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On The Chemical, Botanical, and Chemotaxonomical Evaluation of The Genus *Citrus*

Part I*: Polymethoxyflavones of The Leaf of *Citrus deliciosa* Ten.

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Abstract – Four polymethoxyfavones were isolated from the leaves of *Citrus deliciosa*, three of which (nobiletin, 5-O-demethylnobiletin, and tangeritin) are bioactive. The fourth (7,4'-dihydroxy-5,6,8,3'-tetramethoxyflavone) is reported for the first time in the genus *Citrus* and is a potential chemotaxonomic marker. The structures of these flavones were confirmed by analysing their spectral data and comparison with similar compounds. The previously reported ^{13}C NMR assignment of 5-O-demethylnobiletin has been revised on the basis of 2D NMR experiments (HETCOR, COSY, and COLOC). The chemotaxonomic value of the present finding is verified.

Key words – *Citrus deliciosa, Rutaceae,* Polymethoxyflavones, Nobiletin, 5-demethylnobiletin, tangeritin, 7,4'-dithydroxy-5,6,8,3'-tetramethoxyflavone, ^{13}C NMR, Chemotaxonomy.

Introduction

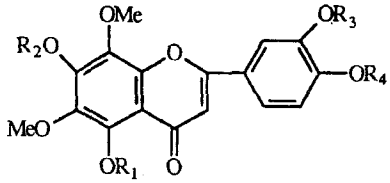
Citrus Plants have been cultivated for over 4000 years and it was early recognized that *Citrus* fruits were important for prevention of scurvy (Davies and Albrigo, 1994).

The genus *Citrus* is well known for its essential oils (Trease and Evans, 1983). It is also a rich source of flavonoids (Vandercook and Tissert, 1989; Mizuno *et al.*, 1991; Kanes *et al.*, 1991; Tatum and Berry, 1972) and recently, there have been increasing numbers of novel bicoumarins from *Citrus* plants (Ito *et al.*, 1990; Ito *et al.*, 1993a; Juichi *et al.*, 1991; Takemura *et al.*, 1993; Ito *et al.*, 1993b; Takemura *et al.*, 1994).

Recent reports on the biological activities of *Citrus* flavonoids included hypotensive (Akiyoshi *et al.*, 1989) and antiallergic (Hideaki *et al.*, 1991) actions. Polymethoxyflavones of *Citrus* peels are sometimes associated with biological activities, as they are reported to be differentiation inducers of myeloid leukemic cells (Sugiyama *et al.*, 1993). In addition, nobiletin (**IV**) which is a highly methoxylated *Citrus* flavonoid, has a potent inhibitory activity on cyclic adenosine monophosphate (AMP) phosphodiesterase, it also has an antifungal activity against *Deuterophoma tracheiphila* which causes a destructive disease of *Citrus* trees (Mizuno *et al.*, 1991).

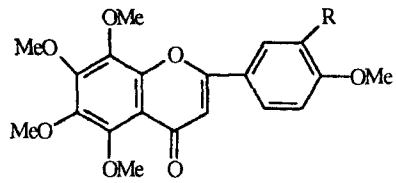
The large numbers of *Citrus* species, cultivars and hybrids reflected some difficulties on the taxonomic evaluation of *Citrus* plants (Davies and Albrigo, 1994; Rehm and Esping, 1984; Sharma, 1993; Bailey, 1949). *Citrus* flavonoids and their glycosides are important secondary metabolites that can aid in making taxonomic decisions (Albach and Red-

*First part of a series of studies on the chemical and botanical characters of *Citrus* plants with the aim of combining these characteristics to serve identification. Partk II: will consider the botanical characteristics of *C. delcisoa* and will be published soon elswhere.



I R_1=H; R_2=R_3=R_4=Me

II R_1=R_3=Me; R_2=R_4=H



III R=H

IV R= OMe

man, 1969). Therefore, chemotaxonomy of this genus has been comprehensively investigated by use of the phenolic or flavonoidal composition as markers (Kanes *et al.*, 1993). Polymethoxyflavones have also been considered as a specific markers for the chemotaxonomy of *Citrus* (Mizuno *et al.*, 1991; Iinuma *et al.*, 1980a).

