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Study of *Citrus taitensis* and radical scavenger activity of the flavonoids isolated

Mostafa Mohamed Saleh,[a] Fatma Abd El-Megeed Hashem[a]* & Karl Werner Glombitza[b]

[a]*Pharmaceutical Science Department, National Research Centre, Dokki, Cairo, Egypt*
[b]*Pharmaceutical Biology Institute, Bonn University, Bonn, Germany*

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The GC-MS study of the head space volatiles of *Citrus taitensis* Risso revealed that the main component, constituting 60% of the oil, is linalool. Three N-containing compounds, benzyl cyanide, indole and methyl anthranilate, constituted a considerable additional percentage of the volatiles (16.9%). Investigation of the flavonoids indicated that the two major components were dihydrorobinetin and genistein. Both compounds give a high percentage inhibition of the chemiluminescence in polymorphonuclear cells stimulated by *N*-formylmethionyl-leucyl-phenylalanine, but a much lower inhibition with stimulation by opsonized zymosan. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Citrus essential oils have been the subject of numerous investigations, since they occupy an important position in the manufacture of perfumery, cosmetics, confectionery and pharmaceutical formulations. This is the first study on *Citrus taitensis* Risso and the main purpose was to isolate volatile components in mild conditions in order to obtain a natural citrus flavour with a minimum of artifacts. Other citrus species were studied in various countries. Mehlitz and Minas (1965) used a combination of TLC and column chromatography to identify a number of oxygenated terpenes in the steam-distilled oil of bitter orange peel. Karawya and Wahba (1962) identified limonene, terpineol and/or linalool, geraniol and/or nerol and methyl anthranilate in oil of sweet orange. Karawya *et al.* (1971) made a comparative chromatographic study of Egyptian citrus species, *C. sinensis* L. and *C. aurantium* L., by cold-pressed and steam-distilled peel oils. Kovats' indices were used as a preselection routine in mass spectra library searches of volatiles of *C. limonia* Christm (leaves) analyzed by GC/MS (Alencar *et al.*, 1984). In Italy, an overview of the chemical composition of the volatile fractions of essential oils from five citrus species was produced, (Dugo, 1994). The history, production, constituents and use of essential oils, obtained using a cold press extraction method in five citrus plants in the USA, were discussed by Shaw (1978). Citrus oils were described as antimicrobials against food poisoning and food spoilage organisms by Dabbah *et al.* (1970). Hesperidin, a flavonoid isolated from orange peel, was found to have anti-inflamatory and analgesic activity (Galati *et al.*, 1994). Two new flavones, 7-hydroxy-3,4,5,6-tetramethoxyflavone and 3-hydroxy-4,5,6,7,8-pentamethoxyflavone, were isolated from the leaves of *Citrus reticulata* Blanco by Mizuno *et al.* (1987). Polymethoxylated flavonoids isolated from *Citrus aurantium* L., were found to affect erythrocyte sedimentation rate (Quarenghi-de-Riera and Seeligmann, 1985).

Radicals have been reported to induce cellular damage, which may play a role in heart diseases, rheumatoid arthritis and inflammatory disorders, as well as in aging processes. Thus, the search for effective, but non-toxic compounds with antioxidative activity has been intensified in the past decade. Among the various kinds of natural radical scavengers, flavonoids have received much attention (Heilmann *et al.*, 1995).

MATERIAL AND METHODS

Collection of volatiles

The fruits (100 g) were sliced and placed in a 0.5 litre glass purge vessel, fitted with an inlet and an exit port. High purity He (99.999%), further purified by passage through an O_2 scrubber and a hydrocarbon trap, was forced by positive pressure into the glass vessel through the inlet port. Connected to the exit port was a sorbent trap, consisting of a stainless-steel tube, fitted with brass nuts and ferrules and containing 0.9 g of Tenax TA (2,6-diphenyl-*p*-phenylene oxide polymer, 60–80 mesh, Chrompack, Raitan, NJ), held in place by silanized glass wool plugs. The Tenax TA trap was initially conditioned

*To whom correspondence should be addressed.

at 340°C for 2 h with He flow at 30 ml min^{-1}. Immediately before trapping, the Tenax TA trap was baked at 225°C for 10 min to ensure no carry-over compounds from previous analyses. He-contacted copper tubing, glass and Teflon only, metal tubing to glass tubing connections being made with brass fittings and Teflon ferrules. The glass purge vessel had a ground-glass closure that was sealed from the outside with Teflon tape and held in place with springs. The flow rate of He 40 ml min^{-1}) was measured at the exit end of the Tenax TA trap using a bubble meter. The plant material was purged for 10 h at 25°C. After sampling, the trap was connected directly to the purified He and purged for 40 min to remove any accumulated moisture.

