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ISOLATION OF A FLAVONOID FROM THE ROOTS OF *CITRUS SINENSIS*

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Citrus sinensis is native to Asia and throughout the Pacific and warm areas of the world. The ethyl acetate extract of the roots of Citrus sinensis yielded a flavonoid. The compound was characterized as 5, 8-dihydroxy-6, 7, 4'-trimethoxyflavone on the basis of UV, I.R, mass and N.M.R (^{1}H, ^{13}C) spectral studies. We believe this is the first report describing the isolation of flavonoid from this plant.

Keywords: *Citrus sinensis, Rutaceae, Flavonoid*

INTRODUCTION

Citrus sinensis is one of the important medicinal plants that is found broadly in the district of Shahjahanpur. This plant is prescribed as a traditional medicine for the treatment of various ailments. It has been used as an anti-diabetic (Hamendra and Anand 2007), antimicrobial (Caccioni *et al.* 1998), antifungal (Stange Jr *et al.* 1993), hypotensive agent (Kumamoto *et al.* 1986), antioxidant (Proteggente *et al.* 2003; Kanaze *et al.* 2008), carminative, insect repellent, antibacterial, larvicidal, antiviral, uricosuric, anti-yeast, antihepatotoxic and antimutagenic agent (Han 1998). The leaves and the peel of the fruit have been used to kill mosquito larvae and mites (Mwaiko 1992). There are strong evidences showing that the essential oil of *Citrus sinensis* have larvicidal, repellent and fumigant activities against *Aedes aegypti L.* mosquitoes (Omobuwajo *et al.* 2005). Leaf extracts of *C. sinensis* have been used in folk medicine to treat neurological disorders and to facilitate the digestion of food (Holdsworth 1992).

Although monoterpenes, sesquiterpenes, gibberellic acid, phytol, amyrin, limonin and its glucosides, nomilin derivatives, carotenoids, alkaloids, brassinolide, castasterone, sitosterol, hydroquinone, sinapic acid, anethole, ferulic acid, etrogol, vitamin C, coumaric acid, citrusins (proteid), caffeic acid, coumarins, pectin, stigmasterol and flavonoids

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have been isolated from *Citrus sinensis* (Han 1998), only flavonoid exhibits a broad spectrum of pharmacological properties (Bylka, Matlawska and Pilewski 2004). The most prevalent flavanones present in *Citrus sinensis* are hesperidin, naringin, tangeretin and nobiletin. Hesperidin was shown to have anti-inflammatory, antihypertensive, diuretic, analgesic and hypolipidemic properties (Gil-Izquierdo *et al*. 2001). It also has antioxidant, anti-allergic, vasoprotective and anti-carcinogenic actions (Proteggente, *et al.* 2003; Kanaze *et al.* 2008). Nobiletin is a novel anti-inflammatory (Lin, Sato and Takayama 2003) and immunomodulatory drug. It has been shown to have an inhibitory effect on phorbol ester-induced skin inflammation, oxidative stress and tumour promotion in mice (Murakami, Nakamura and Torikai 2000). It also has anti-proliferative and apoptotic effects on cancer cell lines (Kandaswami *et al.* 1991). Another flavonoid, naringin showed cytoprotective, anti-ulcer (Martin *et al.* 1994) and antioxidant activity (Pietta 2000). A pharmacological study showed that *Citrus* juices (grape and orange) reduce the risk of forming calcium oxalate stones (kidney stones). There is convincing evidence of the positive effects of dietary intake of *Citrus* fruits on cardiovascular diseases (Hertog *et al.* 1994; Hirvonen *et al.* 2000; WHO 2003). This research on *Citrus sinensis* was carried out as a continuation of our previous investigations on Rutaceous plants (Intekhab, Siddiqui and Aslam 2008a; Intekhab, Siddiqui and Aslam 2008b).

