Antibacterial Activity of *Citrus reticulata* Peel Extracts

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Citrus peels were successively extracted with hexane, chloroform and acetone using a soxhlet extractor. The hexane and chloroform extracts were fractionated into alcohol-soluble and alcohol-insoluble fractions. These fractions were tested against different gram positive and gram negative bacteria. The EtOH-soluble fraction was found to be most effective. Fractionation of EtOH-soluble fraction on silica gel column yielded three polymethoxylated flavones, namely desmethylnobiletin, nobiletin and tangeretin. Their structures were confirmed by UV, ^{1}H, ^{13}C NMR and mass spectral studies. The findings indicated a potential of these natural compounds as biopreservatives in food applications.

Introduction

The annual world production of citrus fruits is 98.4 million metric tons (FAO, 1997) and approximately 34% of the fruits are processed into juices. As the juice yield is about half of the fruit weight, processing of citrus into juices result in large amount of by-products (Bovill, 1996). The volatile oil and non-volatile oil of orange peel are widely used in food, drug and cosmetic industries (Shaw, 1979). Polymethoxylated flavones (PMF) are an interesting group of bioactive compounds present in citrus fruits. PMF are more active than the flavanone glycosides in their antiadhesive effects in red blood cells and platelets (Robbins, 1974). PMF have also been shown to have antiinflammatory properties and they inhibit histamine release thereby reducing allergic reactions (Middleton and Dzrewiecki, 1982). PMF, such as nobiletin and tangeretin are more potent inhibitors of tumour cell growth than hydroxylated flavonoids. This difference in activity may be due to better membrane uptake of the PMF since methoxylation of the phenolic groups decreases the hydrophilicity of the flavonoids (Kandaswami *et al.,* 1991). Nobiletin and sinensetin are effective in decreasing the erythrocyte aggregation and sedimentation in human blood (Robbins, 1976; Bracke *et al.,* 1994). PMF have also been shown to have a cytotoxic effect toward cancerous cell invasion (Kupchan *et al.,* 1965) and to act as antimutagenic agent (Francis *et al.,* 1989). The objective of the present study was to isolate and identify the PMF present in the active fraction determined by evaluating the antibacterial activity of the citrus peel extracts. To our knowledge, this is the first report on the isolation of compounds **1** and **2** from *Citrus reticulata* [Blanco Coorg mandarin] peels and antimicrobial activity of their extracts.

Materials and Methods

Materials

Citrus reticulata [Blanco Coorg mandarin] oranges are cultivated in South India on a large scale in the Coorg district (The Wealth of India, 1992). Dried peels of Blanco Coorg mandarins were procured from a local fruit-processing factory during January-February, 1998. All solvents and chemicals used were of AR and HPLC grades. MP: uncorr. UV spectra were measured using a Genesys-5 UV-visible spectrophometer (Milton Roy, NY, USA). ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). ^{13}C NMR spectral assignments were given on the basis of SEFT spectra. TMS was used as the internal standard. Mass spectra were recorded using Shimadzu QP-5000 Quadrapole Mass Spectrometer (Shimadzu, Tokyo, Japan).

Extraction

Dried peels (100 g) of *Citrus reticulata* were powdered and successively extracted in a soxhlet extractor with hexane, chloroform and acetone for 8 h each. The extracts were filtered, concentrated under vacuum and the yields of hexane, chloroform and acetone extracts were 5.0, 1.6 and 3.6 g, respectively. TLC of hexane and chloroform extracts (hexane:EtOAc, 85:15 v/v) showed three

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spots with different concentrations. Hence, both the extracts were mixed for fractionation.

Fractionation of PMF

One ml each of hexane and chloroform extracts were mixed with 20 ml of EtOH, the precipitate formed was filtered. The supernatant was concentrated under vacuum and used for antimicrobial activity along with the precipitate. Ethanol soluble fraction (1.0 g) was impregnated with 2 g of silica gel and loaded on to the silica gel column. The compounds were eluted with hexane: EtOAc solvent mixtures of increasing polarity. Compound **1** eluted with hexane: EtOAc (78:22 v/v), whereas compounds **2** and **3** were eluted with hexane: EtOAc (70:30 v/v) and (60:40 v/v) respectively. The solvents from the eluates were evaporated under vacuum and recrystallized. Compounds **1**, **2** and **3** were obtained with yields of 50, 110 and 500 mg, respectively. The compounds were dissolved in chloroform, spotted on TLC and developed using hexane: EtOAc (85:15 v/v). TLC plates were sprayed with 10% sulfuric acid in methanol (v/v) and heated at 110 °C for 10 min. The R_f values of the compounds were calculated and compared with reported values. Finally, compounds **1**, **2** and **3** were identified as desmethylnobiletin (6,7,8,3′,4′ pentamethoxy-5-hydroxyflavone), nobeliten (5,6,7,8,3′,4′ hexamethoxy- flavone) and tangeretin (5,6,7,8,4′-pentamethoxyflavone) by ^1H, ^{13}C NMR and mass spectra, respectively.

