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Determination of Limonin and Nomilin Contents in Different Citrus Cultivars Using High Performance Liquid Chromatography

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Abstract. High performance liquid chromatography (HPLC) analysis was done to quantify the amount of limonoids (limonin and nomilin) in seven selected citrus cultivars. According to the HPLC analysis red blood orange (*Citrus sinensis* var *red blood orange*) had maximum amount of limonin (479.77 µg/mL), while rough lemon (*Citrus jambhiri*) had no limonin content. In case of nomilin, rough lemon (*Citrus jambhiri*) had maximum amount of nomilin (54.23 µg/mL), while succari (*Citrus sinensis* var *succari*) had very low amount of nomilin (0.37 µg/mL).

Keywords: limonin, nomilin, HPLC, citrus fruits

Introduction

Plants are considered to have secondary metabolites, which are helpful in defense, healing mechanism and having anticarcinogenic effects. Citrus (Rutaceae) are well known for having such compounds. Secondary metabolites are usually complex and unique carbon structures (Sarker *et al*., 2005), having different chemical groups like alkaloids, terpenoids, phenolic, plant amines, rare amino acids and glycosides (Rohloff, 2003). Monoterpenes (Langenheim, 1994), terpenoids (Gutierrez *et al*., 1997) are used in protecting the plants from herbivore or competitor. Plants use terpenoids in plant-plant interactions which serve as attractants for pollinators (Tholl, 2006). Cyanogenic glycosides, isoflavonoids and alkaloids which are soluble secondary compounds can also be toxic to animals (Morris and Robbins, 1997). These also play an important role in nutrition of food and animal feed with a number of phenolic compounds. Limonoids are a group of chemicals related to terpenoids found in Rutaceae and Meliaceae families of the Order Rutales. Out of 38 limonoids reported to occur in citrus and its hybrids (Jayaprakasha *et al*., 1997), citrus fruits posses a wide variety of bioactive compounds with health promoting disease preventing properties, effective against cancer. Moreover, limonoids from Rutaceae particularly 'Citrus' (Klocke and Kubo, 1982) have attracted greater concern due to their growth regulating activity (Champage *et al*., 1992) besides having anti-carcinogenic effects (Sohail *et al*., 2005) and their ability to treat tumor cell lines (Poulose *et al*., 2005; Tada *et al*., 1999), suggesting their importance as natural chemo preventatives or as therapeutics or nutraceuticals obtained from 'Citrus' by-products (Schoch *et al*., 2002; Widmer and Montanari, 1996). The present study was undertaken for screening of new sources of limonoids among *Citrus* spp. as natural source of metabolites.

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Materials and Methods

Collection of citrus cultivars. Different citrus cultivars i.e., Chakutra (*Citrus grandis*), Kinnow (*Citrus reticulate*), Musambi (*Citrus sinensis* var. *musambi*), Narangi (*Citrus mitis*), red blood orange (*Citrus sinensis* var. *red bloob orange*), rough lemon (*Citrus jambhiri*) and succari (*Citrus sinensis* var. *succari*) were collected from citrus growing areas of Sargodha (Punjab) and Khanpur (Khyber Pakhtoonkhwa) Pakistan.

HPLC analysis. ***Instrument.*** HPLC apparatus was comprised of a Shimadzu (Japan), Shimadzu degasser unit (DGU-12A) and pump (LC-10AT) with a Shimadzu ultraviolet (UV) detector (SDD-10AV). Limonoids were quantified on C18 reverse phase Shim Pack CLC-ODS column, 5 µm particle size (15 cm × 4.6 mm) and detected at 210 nm.

Preparation of mobile phase and standards. HPLC grade methanol, acetonitrile (Merck Germany), standards (nomilin and limonin) from MP Biochemical's (France) and water were used in the preparation of mobile phase with the 10:41:49 ratio. Stock solution was prepared by adding 5 mg of limonin and nomilin in 100 mL of mobile phase.

Chromatographic conditions. The elution was monitored by UV absorption at 210 nm with 1 mL/min flow rate. Millipore filter (0.45 µm) was used for the filtration of both standards and samples. The linearity of method was estimated by analyzing standards. 20 µL of each standard was injected in HPLC and elution was carried out isocratically and peak responses with their respective retention time were obtained. Filtered samples (20 µL) were injected in C18 reverse phase column, eluted isocratically with acetonitrile, methanol, and water (10:41:49).