Apparently, a simultaneous phytochemical and botanical investigations would provide an unambiguous identification of closely related *Citrus* species. It is also quite evident that only few taxonomic characters of the genus *Citrus* are available (Rehm and Esping, 1984; Sharma, 1993; Bailey, 1949). Therefore, it was decided to undertake a series of studies regarding the chemistry and botany of some *Citrus* species. This will facilitate the accomplishment of a straightforward identification of *Citrus* species.

In the present part (Part I), the leaf of one member of *Citrus* (*C. deliciosa*) has been subjected to a phytochemical study, with particular interest in polymethoxyflavones as chemotaxonomic markers.

Experimental

Plant Material-Leaves of *C. deliciosa* Ten. were collected in March and April 1995 from local gardens and from The Agrarian Reform Farms at El-Sharkia Governorate; Egypt, and identified by Prof. Dr. Abdalla M. A.Mohsen, Prof. of Horticulture, Faculty of Agriculture, Zagazig University. A voucher specimen is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

General Experimental-A digital melting point apparatus (Electrothermal LTD England) was used for mp determinations, and are uncorrected; UV spectra were determined with a Shimadzu UV-260 spectrophotometer; ^{1}H and ^{13}C-NMR spectra were recorded in $CDCl_3$ with TMS as internal standard on a Brucker AM-360 spectrometer (360 MHz) or Varian XL-200 spectrometer (200 MHz). Mass spectra were measured on a Finigan Mat 55Q-700 spectrometer, CI (180 eV) and EI (70 eV); IR spectra were recorded on a Perkin Elemer FT-IR 1650 machine; PTLC precoated with Kieselgel 60 (Merck) were used.

Isolation of Flavonoids-The leaves were air dried and ground (1 kg) and macerated with EtOH (4×5 L) at room temperature. The combined extracts were concentrated in vacuo and treatment with 5-fold cold methanol followed by filtration (To remove most of the hydrocarbons and fats). The defatted extract (109 g) was suspended in aqueous alcohol (9:1, 1 L) and succissively extracted with chloroform (5×250 ml) then with ethyl acetate (5×250 ml). TLC examination of both extracts showed the presence of flavonoids only in the chloroform extract (31.5 g). The latter was chromatographed over a silica gel column, packed in benzene, where non-flavonoids were washed down the column with benzene, and a mixture of four flavonoids were eluted together with 5% ethyl acetate in benzene. Rechromatography of the flavonoid containing fraction (4.2 g) over a column (50×3 cm) of

silica gel, packed in benzene and using mixtures of C_6H_6-EtOAc with an increasing ratio of EtOAc. Fractions eluted with C_6H_6-EtOAc (98:02) afforded 65 mg of flavonoid I as yellow needles (from $CHCl_3$/MeOH). Fractions eluted with C_6H_6-EtOAc (96:04) afforded a mixture containing flavonoids **II** and **III**, followed by fractions containing **IV** which was crystallized from $CHCl_3$/MeOH to give 45 mg of faint yellow needles. PTLC of the mixture of **II** and **III** using C_6H_6-EtOAc (75:25) afforded: 30 mg of **II** (Rf, 0.5) as yellow needles ($CHCl_6$/MeOH) and 28 mg of **III** (Rf, 0.4) as colourless needles ($CHCl_3$/EtOAc).

Compound I-Yellow needles crystallized from $CHCl_3$/MeOH, mp 143-144°. IR bands (KBr) at 3448, 1650, 1607, 1587, 1460, 1361, 1109, and 1073 cm^{-1}. UV (λmax, nm): (MeOH) 281, 342; (MeOH-NaOMe) 290 (sh), 316, 401 (sh); (MeOH-NaOAc) unchanged; (MeOH-NaOAc-H_3BO_3) unchanged; (MeOH-$AlCl_3$) 288, 352, 410 (sh); (MeOH-$AlCl_3$-HCl) 288, 354, 410 (sh). CI-MS m/z (rel. int. %): 389 $[M+1]^+$ (100%); EI-MS m/z (rel. int. %): 388 $[M]^+$ (72), 373 (100) 311 (42), 255 (22), 211 (17), 183 (18), 165 (14), 162 (8), 109 (22), 69 (39). ^1H and ^{13}C NMR: Tables 1 and 2, respectively.

