 (ϵ = 9375) nm. On addition of a potassium hydroxide solution to the sample, a bathochromic shift to 300 (ϵ = 5937) and 224 (ϵ = 7812) nm. was observed, indicating the presence of an enolizable moiety in the isolate, perhaps due to a phenol group.

In consideration of these data, a search was made of previously isolated constituents to obtain a list of organic acids having a molecular weight of 198 (as evidenced by a mass spectrum of the isolate) and a melting-point range of 197–200°. Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), and 2,4,5-trihydroxyphenylglyoxylic acid were the only two compounds that fulfilled these criteria. Since the IR spectrum did not show the two different carbonyl absorptions necessary for the substituted phenylglyoxylic acid, and since the literature melting point for 2,4,5-trihydroxyphenylglyoxylic acid was 193°, syringic acid was tentatively assumed to be the isolated compound.

Supporting evidence that this selection was correct is found if one examines the UV spectrum again. Comparing the theoretical maximum for syringic acid, an excellent correlation is found. If one uses a base value of 239 nm. for benzoic acid, adding 25 nm. for a *para*-substituted hydroxyl group and 7 nm. each for two *meta*-substituted methoxyl groups, one arrives at a calculated value of 269 nm. for syringic acid (19). This compares favorably with the observed value of 267 nm. for the isolate.

Mass spectra of the isolate and a reference sample of syringic acid were identical.

In addition to these data, TLC in three different solvent systems gave identical R_f values when the isolate was simultaneously chromatographed with a reference sample of syringic acid. Also, the IR spectra of the isolate and syringic acid were superimposable.

Antitumor Activity of *T. minuta*—Each crude fraction (A–D) was submitted to the Drug Research and Development Branch, National Cancer Institute, for bioassay using established protocols (23) in the PS leukemia, L-1210 leukemia, Walker muscular carcinosarcoma, and Lewis lung *in vivo* tumor systems. In addition, the fractions were evaluated for cytotoxicity using Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Fractions A–C were inactive against all neoplasms and were not cytotoxic. Fraction D had marginal activity against the Lewis lung carcinoma, giving a T/C value[1] of 43% at 266 mg./kg. The monomethyl ester fumaric acid is inactive against the P-388 leukemia. It was also found to be noncytotoxic.

SUMMARY

The aqueous extract prepared from *T. minuta* L. aerial parts has shown reproducible activity against the Lewis lung carcinoma. A phytochemical investigation was initiated in an attempt to isolate and characterize the active antitumor principle(s). During the investigation, quercetagetrin, patuletrin, the monomethyl ester of fumaric acid, and syringic acid were isolated and identified. This is the first report on the occurrence of these four compounds in *T. minuta*. Only the ester was available in quantities sufficient for biological testing, and it was found to be noncytotoxic. Studies are in progress to isolate the active antitumor principle(s) from this plant.

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Imidazolecarbohydrazides IV: Synthesis and Biological Evaluation

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Abstract □ A series of 1-methylimidazole-4,5-dicarboxylic acid hydrazides and a bicyclic compound imidazo[4,5-*d*]pyridazine were synthesized and their structures were elucidated. The compounds were subjected to a limited pharmacological evaluation (MAO inhibitory activity) and the results are reported.

Keyphrases □ 1-Methylimidazole-4,5-dicarboxylic acid hydrazides —synthesis, structure determination, screened for MAO inhibitory activity □ Imidazo[4,5-*d*]pyridazine—synthesis, structure determination, screened for MAO inhibitory activity □ Hydrazides, 1-methylimidazole-4,5-dicarboxylic acid—synthesis, structure determination, screened for MAO inhibitory activity □ MAO inhibitors, potential—synthesis and evaluation of 1-methylimidazole-4,5-dicarboxylic acid hydrazides and imidazo[4,5-*d*]pyridazine

The synthesis and certain biological properties of some imidazole-4(or 5)-mono- and 4,5-dicarboxylic acid esters, hydrazides, and hydrazones were reported previously (1, 2). The present paper reports the investigation of 1-methylimidazole-4,5-dicarboxylic acid derivatives and their bicyclic analogs.

The starting ester, dimethyl 1-methylimidazole-4,5-dicarboxylate (I), was synthesized by methods previously reported (1, 2). The reaction of I with hydrazine (II*a*) or methylhydrazine (II*b*), depending on the reaction conditions, gave the dihydrazide (III*a*) (1) or 1-methylimidazole-4,5-dicarboxylic acid bis(2-methylhydrazide) (III*b*), respectively, or their bicyclic analogs IV*a* (3) and 1,5-dimethyl-7-hydroxyimidazo[4,5-*d*]pyridazin-4-one (IV*b*), respectively. With phenylhydrazine (II*c*), only III*c* was obtained.

As depicted in Scheme I, some analogs of III were synthesized by sodium borohydride reduction of their respective hydrazones (4). In this study, the ethylidene derivative (VI) was found to be resistant to such reduction.

The reaction product of I and II*b* could possibly be IV*b*, IV*c*, or a mixture of the two. A relatively sharp melting point of the product, together with a single spot observed on a thin-layer chromatogram and the presence of only a single NMR peak for the CH_3 protons on the pyridazine, gave convincing evidence that the compound was not a mixture of the two isomers. The elucidation of the molecular structure of the compound was attempted by the following experimental procedure. Mixing stoichiometric quantities of an aqueous solution of silver nitrate and IV*a* gave a precipitate analogous to those reported (5) for purines on whose 6-position exists an OH or NH_2 with the 7 as pyridinic N, or on the 6-position a C=O with the 7 as pyrrolic NH. Separation of the precipitate by centrifugation, its dissolution in perchloric acid, and UV spectrophotometric analysis of both the dissolved precipitate and the supernate revealed a 1:1 silver–IV*a* chelate. Similar experiments on the bicyclic reaction product of I and II*b* did not produce any precipitate. Because the purine analog of IV*c* forms a water-insoluble silver chelate (5), the reaction product was assumed to be IV*b*.

The formation of IV*b* may possibly involve an intermediate half-ester half-hydrazide, 1-methylimidazole-4-carbomethoxy-5-(2-methylhydrazide), although it has not been isolated or identified. The phenyl analog of this compound, imidazole-4-carbomethoxy-5-(2-phenylhydrazide), however, has been shown (1) not to give an analogous bicyclic system. The possibility of III*b* being