Statistical analysis. The compounds were quantified by chromatography station software CSW 32, version 1.4.11.89. The limonoid aglycones were identified on the basis of retention times and quantified (Ohta *et al*., 1993). Peaks were recorded with standards at retention time of 8.15 min (limonin) and 12.66 min (nomilin).

Results and Discussion

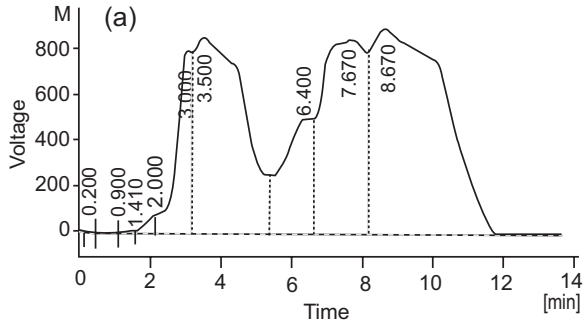
Citrus fruits contain certain compounds (limonoids) that are extremely bitter which are mainly accumulated in seeds (Faisal *et al*., 2011) and by-products (Dandekar *et al*., 2008; Schoch *et al*., 2001; Hasegawa, 2000). Non-volatile collection through HPLC analysis was done and the responsive peaks at specific retention time were recorded (Fig. 1). The highest content of limonin was found in red blood orange (479.77 µg/mL), followed by succari (426.77 µg/mL), kinnow (99.62 µg/mL), musambi (42.63 µg/mL), chakutra (39.87 µg/mL) while narangi and rough lemon had almost 0.77 µg/mL and 0 µg/mL limonin content, respectively as shown in Table 1.

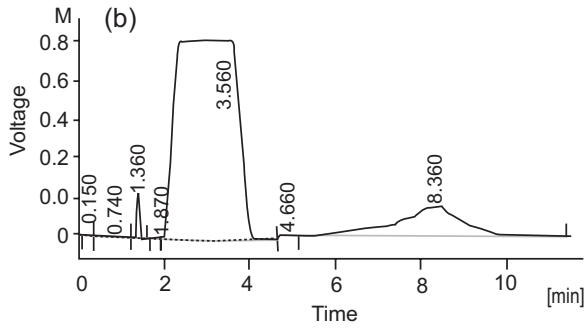
According to Table 1, rough lemon (54.23 µg/mL) has the highest nomilin contents, followed by musambi (41.67 µg/mL), narangi (11.85 µg/mL), chakutra (6 µg/mL), kinnow (3.91 µg/mL) while red blood orange and succari have the lowest level of nomilin contents, i.e., 1.49 µg/mL and 0.37 µg/mL, respectively.

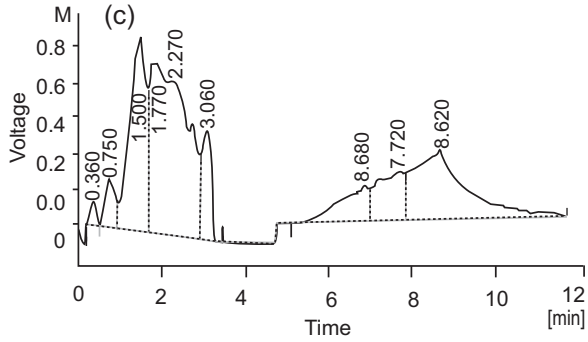
Generally, the citrus oils are characterized by high percentage of limonin (more than 90%) (Hérent *et al*., 2007; Pultrini *et al*., 2006; Merle *et al*., 2004). Among the two limonoids (limonin and nomilin), limonin is the dominant limonoid found in almost all citrus cultivar oils. These results are well supported by the findings of Manners *et al*. (2003), Rouseff and Fisher (1980) and Kefford (1955). Similar studies of other citrus species such as *C. limon*, *C. sphaerocarpa*, *C. sinensis* × *Poncirus trifoliata* and *C. reticulata* also showed that the oil have about 70% limonin as the major components. Meanwhile the oils from lemon, lime, orange and grapefruits had high level of nomilin and limonin (Buettner *et al*., 2003; Miller *et al*., 1994; Lam *et al*., 1994). Vekiari *et al*. (2002) reported that the main components of citrus essential oils were limonene, β-pinene, myrcene, neral, geranial, neryl acetate and β-caryophyllene. Citrus peel oils also have limonin as a major component of essential oils like commercial Brazillian Murcot Tangerines (Feger *et al*., 2003), *C. reticulate, C. sinensis, C. paradisii* (Kamal *et al*., 2011; Ahmad *et al*., 2006). It is concluded from the present study that, among the seven citrus cultivars, red blood orange had the highest level of limonin while rough lemon had the highest level of nomilin.