Inoculum preparation

Strains of *Bacillus cereus, B. coagulans, B. subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the stock culture collection of Food Microbiology Department of the Institute. The bacterial cultures were maintained at 4 °C on nutrient agar slants and subcultured at 15-day intervals. Prior to use, the cultures were grown in nutrient broth at 37 °C for 24 h. A preculture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth and incubated for 48 h at 37 °C. The cells were harvested by centrifugation (1200×*g*, 5 min), washed and suspended in sterilized saline.

Antibacterial activity

The fractions were tested against different micro-organisms by the method of Chen *et al.,* (1998). To flasks containing 20 ml melted nutrient agar, different concentration (200, 400 and 800 μg/ml) of test material in propylene glycol were added. In case of control, equivalent amount of propylene glycol was added. One hundred μl (about 10^3 cfu/ml) of each bacterium to be tested was inoculated into the flasks under aseptic conditions. The contents were mixed thoroughly and media was then poured into sterilized petri dishes in quadruplet and incubated at 37 °C for 20–24 h. The colonies developed after incubation were counted and the inhibitory effect was calculated using the following formula (Rico-Muñoz and Davidson, 1983).

% Inhibition = $(1 - T/C) \times 100$, where T is cfu/ml of test sample and C is cfu/ml of control.

The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium being tested (Naganawa *et al.,* 1996).

Results and Discussion

The antimicrobial activity of different fractions from citrus peel is shown in Table I. All fractions suppressed the growth of grampositive bacteria at concentrations lower than that required for gram-negative bacteria. EtOH soluble fraction was most active against all the bacterial strains. The acetone extract was found least effective of all the tested fractions. In case of EtOH-soluble fraction MIC for *Bacillus cereus* and *Staphylococcus aureus* was observed at 300 μg/ml level, while for *B. coagulans* and *B.subtilis,* 500 μg/ml were required to bring about complete inhibition of growth. In case of gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* MIC of EtOH soluble fraction was found to be 1200 and 600 μg/ml, respectively. MIC levels of EtOH soluble fraction were lower than other fractions probably due to higher polymethoxylated flavone content. Results of the present study are in consistent with the antifungal activity of citrus polymethoxylated flavones reported by earlier workers (Del Rio *et al.,* 1998). They reported antifungal activity of PMF from essential oils of *Citrus* against *Phytophthora cit-*

Table I. Minimum inhibitory concentration (MIC) of citrus peel fractions*.

Bacteria	MIC (μg/ml)				
	Hexane extract	Chloroform extract	Acetone extract	Ethanol soluble fraction	Ethanol in soluble fraction
Grampositive					
Bacillus cereus	500	500	600	300	600
Bacillus coagulans	600	800	800	500	800
Bacillus subtilis	600	700	800	500	800
Staphylococcus aureus	600	800	1000	300	800
Gramnegative					
Escherichia coli	1600	1600	2200	1200	1800
Pseudomonas aeruginosa	1000	1200	1600	600	1000

* Results of four replications.

Table II. ^{1}H NMR chemical shifts (δ_H in $CDCl_3$) of compounds **1–3**.

H	1	2	3
3	6.63 (s)	6.60 (s)	6.63 (s)
2′	7.44 (d) (*J*=1.9)	7.50 (d) (*J*=2.1)	7.89 (d) (*J*=8.9)
3′	–––	––––	7.04 (d) (*J*=8.9)
5′	7.02 (d) (*J*=8.5)	7.10 (d) (*J*=9.0)	7.06 (d) (*J*=8.9)
6′	7.60 (dd) (*J*=1.6,9.0)	7.60 (dd) (*J*=2.0, 9.00)	7.90 (d) (*J*=9.0)
OCH_3	4.13	4.35	4.13
OCH_3	4.00	4.10	4.04
OCH_3	3.99	4.05	3.97
OCH_3	3.97	4.05	3.97
OCH_3	3.96	4.05	3.91
OCH_3	–––––	4.05	–––––
4′-OH	12.56	–––	–––––

Chemical shift values are in ppm and *J* values in parentheses (Hz).
s- singlets.
d- doublets.

Table III. ^{13}C NMR chemical shifts (δ_C in $CDCl_3$) of compounds **1–3**.