Desorption and GC-MS analysis

The volatiles were thermally desorbed (190°C, 15 min) from the Tenax TA trap installed in a Tekmar model 4001 dynamic headspace concentrator and flushed through a heated transfer line (120°C) into a silica WG 11 glass capillary column (50 m length×0.25 mm i.d. ×0.025 μm film thickness; Supelco Inc., Bellafonte, PA). Prior to desorption, a loop of the column, ca. 10 cm at the injection end, was lowered into a liquid N_2 bath, where it remained during desorption in order to focus (cryogenically) the desorbed compounds. During desorption, the carrier gas head pressure was 30 psi, and the septum purge and split vent were closed. After the desorption period, the liquid N_2 bath was removed and oven temperature programming was begun.

Chromatograms were obtained with a Hewlett Packard 5792 gas chromatograph interfaced to a Model 5971 mass selective detector using the following conditions: Carrier gas head pressure 15 psi, carrier gas linear velocity 25 cm s^{-1}, carrier gas flow rate 0.6 ml min^{-1}, electron ionization at 70 eV, electron multiplier voltage 1800 V.

GC oven temperature was programmed from an initial temperature of 30°C to 180°C at 6°C min^{-1}. The column was baked at 195°C for 15 min before use.

The constituents of the oil were identified by comparing their retention indices and mass fragmentation patterns with those of the available references (Adams, 1989) and/or with other published data. The results of GC-MS analysis are recorded in Table 1.

Table 1. Volatile components of *Citrus taitensis* Risso

Identified compound	Kovats index	% Conc.
Myrcene	983	0.12
Limonene	1022	0.12
Cis-Ocimene	1025	0.05
Trans-Ocimene		0.06
Cis-Linalool oxide		0.25
Trans-Linalool oxide		0.42
Linalool	1086	60.2
Benzyl cyanide		2.18
Citronellal		0.18
Terpinen-4ol	1175	0.38
α-Terpineol	1185	4.26
Nerol		0.42
Linalyl acetate		6.25
Indole		9.00
Methyl anthranilate	1232	5.67
Citronellyl acetate	1275	0.29
Geranyl acetate	1303	0.14
Neryl acetate	1352	0.32
β-Caryophyllene	1428	0.19
Trans β-Farnesene		0.06
Bergamotene	1434	0.08
Trans, trans-α-Farnesene	1657	0.17
Cis-Nerolidol	1724	6.00
Heptadecene-1		0.17
Trans-Nerolidol	1796	1.47

Isolation of flavonoids

Fresh plant material (5 kg) was macerated successively in 15, 10 and 5 litres of n-hexane on three succesive days. The extracts were mixed, filtered and the solvent was evaporated on a water bath under vacuum at as low a temperature as possible. The remaining hard solid was extracted with ice-cold absolute ethanol and the extract was filtered through a sintered-glass funnel. The solvent was evaporated under vacuum and the volatile oil was distilled as completely as possible. The remaining residue was chromatographed on Alumina 60 G Neutral (Merck Cat. 1090; 5–40 μm). The mixture (200 mg) was separated by vacuum liquid chromatography (VLC) on 10 g silica gel by successive elution with 30 ml light petroleum (b.p. 40–60) and ether (1:1), ether and ether: ethyl acetate (3:1). The VLC apparatus consisted of a Buchner G4 filter funnel connected to a filter flask and a vacuum pump according to Pelletier *et al.* (1986) and Coll and Bodwen (1986). Compound I was present in the ether eluate while Compound II was present in the ether: ethyl acetate (3:1) eluate.