Compound II-Pale yellow needles, mp 125-126° ($CHCl_3$/MeOH). IR bands (KBr) at 3377, 1732, 1649, 1603, 1574, 1434, 1369, 1107, 1068 and 1031 cm^{-1}. UV (λmax, nm): (MeOH) 279, 345; (MeOH-NaOMe) 286, 320, 405; (MeOH-NaOAc), 274, 345, 410 (sh); (MeOH-NaOAc-H_3BO_3) unchanged; (MeOH-$AlCl_3$), unchanged; (MeOH-$AlCl_3$) unchanged. EI-MS m/z (rel. int. %) 374 $[M]^+$ (78), 359 (100), 211 (12), 187 (5), 183 (15), 165 (7), 151 (8), 148 (4), 133 (10), 105 (8). ^1H NMR (200 MHz, $CDCl_3$: Table 1.

Compound III-Colourless needles crystallized from $CHCl_3$/EtOAc, mp. 152°. IR bands (KBr) at 1661, 1587, 1518, 1483, 1373, 1174, 1073, 1034 and 1012 cm^{-1}. UV (λmax, nm): (MeOH) 270, 323; unchanged upon addition of the standard sift reagents (Habrorne *et al.*, 1975). EI-MS m/z (rel. int. %): 372 $[M]^+$ (32) 357 (100), 225 (4), 197 (12), 182 (8), 135 (6), 132 (14), 83 (13). ^1H NMR (200 MHz, $CDCl_3$): Table 1.

Compound IV-Faint yellow microneedles ($CHCl_3$/MeOH), mp 135°. IR bands (KBr) at 1645, 1588, 1519, 1463, 1370, 1334, 1171 and 1037 cm^{-1}. UV (λmax, nm): (MeOH) 256, 270, 334; unchanged upon addition of the standard shift reagents (Habrorne *et al.*, 1975). EI-MS m/z (rel. int. %): 402 $[M]^+$ (42), 387 (100%), 371 (6), 344 (12), 225 (5), 197 (13), 165 (4), 162 (7). ^1H NMR (200 MHz, $CDCl_3$): Table 1.

Table 1. ^1HNMR spectral data of flavones **I-IV** (in $CDCl_3$, TMS as int. st.)*

H	I	II	III	IV
3	6.62 (*s*)	6.60 (*s*)	6.71 (*s*)	6.66 (*s*)
2'	7.42 (d, 2.0 Hz)	7.42 (*brs*)	7.89 (d, 9.0 Hz)	7.40 (*brs*)
6'	7.59 (dd, 9.5, 2 Hz)	7.54 (brd, 8.4 Hz)		7.56 (brd, 8.5 Hz)
5'	7.01 (d, 9.5 Hz)	7.05 (d, 8.4 Hz)	7.03 (d, 9.0 Hz)	6.98 (d, 8.5 Hz)
3'	–	-	-	-
OH-5	12.56 (*s*)	-	-	-
OH-7	-	4.69 (*s*)	-	-
OH-4'	-	8.10 (*s*)	4.11 (*s*)	4.09 (*s*)
OMe	4.12 (*s*)	4.11 (*s*)	4.02 (*s*)	4.01 (*s*)
	3.99 (*s*)	4.00 (*s*)	3.95 (*s*)	3.99 (*s*)
	3.98 (*s*)	3.98 (*s*)	3.95 (*s*)	3.96 (*s*)
	3.98 (*s*)	3.95 (*s*)	3.89 (*s*)	3.94 (*s*)
	3.96 (*s*)			3.94 (*s*)

*Run at 200 MHz, except I run at 360 MHz.

