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FUNGISTATIC CONSTITUENTS IN CITRUS VARIETIES RESISTANT TO THE MAL-SECCO DISEASE

J. PINKAS,*· D. LAVIE and MATHILDA CHORIN

Division of Plant Pathology, Volcani Institute of Agricultural Research Bet Dagan, and Department of Chemistry, Weizmann Institute of Science, Rehovoth, Israel

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Abstract—The resistance mechanism of mandarin varieties towards *Deuterophoma tracheiphila* Petri, the causal agent of Mal-secco, (a citrus disease) has been investigated. From two mandarin varieties, Cleopatra and Avana, five flavones were isolated, identified and tested for their fungistatic activity. Four of the flavones were found potent, one of which, 5, 4′-dihydroxy-6, 7, 8, 3′-tetramethoxy flavone, is a new compound, while a second, 5, 4′-dihydroxy 6, 7, 8-trimethoxy flavone, is reported for the first time to occur in citrus.

INTRODUCTION

ONE of the most destructive diseases of citrus trees, especially of the lemon in the Mediterranean region, is known as Mal-secco. This disease is caused by *Deuterophoma tracheiphila* Petri, a fungus which invades the conducting vessels of the xylem, inducing defoliation and progressive drying of the branches from the top towards the main trunk. Ultimately, the tree dies.[1] The disease can be easily identified by a pink orange colour seen in a cross-section of the stem. Several species of citrus trees, such as mandarins, sweet orange[2] and grapefruit,[3] have been reported to be resistant to the fungus.

In 1930, Petri assumed the occurrence of some fungistatic principles in different species of citrus;[4] subsequently, an antifungal activity was reported[5] for certain fractions extracted from leaves and bark of the Cleopatra mandarin, a variety known to be highly resistant to Mal-secco. Extracts from susceptible lemon varieties failed to show such activity. In the present study we describe the isolation of four fungistatic flavones which were obtained from two mandarin varieties, one of which is described for the first time, while another has not yet been reported to occur in Rutaceae. A fifth flavone of known structure, which was also isolated, was not found to have fungistatic properties in our tests.

RESULTS

Isolation and Identification of the Compounds

Dried leaves of Cleopatra mandarin were extracted consecutively with ether, chloroform and methanol. The crude extracts were tested for fungistatic activity on potato dextrose agar

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[2] L. PETRI, *Boll. Real. Staz. Pat. Veg. Firenze* **10**, 63 (1930); G. GASSNER, *Phytopath. Z.* **13**, 1 (1940).
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[5] A. BEN-AZIZ, M. CHORIN, S. P. MONSELISE and I. REICHERT, *Science* **135**, 1066 (1962).

media inoculated with the fungus *Deuterophoma tracheiphila*, and the activity was determined by the degree of inhibition of the fungal growth. Both ether and chloroform extracts were found to inhibit fungal development; however, the former was more potent. This fraction was therefore selected for further purification. Chromatography of the crude extract gave in the benzene-ether eluate, a crystalline mixture which on subsequent rechromatography, yielded the flavones tangeritin[6] and nobiletin.[7] Tangeritin (I) m.p. 152°, λ_{max} 271 and 323 nm showed the following NMR spectrum: set of signals at about δ 4 accounting for 15 protons, due to five methoxyl groups and at lower field a singlet at δ 6·50 for the C-3 proton. Two signals centered at δ 6·91 and 7·80 represent the superimposition of two doublets each for the C-3′, C-5′ and C-2′, C-6′ protons, respectively. Additional *meta* splitting resulted in diffuse signals. The NMR spectra of flavonoids have been extensively analysed, enabling the allocation and the identification of the various signals.[8] Comparison with an authentic sample of tangeritin afforded unequivocal identification of the compound. Nobiletin (II), m.p. 136°, λ_{max} 250, 270 and 333 nm, showed the following NMR spectrum: at about δ 4, signals accounting for 18 protons disclosed six methoxyl groups in the molecule, a singlet at δ 6·60 is attributed to the C-3 proton, and a doublet centered at δ 7·0 ($J_{H5',6'}=8{\cdot}5$ c/s) is related to the C-5′ proton; the signal of the C-6′ proton is centered at δ 7·59 and appears as a double doublet ($J_{H6',5'}=8{\cdot}5$ c/s, $J_{H6',2'}=2$ c/s). The signal of the C-2′, proton is a narrow doublet at δ 7·40 ($J_{H2',6'}=2$ c/s), as expected from *meta* splitting. All these data clearly indicate that the structure of the compound is 5, 6, 7, 8, 3′, 4′ hexamethoxy flavone, i.e., nobiletin. Similar results have recently been reported for these two compounds as well as for their fungistatic activity.[9]