METHODS

Plant Material

The roots and leaves of *Citrus sinensis* were collected from the rural areas of Shahjahanpur district in the month of January 2007. The plant was identified by the Department of Botany of G. F. College (Rohilkhand University) Shahjahanpur, where a voucher specimen has been deposited. Fresh or dried plant material can be used as source for the extraction of secondary plant components. Both freshly harvested and dried materials are commonly used since old dried material stored for a period of time may undergo some qualitative changes. Roots and leaves were carefully examined. Old, insect-damaged and fungus-infested roots and leaves were removed. Healthy roots and leaves were spread out and dried in the laboratory at room temperature until they broke easily by hand. Air-dried

plant material (about 1 kg) was ground into fine powder and extracted successively with hexane, chloroform, ethyl acetate and methanol.

Instrumentation

Ultraviolet absorption spectrum was recorded on a Perkin-Elmer Lambda Bio 20 UV spectrometer. IR spectroscopy was performed on a Perkin-Elmer 1710 infrared fourier transformation spectrometer. NMR spectra were recorded on a Bruker AVANCE DRX- 300(300, 100 Hz). Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. FEBMS was recorded on a JEOLSX 1021/DA-6000 mass spectrometer. Column chromatography was performed using silica gel (Merk 7749).

Extraction and Isolation

Air-dried roots of *Citrus sinensis* were first defatted with hexane (3 L x 5 times) and then soxhleted with chloroform, ethyl acetate (EtOAc) and methanol (3 L x 5 times each). The EtOAc extract was then evaporated under vacuum on rotatory evaporator below 50˚C to yield a brownish mass (60 g). A well-stirred suspension of silica gel (100–150 g in petroleum ether at 60˚C–80˚C was poured into a column (150 cm long and 50 mm in diameter). When the absorbent was well settled, the excess of petrol-ether was allowed to pass through the column. The slurry was passed through the silica gel in petrol-ether and was digested to well stirred column. The column was successively eluted with hexane, chloroform, EtOAc, methanol, and their mixtures of increasing polarity. Elution with $CHCl_3$:MeOH (7:1) afforded a yellow powder (1.23 g). The compound yielded a positive Shinoda test, and alcoholic solution $FeCl_3$ (alc.) yielded a green colour.

m.p.: 170˚C–172˚C.
UV (MeOH) λ_{max}: 272 and 334 nm.
IR (KBr) ν_{max} cm^{-1}: 3488, 1632, 1591, 1498, 1039, 811.
^1H NMR (300 Hz, $CDCl_3$) δ: 12.68 (1H, s, 5-OH), 8.59 (1H, s, 8-OH), 3.90-4.01 (9H, s, 3x –OCH_3), 7.86 (2H, d, J=8.6 Hz, H-2′, H-6′), 6.98 (2H, d, J= 8.6 Hz, H-3′, H-5′), 6.53 (1H, s, H-3).
^{13}C NMR (100 Hz, $CDCl_3$) δ: 162.9 (C-2), 101.5 (C-3), 183.3 (C-4), 151.4 (C-5), 129.9 (C-6), 158.2 (C-7), 128.1 (C-8), 139.9 (C-9), 108.4 (C-10), 125.4 (C-1′), 129.2 (C-2′/6′), 113.9 (C-3′/5′), 161.4 (C-4′), 56.4 (6 –OCH_3), 56.1

(7 –OCH_3), 55.5 (4′ –OCH_3).
MS (m/z) 344 $[M+H]^+$, 329 $[M -CH_3]^+$, 316 $[M -CO]^+$, 314 $[M -2xCH_3]^+$, 301 $[M -CO-CH_3]^+$, 197 [r. D. A. cleavage], 169 [197 –CO]$^+$, 153 [169 –CH_3]+, 141 [169 –CO]+, 135 [fragmented ion peak], 126 [141 –CH_3]+, 113 [141 –CO]$^+$ and 107 [135 –CH_3]$^+$.

RESULTS AND DISCUSSION

The compound was isolated from the EtOAc extract by eluting the column with a chloroform:methanol (7:1) mixture. The compound showed a positive ferric chloride and Shinoda test for flavonoids, indicating that the compound may be a flavonoid (Geissman 1962; Markham 1982). These results also suggested that the compound is a flavonoid derivative with a free hydroxyl group at C-5 (Geissman 1962). In its electrospray mass spectrum, the molecular ion peak of the compound at m/z 344 $[M+H]^+$ corresponded to the molecular formula $C_{18}H_{16}O_7$. The UV spectrum exhibited absorption maxima at 272 and 334, suggesting that the compound belongs to the flavone family, unsubstituted at the 3-position (Markham and Mabry 1975). The IR spectra of the compound showed absorption bands for hydroxyl group (3488 cm^{-1}), chelated α, β –unsaturated carbonyl attached with aromatic nucleus (1632, 1591, 1448 cm^{-1}), methoxy group (1039 cm^{-1}), and *p*-substituted benzene ring (811 cm^{-1}) functionalities (Mabry, Markham and Thomas 1970).