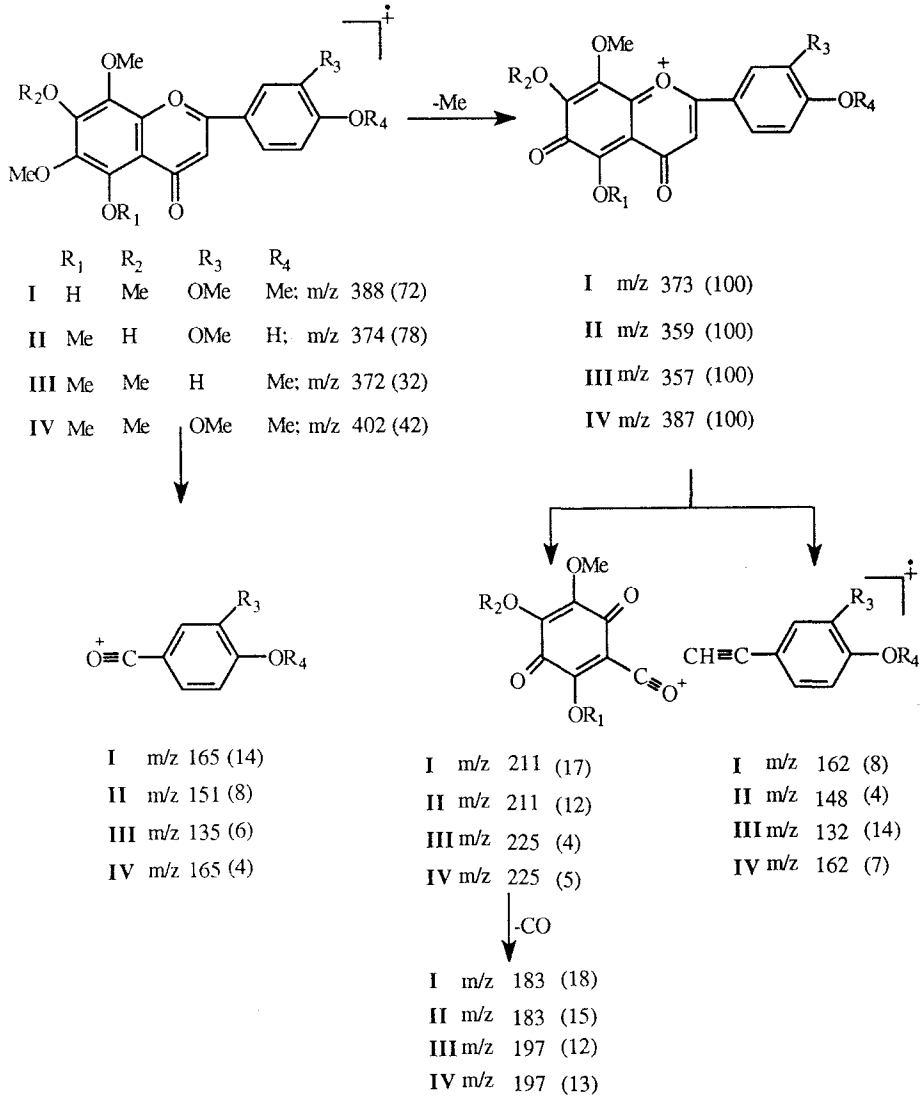
Results and Discussion

The defatted and concentrated extract of the dried leaves of *C. deliciosa* was extracted with chloroform. Repeated column chromatography of the chloroform extract followed by PTLC resulted in the isolation of the polymethoxyflavones **I-IV**. UV spectra which were carried out with diagnostic reagents using standard procedures (Mabry *et al.*, 1970; Harborne *et al.*, 1975), as well as the ^{1}H NMR data revealed a great similarity between compounds **I-IV**. The UV spectra in methanol are typical for flavones or 3-substituted flavonols. The presence of the H-3 singlet at *ca* δ 6.60 in the ^{1}H NMR excluded the presence of flavonols leaving flavones as the only possibility (Harbone *et al.*, 1975; Gonzalez *et al.*, 1991).