In a second series of experiments, leaves of Avana mandarin were extracted with chloroform, and the residue was found to be highly fungistatic. Following chromatography, the activity was disclosed in all the fractions by gradual elution with the solvent mixtures ethyl acetate-benzene (1:9) up to pure ethyl acetate. All these fractions were combined, treated with charcoal and rechromatographed over silica gel using benzene-ethyl acetate 6:4 as the only eluting solvent mixture, and five pure compounds were obtained and identified as follows:

The first compound was identified as 5-hydroxy-6, 7, 8, 3′, 4′-pentamethoxy flavone[10] by its m.p. 145°, u.v. spectrum, molecular ion peak M^+ 388 in accordance with the formula $C_{20}H_{20}O_8$ and the NMR spectrum. The latter showed the expected 5 methoxyl group signals (δ 3·98, $4\times CH_3O$ and δ 4·11, $1\times CH_3O$), and a singlet at about δ 12·5 characteristic for the C-5 hydroxyl which is hydrogen bonded to the C-4 carbonyl group.[8] This group was further confirmed by a bathochromic shift in the u.v. spectrum of 21 nm upon addition of drops of an aluminium chloride solution (λ_{max} 341 → 362 nm).

The second purified sample, following methylation with dimethyl sulphate, yielded tangeritin. Its mass spectrum had a molecular ion peak of M^+ 344 in accordance with an empirical formula of $C_{18}H_{16}O_7$, disclosing two hydroxyl and three methoxyl groups in the flavonoid skeleton. One hydroxyl group at C-5 could easily be detected by observing in the u.v. the specific bathochromic shift of Band I upon addition of a few drops of an $AlCl_3$ solution (λ_{max} 330 → 352 nm). The location of the second hydroxyl group at C-4′ in ring B was shown by alkali fission of the diethoxy derivative. Following extraction of the acidified

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reaction product with aqueous sodium hydrogen carbonate and reacidification, the organic acid was re-extracted with chloroform and esterified to methyl-4-ethoxy benzoate identified by its retention time on GLC using as reference an authentic sample. The compound was therefore 5, 4′-dihydroxy-6, 7, 8-trimethoxy flavone (IV), which has been previously isolated from *Satureia douglassii* (Labiatae).[11]

The third compound eluted was exhaustively methylated with dimethyl sulphate and yielded nobiletin. However, partial methylation with diazomethane afforded a mixture of two compounds, nobiletin and 5-desmethyl-nobiletin, inferring the presence of at least two hydroxyl groups. Mass spectrum analysis of the compound indicated a molecular ion peak M^+ 374 in accordance with an empirical formula of $C_{19}H_{18}O_8$. The fragmentation pattern showed a peak m/e 211 for fragment VI of the flavone molecule containing one hydroxyl group. A second fragment m/e 123 accounts for ring B with one hydroxyl and one methoxyl group as shown in (VII). The NMR spectrum at low field indicated signals accounting for three aromatic protons, while the signal at δ 6·63 is related to the C-3 vinylic proton. All other positions are therefore substituted. Two signals, one at δ 4·09 for nine protons ($3 \times CH_3O$), and the second at δ 4·2 for three protons ($1 \times CH_3O$) could be observed. Of the two hydroxyl groups which had to be accounted for, one could be detected at δ 12·5 for the C_5-OH. Since no bathochromic shift could be observed either for Band II of the flavone in the u.v. spectrum of the compound after the addition of sodium acetate,[12] or for Band I upon addition of a sodium acetate-boric acid mixture,[13] no hydroxyl group could be present at positions 6 or 7. Furthermore, treatment with *p*-benzoquinone did not result in the formation of red colour or of a precipitate (gossypetone reaction), thereby discarding the possibility of the second hydroxyl group being in position 8.[14] The second hydroxyl group is therefore in ring B and its location was determined by alkali fission of the diethyl ether derivative which afforded 3-methoxy-4-ethoxy-benzoic acid, the methyl-ester of which was identified by its retention time in GLC with an authentic sample. This hydroxyl group is therefore at C-4′ and the compound was identified as 5, 4′-dihydroxy-6, 7, 8, 3′-tetramethoxy flavone. The last two compounds to be obtained were again nobiletin and tangeritin. During the identification of the various flavones, a colour reaction was developed for chromatoplate detection by spraying the plates previously treated with iodine vapour with aqueous sodium hydrogen carbonate. Coloured complexes of various shades were obtained for the different flavones.