Radical scavenger activity of the isolated flavonoids (I and II)

Polymorphonuclear cells (PMNs) were suspended in antibiotic-free Hank's balanced salt solution (HBSS) without Phenol Red. *N*-Formylmethionyl-leucyl-phenylalanine (FMLP) and zymosan were used as chemoattractants. Human PMNs of 100 healthy volunteers were isolated from 10 ml of heparinized venous blood by the polymorphprep. (aqueous solution of 13.8% sodium metrizoate and 8% dextran 500) density gradient, centrifugation method at 500 g for 30 min at 20°C. The cells were washed twice and adjusted to a concentration of 5×10^6 ml^{-1} in HBSS. These cell preparations contained 95–98% of PMNs and their viability was more than 95% as determined by Trypane Blue exclusion.

Chemiluminescence assay with PMNs

The flavonoid solutions were prepared by dissolving 1 mg of a compound in 1 ml DMSO and diluting with

HBSS to four different molar concentrations. The reaction was initiated by adding 50 μl of each of the stimulants: FMLP (5×10^{-7} mol.) and opsonized zymosan (5 mg ml^{-1}) to each concentration of Compounds I and II. Light emission was recorded continuously in counts per min (c.p.m.) for 30 min. All results were calculated as a percentage of the response of the control (chemiluminescence reaction in the absence of the compound).

Data are expresed as mean $\pm$ standard deviation. Statistical analysis was done using the Wilcoxon test.

RESULTS AND DISCUSSION

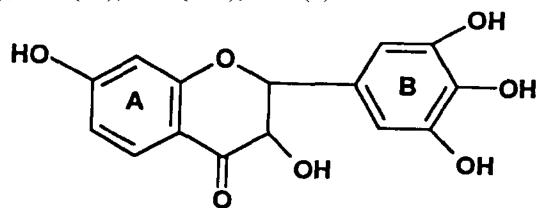
GC/MS examination of the headspace over *Citrus taitensis* Risso fruit revealed the presence of 33 components, 25 of which were identified (Table 1). The main components were linalool, indole, linalyl acetate, methyl anthranilate, α-terpineol and benzyl cyanide (60.2, 9.0, 6.25, 5.67, 4.26 and 2.18%, respectively). All of them were previously identified in *C. sinensis* L. and *C. aurantium* L. by Karawya *et al.* (1971), except benzyl cyanide and indole, which are found to be characteristic for *C. taitensis* Risso.

Compound I, isolated from the alumina column, when treated with methanol, afforded a yellow solid, which was recrystallized from methanol to give dihydrorobinetin. Its chromatographic data were: R_f values produced on paper chromatography Whatman No. 1, with systems, butanol: acetic acid: water 3:1:1 and 15% acetic acid were 0.36 and 0.58. Colour in UV at 365 nm was pale yellow; in UV/NH_3 yellow; colour with $AlCl_3$ was yellow.

Spectroscopic data for Compound I

UV: λ_{max} (MeOH): 275, 307 nm; λ_{max} (MeOH. MeONa): 251, 334; λ_{max} (MeOH-$AlCl_3$); 280, 308, 345 Sh. λ_{max} (MeOH-$AlCl_3$/HCl): 275, 308; λ_{max} (MeOH-Sod. acetate): 257 Sh, 280, 333; λ_{max} (MeOH–Sod.acetate/H_3BO_3): 278, 312 Sh.

^{1}H-NMR (DMSO): δ ppm 7.8 (1H, d, J = 9 Hz, H-5). 5.6 (2H, s, H-'2, H-'6), 6.4 (1H, d, J = 2.5, H-8), 6.3 (1H, d, J = 2.5 Hz, H-6), 4.9 (1H, d, J = 11, H-2), 4.3 (1H, d, J = 11, H-3). MS: M^+304 (18), 275 (20), 168 (8), 149 (18), 139 (25), 137 (100), 121 (5).



Compound I: Dihydrorobinetin
3,7-hydroxy-2,3 dihydro, 3',4',5' trihydroxyflavonol

Dihydrorobinetin has been isolated previously from the stemwood of *Robinia pseudoacacia* L. by Mogel *et al.* (1994) and several species of *Cordia* by Ficarra *et al.* (1995).

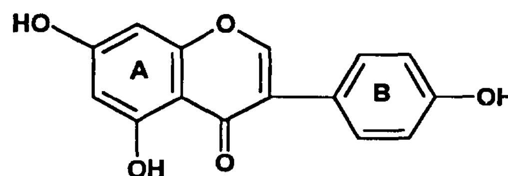
Compound II, when treated with methanol, also afforded a yellow solid, which, recrystallized from methanol and had m.p. 297°C. Its chromatographic data were: R_f values produced on paper chromatography, Whatman No. 1, with systems, butanol: acetic acid: water 3:1:1 and 15% acetic acid, were 0.85 and 0.30; colour in UV at 365 nm was deep purple; in UV/NH_3 deep purple; colour with $AlCl_3$ was yellow.