The MS of compound **I** exhibited a molecular ion peak at m/z 388 (72%) indicating a monohydroxypentamethoxyflavone ($C_{20}H_{20}O_8$). The low UV shift (+12 nm) of band I upon addition of $AlCl_3$+HCl suggested a 5-hydroxyflavone which is oxygenated at C-6 (Harborne *et al.*, 1975; Van Den Broucke *et al.*, 1982). The lone hydroxyl was located on C-5 [chelated hydroxyl signal in the ^{1}H NMR at δ 12.5 and a highly deshielded ^{13}C NMR singlet at δ 182.93 (C-4), chelated with an adjacent-OH group at C-5] (Harborne *et al.*, 1975; Gonzalez *et al.*, 1991; Agrawal, 1989). The ^{1}H NMR showed a singlet proton at δ 6.62 assigned to H-3, five aromatic methoxyl groups and aromatic protons showing a typical pattern for 3',4'-disubstitution at δ 7.59 (*dd*), 7.42 (*d*) and 7.01 (*d*) assigned to H-6', H-2' and H-5', respectively (Harborne *et al.*, 1975; Van Den Broucke *et al.*, 1982; Herz and Kulanthaivel, 1982). All the these facts together with the MS fragmentation (Scheme 1), indicated that the five metho- xyls are distributed over C-6, C-7, C-8, C-3' and C-4' of the ring system.

The ^{13}C NMR spectrum of **I** (Table 2) showed 20 carbon signals, confirmed the presence of 5 methoxyls and exhibited signals at δ 163.87 (C-2) and 103.95 (C-3) typical for flavones (Markhan, 1982). The highly deshielded carbonyl singlet at δ 182.93 confirmed the location of the-OH group at C-5 (Agrawal, 1989; Markham, 1982). The previous data besides comparison with published data for similar polymethoxy flavones (Iinuma *et al.*, 1980a; Mabry *et al.*, 1970; Harborne *et al.*, 1975; Van Den Broucke *et al.*, 1982; Gonzalez *et al.*, 1991; Agrawal, 1989; Herz and Kulanthaivel, 1982; Markham, 1982; Vyas and Bulchandani, 1986; Quijano *et al.*, 1985). confirmed that compound **I** has to be 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5-*O*-demethylnobiletin) (Iinuma *et al.*, 1980 a&b; Sugiyama *et al.*, 1993).

However, previous ^{13}C NMR assignment based only on chemical shift values (Iinuma *et al.*, 1980b) showed differences between reported values (δ 119.8, 122.5 and 114.6) and the present values (δ 123.62, 120.11 and 106.94) for C-1', C-6' and C-10, respectively, (Table 2). Therefore, a series of 2D NMR experiments (including COSY, HETCOR and COLOC) was performed to provide solid evidence and unambiguous assignments of the ^{13}C NMR of **I**. First, the ^{1}H-^{13}C HETCOR spectrum exhibited a clear correlation between H-6' (δ 7.59) and the relatively up-field ^{13}C signal at δ 120.11 (C-6'), not with that at δ 123.62. This gave clear evidence that the protonated carbon (C-6') should be at lower shift value relative to the non-protonated carbon (C-1'). Subsequently, the reported (Iinuma *et al.*, 1980b) assignments for C-1' (δ 119.8) and C-6' (δ 122.5) should be exchanged. Secondly, the COLOC spectrum showed a clear long range coupling correlating the ^{13}C-singal at δ 106.94 and the H-3 proton, confirming its assignment to C-10. In addition, consulting the many published ^{13}C-NMR spectra of 5-hydroxy polymethoxyflavones (Van Den Broucke *et al.*, 1982; Agrawal, 1989; Markham, 1982; Liu *et al.*, 1992) it was quite clear that C-10 always



	R_1	R_2	R_3	R_4
I	H	Me	OMe	Me; m/z 388 (72)
II	Me	H	OMe	H; m/z 374 (78)
III	Me	Me	H	Me; m/z 372 (32)
IV	Me	Me	OMe	Me; m/z 402 (42)

Scheme 1. Selected mass spectral fragments of flavones I-IV.

resonates at *ca* δ 101-107 and reaches *ca* δ 114 for 5-methoxyflavones only (Gonzalez *et al.*, 1991; Agrawal, 1989). This confirmed the present assignments of **I** and confers some doubt on the reported (Iinuma *et al.*, 1980b) assignments which should be revised.