Fungitoxicity

From these five compounds, four were found to possess fungistatic activity towards *Deuterophoma tracheiphila*. With tangeritin and 5, 4′-dihydroxy-6, 7, 8-trimethoxy flavone, growth reached about 80–90 per cent of the control, while with nobiletin and 5, 4′-dihydroxy-6, 7, 8, 3′-tetramethoxy flavone, which were found to be much more active, growth was reduced to about 50 per cent in 200 ppm concentration. Interestingly, higher concentrations of up to 500 ppm of nobiletin and 600 ppm of 5, 4′-dihydroxy-6, 7, 8, 3′-tetramethoxy-flavone did not increase their inhibitory activity.

Nobiletin and tangeriten were also tested against three other plant parasitic fungi, *Fusarium moniliforme* Sheldon, *Sclerotium rolfsii* Sacc. and *Verticillium alboatrum* Reinke and Berth. Activity was observed only against the first two at a concentration of 250 ppm

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[12] L. Jurd and R. M. Horowitz, *J. Org. Chem.* **22**, 1618 (1957).
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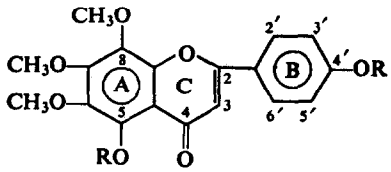
as shown in Table 2. Tangeritin, which was found to exhibit low fungitoxicity against *Deuterophoma tracheiphila* was very active against the *Fusarium moniliforme* fungus. This last plant pathogen tends to produce aerial mycelium on the medium containing antifungal compounds. The crude extracts of both varieties of mandarins were much more active than either of the compounds alone.

TABLE 1. GROWTH OF *Deuterophoma tracheiphila* Petri AT DIFFERENT CONCENTRATIONS OF FOUR FUNGISTATIC COMPOUNDS EXPRESSED AS PER CENT OF CONTROL

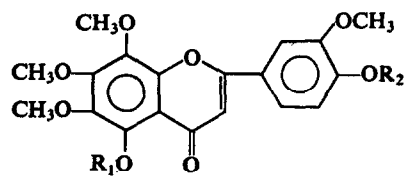
Concentration	Nobiletin	Tangeritin	5, 4′-Dihydroxy 6, 7, 8, 3′-tetramethoxy flavone	5, 4′-Dihydroxy 6, 7, 8-trimethoxy flavone
50 ppm			41	89
100 ppm	65	79	49	80
200 ppm	53	81	48	85
300 ppm	49	81	44	90
400 ppm	49	85		
500 ppm	49	77		
600 ppm			44	89

TABLE 2. GROWTH OF THREE PLANT PATHOGENIC FUNGI AT 250 PPM OF NOBILETIN AND TANGERITIN EXPRESSED AS PER CENT OF CONTROL

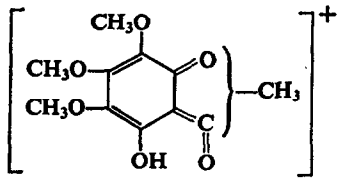
	Nobiletin	Tangeritin
Fusarium moniliforme	40	42
Sclerotium rolfsii	52	76
Verticillium albo-atrum	100	100