Spectroscopic data for Compound II

UV: λ_{max} (MeOH): 262, 326 Sh; λ_{max} (MeOH-MeONa): 276, 327 Sh; λ_{max} (MeOH-$AlCl_3$): 274, 307 Sh, 372; λ_{max} (MeOH-$AlCl_3$/HCl): 273, 308 Sh, 372; λ_{max} (MeOH–Sod.acetate): 271, 325; λ_{max} (MeOH–Sod.acetate/H_3BO_3) 263, 336 Sh.

^{1}H-NMR (DMSO): δ ppm: 7.2 (1H, s, H-2), 6.5 (2H, d, J = 8.5 Hz, H-'2, H-'6), 6.0 (2H, d, J = 8.5, H-'3, H-'5), 5.4 (2H, d.d, J = 2.5, 1.0 Hz, H-6, H-8).

MS: M^+ 270 (100), 269 (38), 152 (20), 124 (10), 118 (15).



Compound II: Genistein
5,7 4' hydroxyisoflavone

Genistein is an isoflavone isolated before from *Prunus cerasoides* D.Don. by Bahuguna *et al.* (1987) and from the flowers of *Cytisus scoparius* by Link, Kurihara and Kekuchi (1980) and flowers of *Genista lydia* Boiss, by Ulubelen and Doguc (1974).

The percentage inhibition of chemiluminescence in polymorphonuclear cells, stimulated by FMLP or opsonized zymosan, was increased greatly by increasing concentration of dihydrorobinetin. Genistein also inhibited chemiluminescence, but to a lesser extent than dihydrorobinetin (Table 2). Heilmann *et al.* (1995) studied the radical scavenger activity of 3', 4'-dihydroxyflavonols differing in the substitution of the A and C rings.

Structural features are very important in conferring activity, e.g. the *o*-dihydroxy structure of the B-ring, the 2,3-double bond in conjugation with a 4-oxo-function and the additional presence of either a 3- or a 5-hydroxy group. The present paper makes a further contribution, as Compound I containing the *o*-dihydroxy structure of the B ring, also has 3,7-hydroxy groups in Rings A and C while, in Compound II, the 2,3-double bond is conjugated with the 4-oxo-function and there are additional hydroxy groups at the 5 and 7 positions. The radical scavenging activity was evaluated by quantifying the

Table 2. Percentage inhibition of chemiluminescence in polymorphonuclear cells stimulated by FMLP or opsonized zymosan (OZ)

Compound	Concentration μmol l^{-1}	FMLP	SD	OZ	SD
I, (Dihydrorobinetin)	0.25	43.0	±2.9	5.4	±3.7
	0.50	72.3	±3.0	8.2	±4.0
	1.00	85.2	±3.1	12.0	±4.0
	1.25	90.3	±3.1	12.9	±5.1
II, (Genistein)	0.25	33.4	±3.3	2.8	±2.9
	0.50	66.3	±3.4	6.4	±3.2
	1.00	75.0	±4.2	11.0	±3.2
	1.25	86.2	±4.2	11.8	±4.2

inhibition of luminol-enhanced chemiluminescence in FMLP- and zymosan-stimulated human PMNs. Depending on the stimulant, different enzymes are influenced. FMLP, as the major peptide neutrophil chemoattractor factor, binds to its specific membrane receptors on phagocytes and activates a guanine nucleotide regulatory (G) protein. Other enzymes include the activated phospholipase C, which hydrolyses phosphatidyl-inositol 4,5-biphosphate (PIP_2) into the calcium mobilizer, inositol 1,4,5-triphosphate (IP_3) and the protein kinase activator, 1,2-diacylglycerol (DAG). Thus binding of FMLP causes an increase in intracellular calcium concentration and activation of protein kinase C, leading finally to activation of NADPH-oxidase. Zymoan stimulates the Fc (3b receptor). Its post-receptor signal transduction is less dependent on GTP binding regular protein than that of FMLP and the magnitude of DAG generation is greater.

The two compounds tested were able to reduce chemiluminescence in human PMNs after stimulation either by FMLP or opsonized zymosan.