Compound **IV** was obtained as faint yellow needles (mp. 131-132°). MS gave the molecular ion peak at m/z 402 (42%), corresponding to a flavone containing 6 methoxyls ($C_{21}H_{22}O_8$). The UV spectra of **IV** showed no change upon addition of the different shift reagents indicating the absence of free hydroxyl groups (Mabry *et al.*, 1970; harborne *et al.*, 1975). No hydroxyl bands were observed in the IR spectrum. The ^{1}H NMR spectrum showed signals for six aromatic methoxyls, three aromatic protons and H-3 singlet at δ 6.66. In fact, the ^{1}H-NMR data (Table 1), which were very similar to that of **I** showed the following differences from **I**. The chelated hydroxyl signal at δ 12.56 was missing and, instead an extra methoxyl signal appeared, indicating that an extra meth-

Table 2. ^{13}C-NMR data of compound **I*** in comparison with reported data

Carbon atom	**I**	Reported data**
2	163.87 (*s*)	163.2
3	103.95 (*d*)	105.9
4	182.93 (*s*)	182.0
5	145.74 (*s*)	144.7
6	136.51 (*s*)	135.6
7	152.96 (*s*)	152.0
8	132.89 (*s*)	132.3
9	149.29 (*s*)	148.2
10	106.94 (*s*)	114.6
1'	123.62 (*s*)	119.8[a]
2'	108.62 (*d*)	109.3
3'	149.50 (*s*)	148.9
4'	152.39 (*s*)	152.0
5'	111.18 (*d*)	111.9
6'	120.11 (*d*)	122.5[a]
OMe (3',4')	55.10(*q*), 55.95(*q*)	55.5
OMe (6,7,8)	61.13(*q*), 61.71(*q*), 62.05(*q*)	61.4, 60.2

*Run in $CDCl_3$ as a solvent and TMS as int. st.; Assignments and multiplicities were determined by the aid of 2D NMR experiments (COSY, HETCOR, COLOC).
**Iinuma *et al.*, 1980b
*Assignments based on chemical shifts only (using DMSO-d_6 and TMS as int. st.)
[a]Assignment should be exchanged (see text).

oxyl group must be at C-5. On the basis of these observation along with the characteristic MS fragmentation (Iinuma *et al.*, 1980a; Mabry *et al.*, 1970) (Scheme 1) and also comparison with data of similar compounds (Sugiyama *et al.*, 1993; Gonzalez *et al.*, 1991; Herz and Kulanth- aivel, 1982; Vyas and Bulchandani, 1986), compound **IV** was assigned the structure 5,6,7,8,3',4'-hexamethoxyflavone [nobiletin, 1986) and also called citromitin].

Compound **II** was isolated as pale yellow crystals. Both UV (279, 345 nm) and IR (3377, 1649 cm^{-1}) absorptions are typical of flavones. (Harborne *et al.*, 1975). The MS of **II** exhibited a molecular ion peak at 374 (78%) in accord with a dihydroxy-tetramethoxyflavone ($C_{19}H_{18}O_8$). Other MS fragments at m/z 211 and 183 (Scheme 1) indicated a monohydroxy-trimethoxy substituted ring A, while those at m/z 151 and 148 placed the second hydroxyl and the remaining methoxyl on ring B. The ^{1}H NMR data (Table 1) confirmed the presence of four methoxyl, an olefenic proton at δ 6.60 assigned to H-3 and three aromatic protons coupled with ABX type (as in **I** and **IV**) at δ 7.42, 7.05 and 7.54 assignable to H-2', H-5' and H-6', respectively, confirming a 3',4'-dioxygenation in ring B. It also revealed the presence of two non-chelated -OH signals at δ 4.69 and 8.10 assignable (Liu *et al.*, 1992) to 7-OH and 4'-OH, respectively, as also confirmed by the UV analysis. The UV spectrum of **II** on addition of NaOMe exhibited a shift (+60 nm) in Band I, placing a hydroxyl group at C-4', (Harborne *et al.*, 1975; Markham, 1982) and hence a methoxyl group must be located at C-3'. The absence of a free 5-OH is deduced: firstly from the absence of a chelated-OH signal in the ^{1}H NMR; and secondly from the difference between the obtained data for compound **II** and those reported for 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone. (Van Den Broucke *et al.*, 1982). The UV spectrum (NaOMe) of **II** showed a new band at 320 nm (cf MeOH) indicating a free 7-OH group (Mabry *et al.*, 1970; Harborne *et al.*, 1975; Herz *et al.*, 1980). From the previous data together with comparison with the enormous published data (Sugiyama *et al.*, 1993; Iinuma *et al.*, 1980a; Liu *et al.*, 1992; Herz *et al.*, 1980; Faini *et al.*, 1982) for similar compounds, it was concluded that compound **II** is 7,4'-dihydroxy-5,6,8-3'-tetramethoxyflavone. This compound has not been reported in the genus *Citrus* before and also could not be found in the available literature and is very likely to be a new flavonoid. However, ^{13}C NMR assignment of this compound will be addressed upon isolation of further quantity of it.

