

## Coumarin Content and Anti-Bilharzial Activity of Extracts of Leaves of *Citrus aurantifolia* (Lime) and *Citrus limonia* (Lemon)

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Coumarins were isolated and identified from the leaves of *Citrus aurantifolia* and *C. limonia*; the percentage yields were 3.1 and 2.7%, respectively. Bergapten, limettin, isopimpinellin and umbelliferone were obtained from both species; however, this is the first report of the four compounds from lemon leaves. Xanthotoxin, found only in lime leaves is reported here for the first time from the genus *Citrus*. The anti-bilharzial activity of the crude coumarin concentrates from the alcohol extracts of both species was found to be just slightly less than pure isopimpinellin, a coumarin with known high activity.

### INTRODUCTION

*Citrus aurantifolia* Swingle (lime) and *Citrus limonia* Osbeck (lemon), Family Rutaceae, have not been investigated for coumarins in leaves to the same degree as in fruits, barks, roots and seeds.<sup>1</sup> Coumarins are well known for their anticoagulant, estrogenic, vasodilatory, diuretic, hepatotoxic, dermal photosensitizing, respiratory stimulant and antibacterial properties,<sup>1</sup> as well as molluscicidal and anthelmintic activities.<sup>2</sup> This latter property led to our investigation of the coumarins in the abundant leaves of these two *Citrus* species in order to test the concentrates from the extracts for activity against the *Schistosoma* parasite, which causes bilharziasis. Endemics of this disease exact a significant drain on the economy of Egypt.<sup>3</sup>

### EXPERIMENTAL

**Plant material:** Leaf samples of *Citrus aurantifolia* (lime) and *Citrus limonia* (lemon) were obtained from fruiting trees cultivated at the Agricultural Crops Research Institute, Giza, Egypt in November 1986-87. The plants were identified by Dr. El-Hadidy and herbarium specimens are deposited in the Agricultural Crops Research Institute.

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*Authentic reference compounds:* Bergapten, limettin, isopimpinellin, umbelliferone, and xanthotoxin were obtained from the Natural Product Department, National Research Centre, Dokki, Cairo, Egypt.

*Animals for anti-bilharzial study:* Albino mice weighing 18–20 g were used.

*Special reagents and instruments:* Iodine-potassium iodide spray reagent<sup>4</sup>: 0.2 g I<sub>2</sub> and 4 g KI were dissolved in 100 mL H<sub>2</sub>O. Kofler's hot stage microscopic apparatus was used for determining melting points. Other equipment utilized included: ultraviolet visible recording spectrometer Shimadzu UV 240; EI-MS MAT 112, 70 eV; Varian <sup>1</sup>H-NMR T-60; Pye-Unicam Infrared Spectrometer SP 1100; and a Photo Binocular Nikon AFX Microscope and Stereoscope.

*Extraction, isolation, and identification:* Coumarins were extracted<sup>5</sup> from dried powdered leaves (1000 g) with 95% ethanol (10 L); each extract was concentrated to 500 mL under reduced pressure and treated with an equal volume of 10% aqueous KOH. After 1 h each alkaline alcoholic extract was diluted with 500 mL of water and defatted with ether (4 × 250 mL). The aqueous mother liquor was acidified with dilute HCl and the acidic solution was refluxed for 1.5 h, cooled, and extracted with ether (4 × 250 mL). The ether layer (1 L) was washed with water and then dried over anhydrous sodium sulfate before being taken to dryness, to give 30.7 and 26.8 g of lime and lemon crude coumarin concentrates. The two coumarin concentrates were compared with standards by TLC on silica gel G plates using benzene : ethyl acetate 8 : 2 as a solvent system; the compounds were visualized under UV at 365 nm, and then the plates were sprayed with I<sub>2</sub>/KI spray reagent. The crude coumarin concentrates were each fractionated on a silica gel column (800 g, 1.5 m × 3.5 cm); elution solvents used in sequence until nothing detected by TLC : petroleum ether : benzene mixtures (4 : 1, 1 : 1 and 1 : 4); benzene and benzene : methanol mixtures (99 : 1 and 99 : 3), and methanol, all with a flow rate of 4 mL/min. Fractions with similar TLC patterns were combined. Crystallization from methanol gave pure compounds allowing identification of each coumarin using UV, NMR, IR and MS spectral data and also by direct TLC comparison with authentic samples and by melting points and mixed melting points. The percentage yield of each coumarin was determined, and the results are recorded in Table-1.

TABLE-1  
PERCENTAGE YIELD OF COUMARINS FROM DRY LIME AND LEMON LEAVES

	Lime(%)	Lemon(%)
Bergapten	0.22	0.11
Limettin	0.13	0.11
Xanthotoxin	0.97	—
Isopimpinellin	0.26	0.18
Umbelliferone	0.32	0.37

*Anti-bilharzial activity:* Four groups of albino mice (10 mice each) were infested with cercaria of the Egyptian strain of *Schistosoma mansoni* using the tail method technique<sup>6</sup> involving isolating each mouse in a jar containing 100

cercaria. After 1 h the water was examined to assure the absence of cercaria, hence confirming cercaria penetration into the mouse through the skin. After 6–8 weeks the stools were examined for the presence of *Schistosoma* eggs, indicating that the parasite had reached the adult stage. Two groups of mice were then treated each with 1 mL of distilled water containing 1 mg of the concentrate from each of the two 95% ethanolic leaf extracts; a third group was treated with 1 mg of pure isopimpinellin in 1 mL of distilled water, and a fourth group was not treated to serve as a control. The LD<sub>50</sub> of the samples was determined to be approximately 50 mg per kg mouse body weight for each sample. Since the average weight of the mice was 20 g, each mouse was intraperitoneally injected with 1 mg of the sample under investigation, each in 1 mL of distilled water. Stool examinations were carried out after 3, 5 and 7 days of treatment for observation of the egg state and reduction in the number of eggs.<sup>7,8</sup> After stool examination all mice were sacrificed and the percentage reduction of the number of worms was calculated relative to the number of worms in the mice control group.

## RESULTS AND DISCUSSION

Five coumarins, bergapten, limettin, isopimpinellin, umbelliferone and xanthotoxin, were isolated from *Citrus aurantifolia* (lime) and the latter four were also obtained from *Citrus limonia* (lemon) and are reported here for the first time from lemon leaves. Xanthotoxin had not been previously detected in *Citrus*. The percentage yield of crude coumarin concentrates from leaves of lime was 3.1% and from lemon leaves 2.7%. All compounds were identified by comparison of their m.p.s, UV, IR, NMR and MS data with those of authentic samples.

Because bilharziasis is a major disease in Egypt and coumarins have well known antiwormal activity, the coumarins occurring in the leaves of lime and lemon trees were isolated and identified and the concentrate of the alcohol extracts of these leaves were tested in mice infested with *Schistosoma mansoni*. The mice were sacrificed and the number of worms present in the mesentery and liver was determined by comparing with the control group. The percentage reductions for pure isopimpinellin, a coumarin known to have high activity and one of the major components of lemon and lime leaves, was 88%, while for the concentrates of the alcohol extracts of lime and lemon the reduction was 61% and 53%, respectively. These activity results for the coumarin concentrates are significantly high because they represent crude samples.

Lime and lemon trees are important crops in Egypt, and their leaves represent a major available resource. The relatively large amount of highly active coumarins in the leaves of these *Citrus* species could lead to the medicinal use of this resource.

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