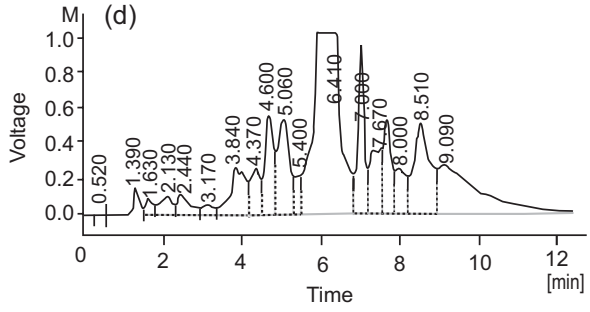
Table 1. Quantities of limonin and nomilin in citrus varieties

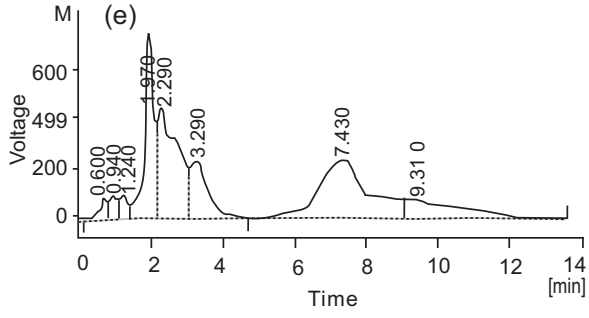
Sr. no.	Common name	Botanical name	Nomilin (µg/mL)	Ret. time (Min)	Limonin (µg/mL)	Ret. time (Min)
1	Chakutra	(*Citrus grandis*)	6.01	1.88	39.87	3.26
2	Kinnow	(*Citrus reticulate*)	3.91	1.24	99.62	3.29
3	Musambi	(*Citrus sinensis* var *musambi*)	41.67	1.63	42.63	3.17
4	Narangi	(*Citrus mitis*)	11.85	1.50	0.77	-
5	Red blood orange	(*Citrus sinensis* var *red blood orange*)	1.49	1.87	479.77	3.54
6	Rough lemon	(*Citrus jambhiri*)	54.23	1.70	0.00	-
7	Succari	(*Citrus sinensis* var *succari*)	0.37	1.41	426.77	3.55

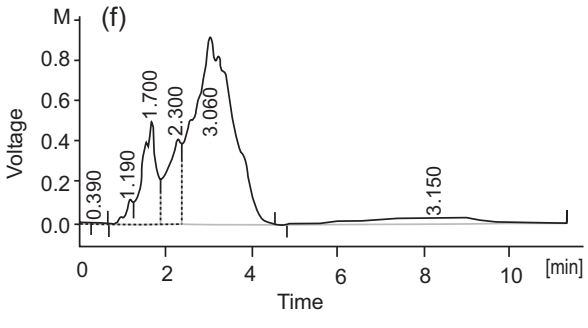












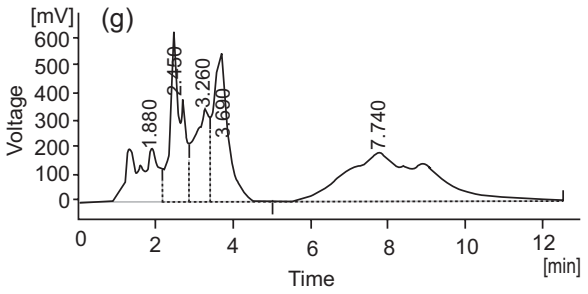


Fig. 1(a-g). HPLC analysis (a) *C. sinensis* var *succari*; (b) *C. sinensis* var *red blood orange*; (c) *C. mitis*; (d) *C. sinensis var musambi*; (e) *C. reticulate*; (f) *C. jambhiri*; (g) *C. grandis*.

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