Compound **III** was obtained as colourless needles, mp 152°. Both the UV (270, 323 nm) and IR (1661, 1587 cm^{-1}) absorptions, besides the proton signal at δ 6.71 (H-3) in the ^{1}H NMR spectrum are typical of flavones

(Mabry *et al.*, 1970; Harborne *et al.*, 1975; Markham, 1982). The MS spectrum showed a molecular ion at m/z 372 (32%) indicating a pentamethoxyflavone ($C_{20}H_{20}O_7$). The ^1H NMR data (Table 1) confirmed the presence of five methoxyls and revealed a typical A_2B_2 signals of 4'-substitution in ring B (Iinuma *et al.*, 1980a; Harborne *et al.*, 1975). The IR spectrum showed the abscence of any -OH groups; this was confirmed by the UV spectra which did not change upon addition of the different shift reagents (Mabry *et al.*, 1970; Harborne *et al.*, 1975; Markham, 1982). The MS fragments (Scheme. 1) at m/z 225 and 197 indicated a fully methoxylated ring A, and those at m/z 132 and 135 confirmed the presence of a 4'-methoxyl (Harborne *et al.*, 1975; Herz and Kulanthaivel, 1982). On the basis of these observation, flavone **III** was assigned the structure 5,6,7,8.4'-pentamethoxyflavone (tangeritin). The obtained data are in a good agreement with those reported for tangeritin which is also called penkanetin (Kanes *et al.*, 1993; Iinuma *et al.*, 1980a), previously isolated from the rinds of *C. reticulata*. (Mizuno *et al.*, 1991; Iinuma *et al.*, 1980a).

The previous reults indicate that *C. deliciosa* elaborates a number of polymethoxyflavones some of them are of variable bioactivity. Nobiletin **IV**, 5-O-demethylnobiletin **I** and tangeritin **III** are currently being tested for their differentation inducer activity for myeloid leukemic cells (Sugiyama *et al.*, 1993). The presence of a free-OH group at C-7 in flavone **II** strongly suggests it as an effective polymethoxyflavone regarding inducing of leukemic cells (MI) to have phagocytic activity (Sugiyama *et al.*, 1993) Nobelitin IV has also been reported as an antifungal agent which protect *Citrus* trees against destructive fungi (Mizuno *et al.*, 1991).

Although flavones **I**, **III** and **IV** have been reported in the closely related species *C. reticulata* (Iinuma *et al.*, 1980a), the isolation of the polymethoxyflavone **II** for the first time from *C. deliciosa* would possess a significant chemotaxonomic value for the identification of this species.

It should also be noted that almost all the previous chemical investigation of *Citrus* were concerned with the peels or the roots of *Citrus* species. Investigation of other organs (e.g. the leaf) would provide a new source for specific secondary metabolites that can aid in the chemotaxonomic evaluation of this genus.

Finally, our results suggests that the presence of 5-*O*-demethylnobeletin **I** as a major polymethoxyflavone besides flavone **II** that can be considered as a specific chemotaxonomic marker for this species will, certainly help the chemotaxonomic evaluation of *C. deliciosa*. This in addition to the botanical characteristics and the different numerical values (which will be discussed in Part II) would certainly provide a good tool for an unambiguous identification of this species.

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