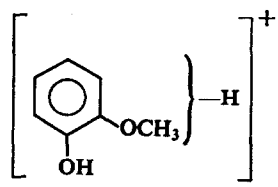
(I) Tangeritin; R=CH_3
(IV) R=H



(II) Nobiletin; $R_1=R_2=CH_3$
(III) $R_1=R_2=H$
(V) $R_1=H$, $R_2=CH_3$



m/e 211
($C_9H_7O_6$)
(VI)



m/e 123
($C_7H_7O_2$)
(VII)

EXPERIMENTAL

The melting points are uncorrected and were taken on a Fisher-Johns apparatus. The u.v. spectra were recorded on a Cary 14 spectrometer in ethanolic solution. The NMR spectra were recorded on a Varian A-60 instrument. Silica gel G (Merck) coated plates were used for T.L.C.

Isolation of Nobiletin and Tangeritin from Cleopatra Mandarin, Citrus reticulata

Crushed, dried leaves (1 kg) of Cleopatra mandarin were extracted continuously in a soxhlet with ether, chloroform and methanol for 48 hr each.

The ether extract was filtered and evaporated to dryness. The green residue was collected (20 g) and chromatographed on a column packed with acid-washed alumina (Merck) in benzene solution. Elution was performed first with benzene, then with a mixture of benzene-ether of increasing concentration. With ether-benzene 4:6 (about 1·5 l.) a crystalline mixture emerged containing two main compounds, as seen on a chromatoplate (developing solvent, benzene-ethyl acetate 6:4).

Rechromatography of the mixture with the same solvent system yielded two pure compounds: the first to emerge was an oil which crystallized from ethyl acetate as long needles, m.p. 152°; compared with an authentic sample of tangeritin, it showed no depression on mixture m.p. and had identical infrared spectra throughout the whole range.

The second compound to appear was nobiletin, crystallized from ether as small thin needles, m.p. 136°, λ_{max} 250, 270 and 333 nm (ϵ 21, 780; 18, 090; 27, 020). Anal. Found: C, 62·9; H, 5·8; M^+ 402. $C_{21}H_{22}O_8$ requires: C, 62·7; H, 5·5%; mol. wt. 402·39.

Isolation of Five Flavones from Avana Mandarin, Citrus reticulata

Dry leaves of Avana mandarin were ground and extracted as above with chloroform for 92 hr. The dry extract was chromatographed on silica gel (0·05–0·20 mm Merck) packed in benzene. Elution was performed with solvent mixtures containing an increasing concentration of ethyl acetate in benzene. The active fractions which were eluted with 10 per cent ethyl acetate to pure ethyl acetate were combined, evaporated and taken in chloroform, treated with activated charcoal, and re-evaporated to dryness. The residue was rechromatographed on a silica gel H (Merck) column using benzene-ethyl acetate 6:4 as eluent. Fractions of 50 ml were collected. They were combined according to TLC indication using a benzene-methanol-acetic acid mixture (45:3:2). Five compounds were obtained in the following sequence:

(a) *5-Hydroxy-6,7,8,3′,4′-pentamethoxy-flavone(5-O-desmethyl-nobiletin)* (*V*), yellow needles from methanol, m.p. 145°; λ_{max} 252, 287 and 341 nm (ϵ 12,700; 14,500 and 16,900), with three drops of aqueous $AlCl_3$, λ_{max} 260, 292, 303 and 362 nm (ϵ 7760; 10,800; 10,700 and 15,100). Mass spectrum: M^+ 388, $C_{20}H_{20}O_8$, mol. wt. 388·36.