C	1	2	3
2	164.5	161.6	162.4
3	104.5	107.4	106.8
4	183.6	177.7	177.4
5	150.1	144.7	144.2
6	137.2	138.6	138.2
7	153.6	151.9	151.5
8	133.6	148.2	148.5
9	146.4	148.9	147.8
10	107.6	115.4	114.9
1′	124.3	124.6	123.9
2′	111.9	109.4	127.8
3′	150.0	149.9	114.6
4′	153.1	152.6	161.3
5′	109.4	111.9	114.5
6′	120.8	120.2	127.7
6-OCH_3	62.7	62.7	62.3
7-OCH_3	62.3	62.4	62.0
8-OCH_3	61.7	62.3	61.9
5-OCH_3	––––	61.7	61.7
3′-OCH_3	56.6	56.6	––––
4′-OCH_3	56.7	56.5	55.6

rophthora, Penicillium digitatum and Geotrichum species. Piatelli and Impellizzeri (1971) also reported antifungal activity of tangertin against *Deuterophoma tracheiphila,* the fungus responsible for the highly destructive citrus disease known as Mal-secco.

Spectral and chemical characteristics

Compound **1**

Pale yellow needles (MeOH); mp 144–145 °C; R_f 0.65; UV (MeOH) λ_{max} cm^{-1} 245, 284, 341; $AlCl_3$ 263, 363, 368; $AlCl_3$ + HCl 290, 356; NaOMe 291, 314, 398; NaOAc 244, 284, 344; NaOAc + H_3BO_3 244, 284, 341. MS, *m/z* (%) 388 (M^+, 100%), 373 (M^+ -CH_3, 18%), 358 (M^+- OCH_3, 100%), 327 (6%), 259 (4%), 194 (10%), 186 (20%), 165 (8%), 156 (5%), 147 (5%), 127 (14%), 91 (14%), 60 (60%). Compound **1** was identified as desmethylnobiletin (6,7,8,3′,4′ pentamethoxy-5-hydroxy-flavone) from these spectral data, chemical and physical properties (Kinoshita and Firman, 1996).

Compound **2**

Pale yellow needles (MeOH); mp 138–139 °C; R_f 0.30; UV (MeOH) λ_{max} cm^{-1} 247, 271, 324;

$AlCl_3$ 270, 324; $AlCl_3$ + HCl 270, 324; NaOAc 270, 324. MS, *m/z* (%) 402 (M^+, 25%), 387 (M^+ -CH_3, 100%), 371 (M^+ -OCH_3, 5%), 359 (4%), 344 (9%), 182 (15%), 162 (9%), 153 (5%), 147 (6%), 119 (7%), 91 (10%), 83 (24%). Compound **2** was identified as nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) from these spectral data, chemical and physical properties (Del Rio *et al.,* 1998).

Compound **3**

Colourless needles (MeOH); mp 156–157 °C; R_f 0.35; UV (MeOH) λ_{max} cm^{-1} 244, 271, 322; $AlCl_3$ 244, 271, 322; $AlCl_3$ + HCl 242, 271, 322; NaOAc 248, 271, 322. MS, *m/z* (%) 372 (M^+, 20%), 357 (M^+ -CH_3, 100%), 341 (M^+ -OCH_3, 3%), 329 (5%), 314 (16%), 296 (14%), 225(10%), 197 (50%), 182 (25%), 167 (10%), 153 (8%), 135 (24%), 132 (40%), 117 (20%), 89 (30%), 83 (60%). Compound **3** was identified as tangeretin (5,6,7,8,4′-pentamethoxyflavone) from these spectral data, chemical and physical properties (Del Rio *et al.,* 1998).

Fractionation of hexane and chloroform extracts by EtOH precipitation provides the enrichment of the antimicrobial activity. This enriched fraction was subjected to silica gel column chromatography using hexane and EtOAc with increasing polarity to obtain three compounds **1–3** in crystalline form. Based on the spectral data compounds **1**, **2** and **3** (Fig. 1) are identified as desmethylnobiletin, nobiletin and tangeretin, respectively (Roitman and James, 1985; Horie *et al.,* 1998; Sugiyama *et al.,* 1993).

Conclusions

Hexane, chloroform and acetone extracts of peels of *Citrus reticulata* were found to posses antibacterial activity. Active principles were enriched in to the EtOH soluble fraction of hexane and chloroform extracts. EtOH soluble fraction was found to exhibit a high degree of antibacterial activity and has the potential to be used as a biopreservative.

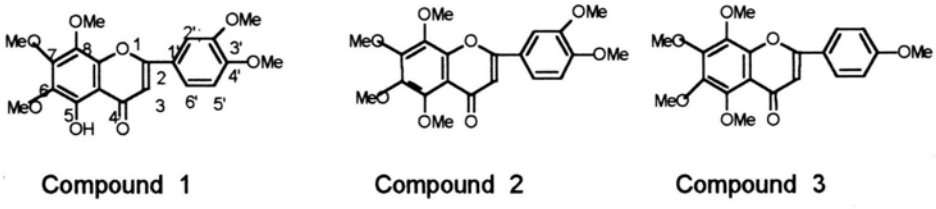

Fig. 1. Isolated polymethoxylated flavones from citrus peel.

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