The ^1H NMR spectrum of the compound exhibited a signal at δ 12.68 (1H, s), attributed to a chelated hydroxyl group. Further, a signal observed at δ 8.59 (1H, s) was due to a phenolic hydroxyl group. The ^1H NMR displayed one singlet at δ 6.53 that could be assigned to an H-3 proton (Markham and Mabry 1975). The three singlets were observed in the range of δ 3.90 - δ 4.01 (9H, s) assigned to the three methoxy groups. The ^1H NMR also demonstrated two protons doublets at δ 7.86 (2H, d, J, = 8.6 Hz), 6.98 (2H, d, J,= 8.6 Hz), assignable to H-2′/H-6′ and H-3′/H-5′ protons (Mabry, Markham and Thomas 1970). The appearance of two doublets and their coupling constant values are further in agreement with the methoxy group at C-4′. The mass spectrum of the compound showed important mass peaks at m/z 344 [M+H]+, 329 $[M -CH_3]^+$, 316 $[M -CO]^+$, 314 $[M -2XCH_3]^+$, 301 $[M -CO-CH_3]^+$, 197 [r. D. A. cleavage], 169 [197 –CO]$^+$, 153 [169 –CH_3], 141 [169 –CO], 135 [fragmented ion peak], 126 [141

–CH3], 113 [141 –CO]$^+$ and 108 [135 –CH_3]$^+$. The MS fragmentation pattern clearly indicated that two methoxy and two hydroxyl groups were attached to the ring-A, while the remaining methoxy group was linked with the ring-B at C-4′ (Harborne and Baxter 1999). The UV spectrum of the compound, in the presence of aluminium chloride, remained unchanged upon the addition of hydrochloric acid, which confirmed the presence of a hydroxyl function at C-5 and one of the methoxy groups at C-4′ (Markham and Mabry 1975). The mass fragmentation pattern also confirmed that the C-6 position was blocked by a methoxy group. The compound gave a negative Gibb's test, indicating the other hydroxyl group was at C-8, and, consequently, the remaining methoxy group must be at the C-7 position (Porter 1988; Porter 1994; Harborne and Wiliams 2000). In view of these spectral data, the compound was identified as 5, 8-dihydroxy-6, 7, 4′-trimethoxyflavone (Fig. 1). This structure was further confirmed by ^{13}C NMR spectral studies. The ^{13}C NMR spectrum of the compound showed a total of 16 signals for 18 carbons. A signal was observed at δ 183.3 and was allocated to C-4. Signals observed at δ 56.4, 56.1 and 55.5 were ascribed to 3 methoxy groups at C-6, C-7 and C-4′. An additional 2 signals were observed resonating at δ 129.2 and δ 113.9 attributed to C-2′/C-6′ and C-3′/C-5′, respectively (Agrawal 1989).

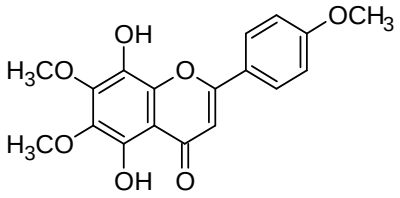

Fig 1: 5, 8-dihydroxy-6, 7, 4′-trimethoxyflavone

All these spectral data were in good concurrence with those reported in the literature (Suksamran *et al*. 2003; Brahmachari *et al.* 2004). This compound was previously reported from *Citrus jambhiri* (Chaliha, Sastry and Rao 1965).

CONCLUSION

We have successfully isolated a flavonoid from the plant *Citrus sinensis*. On the basis of these spectral data, the compound was identified as 5, 8-dihydroxy-6, 7, 4′-trimethoxyflavone.

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