(b) *5,4′-Dihydroxy-6,7,8-trimethoxy-flavone* (*IV*), yellow needles from methanol, m.p. 228°; λ_{max} 281, 293 and 330 nm (ϵ 17,000; 16,800 and 20,400) with three drops of aqueous AlC.$_3$, λ_{max} 288, 311 and 352 nm (ϵ 14,380; 16,750 and 22,350). Mass spectrum M^+ 344, $C_{18}H_{16}O_7$, mol. wt. 344·31.

(c) *5,4′-Dihydroxy-6,7,8,3′-tetramethoxy-flavone* (*III*), yellow needles from benzene, m.p. 192°, λ_{max} 255, 280 and 341 nm (ϵ 15,400; 18,000 and 20,800) with three drops of aqueous $AlCl_3$, λ_{max} 254, 288, 302 and 360 nm (ϵ 11,200; 14,850; 14,950 and 19,200). Anal. Found: C, 60·5; H, 5·0; M^+ 374. $C_{19}H_{18}O_8$ requires: C, 60·9; H, 4·8%, mol. wt. 374·33.

(d) *Tangeritin*; and (e) *Nobiletin*, the two were identified as described above.

Alkali Fission of the Diethyl Ether of (*III*)

Compound (III, 5 mg) dissolved in acetone (15 ml) was ethylated by heating under reflux with silver carbonate (500 mg) and ethyl iodide (0·5 ml) for 20 hr. The reaction mixture was filtered and evaporated to dryness. The residue was dissolved in ethanol (80% 3 ml) and heated with KOH (100 mg) for 6 hr, then acidified with HCl and the solution was extracted with 2% aq. $NaHCO_3$. The solution was reacidified and extracted with ether several times. The combined extracts were concentrated and a solution of CH_2N_2 added. Evaporation of the solvent afforded the methyl ester which was analysed by gas liquid chromatography on a column of stainless steel 5 mm × 180 cm packed with 15% Apiezon N on Fluoropak 80 at 235°. The instrument was an Aerograph A-90-P, the carrier gas helium, at a flow of 1 ml/sec at 30 psi. The retention time of the above ester was 4′44″, which was identical with that of *methyl-4-ethoxy-benzoate.*

Alkali Fission of the Diethyl Ether of (*IV*)

Compound (IV, 5 mg) was treated as above and the resulting ester injected on the same chromatographic column. The retention time was 7′ 52″, which was identical with that of *methyl-3-methoxy-4-ethoxy-benzoate.*

Spot Tests for Chromatoplate for the Five Flavones

The standard procedure for developing the spots on the chromatoplates was to introduce the plates into I_2 vapours. Following the I_2 treatment, spraying the plates with a 2 per cent solution of aqueous ($NaHCO_3$) induced the formation of a coloured complex differing in intensity, shade and speed of appearance, according

to the substance tested. Tangeritin and nobiletin appeared as dark blue spots, the former being darker and appearing first. 5-O-desmethyl nobiletin appeared as a violet spot, while the other two compounds gave pale brown colors. Whenever acids were present in the developing solvent mixture, the plates first had to be thoroughly air dried.

Formation of Tangeritin Iodine Complex

Tangeritin (16 mg) in acetone solution (1 ml) was added to a 5% I_2 and 5% KI aq. solution (1 ml). The dark precipitate which was formed was collected, washed with 1% aq. KI and dried to constant weight. Anal. Found: C, 41·9; H, 3·6; I, 33·6 $(C_{20}H_{20}O_7)_2I_3$ requires: C, 42·7; H, 3·6; I, 33·8%.

Bioassay

Each fraction (10 mg) was dissolved in acetone (or methanol if appropriate) and added to hot potato dextrose agar medium. The mixture was thoroughly shaken and then heated in order to evaporate the organic solvent. Following sterilization, petri dishes were poured, and the fungus *Deuterophoma tracheiphila* was inoculated by introducing agar disks with the fungus (4 mm diameter) in the middle of the dish. Incubation was carried out at 20° for 14 days. The results of each treatment (Tables 1 and 2) represent an average of four replicates (three petri dishes each). Whenever active principles were present, inhibition of the growth could be observed clearly. In preliminary tests the active principles were found to be heat stable and were not affected by sterilization at 121° for 20 min.

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