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REFERENCES

Adams, R. P. (1989) Identification of Essential Oils by Ion Trap Mass Spectroscopy, UK edn. Academic Press, London.

Alencar, J. W., Craveiro, A. A. and Matos, J. A. (1984) Kovats' indices as a preselection routine in mass spectra library searches of volatiles. *J. Nat. Prod.* **47**, 890–892.

Bahuguna, R. P., Jangwan, J. S., Kaiya, T. and Sakakibara, J. (1987) Puddumin-A, a new flavanone glucoside from *Prunus cerasoides* D. *Don. J. Nat. Prod.* **50**, 232–234.

Coll, J. C. and Bodwen, B. F. (1986) The application of VLC to the separation of terpene mixtures. *J. Nat. Prod.* **49**, 934–936.

Dabbah, R., Edward, V. M. and Moats, W. A. (1970) Antimicrobial action of some *citrus* fruit oils on selected food-borne bacteria. *Appl. Microbiol.* **19**, 27–31.

Dugo, G. (1994) Composition of the volatile fraction of the Italian *Citrus* essential oils. *Perfum and Flavor* **19**, 29–42, 44–48, 50–51.

Ficarra, R., Ficarra, P., Tommasini, S., Calabro, M. L., Rogusa, S., Barbera, R. and Rapisard, A. (1995) Leaf extracts of some *Cordia* species: analgesic and anti-inflammatory activities as well as their chromatographic analysis. *Farmaco, Edizione Scientifica* **50**, 245–256.

Galati, E. M., Monfort, M. T., Kirjavainen, S., Forertieri, A. M. and Tripodo, M. M. (1994) Biological effects of hesperidin, a *citrus* flavonoid. Part I. Anti-inflammatory and analgesic activity. *Farmaco* **49**, 709–712.

Heilmann, J., Merfort, I. and Weiss, M. (1995) Radical scavenger activity of different 3′,4′-dihydroxyflavonols and 1,5-dicaffeoylquinic acid studied by inhibition of chemiluminescence. *Planta Med.* **61**, 435–438.

Karawya, M. S., Balbaa, S. I. and Hifnawy, M. S. (1971) Peel oils of different types of *Citrus sinensis* L. and *C.aurantium* L. growing in Egypt. *J. of Pharm. Science* **60**, 381–385.

Karawya, M. S. and Wahba, S. K. (1962) Chromatographic analysis of oil of sweet orange, oil of orange flower, oil of bergamot and oil of lavender. *Egypt. Pharm. Bull.* **44**, 31–41.

Kurihara, T. and Kekuchi, M. (1980) Studies on the constituents of flowers. Part 13. On the components of the flower of *Cytisus scoparius* Link. *J. pharm. Soc. Japan* **100**, 1054–1057.

Mehlitz, A. and Minas, T. (1965) Bitter orange peel oil. II. Terpenoids (oxygen-containing compounds) *Riechstoffe, Aromen, Koerperpflegemittel* **15**, 204; through *Chem. Abstr.*, (1965) **63**, 11243.

Mizuno, M., Matoba, Y., Tanaka, T., Tachibana, H. and Iwamasa, M. (1987) Two new flavones in *Citrus reticulata. Blanco. J. Nat. Prod.* **50**, 751–753.

Mogel, E., Jay-Allemand, C. and Ziegler, H. (1994) Formation of heartwood substances in the stemwood of *Robinia pseudoacacia* L. II. Distribution of nonstructural carbohydrates and wood extractives across the trunk. *Structure and Function* **8**, 165–171.

Pelletier, S. W., Joshi, B. S. and Desai, H. K. (1986) Separation of diterpenoid alkaloid mixtures using VLC. *J. Nat. Prod.* **49**, 892–900.

Quarenghi-de-Riera, M. V. and Seeligmann, P. (1985) Polymethoxylated flavonoids from *Citrus aurantiun* L. (Rutaceae) and their effect on the erythrocyte sedimentation rate. *Acta Farmacutica Bonearense* **4**, 33–35.

Shaw, P. E. (1978) *Citrus* essential oils. *Perfum. and Flavor* **3**, 35–40.

Ulubelen, A. and Doguc, T. (1974) Flavonoid compounds from the flowers of *Genista lydia* Boiss. *Planta Med.* **25**, 39–42.