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Original Article

Comparative study of the hypocholesterolemic, antidiabetic effects of four agro-waste *Citrus* peels cultivars and their HPLC standardization

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ABSTRACT

Citrus is an economically important fruit for Egypt, but its peel also is one of the major sources of agricultural waste. Due to its fermentation, this waste causes many economic and environmental problems. Therefore it is worthwhile to investigate ways to make use of this citrus waste generated by the juice industry. This study was aimed to explore the hypocholesterolemic, antidiabetic activities of four varieties of citrus peels agrowastes, to isolate the main flavonoids in the active fractions and to quantify them by HPLC method for nutraceutical purposes. All the tested samples of the agro-waste *Citrus* fruits peels showed significant decrease in cholesterol, triacylglyceride and glucose. The most decrease in cholesterol level was observed by mandarin peels aqueous homogenate and its hexane fraction (59.3% and 56.8%, respectively) reaching the same effect as the reference drug used (54.7%). Mostly, all samples decrease triacylglyceride (by 36%–80.6%) better than the reference drug used (by 35%), while, glucose was decreased (by 71.1%–82.8 and 68.6%–79.6%, respectively) mostly by the aqueous homogenates (except lime) and alcoholic extracts (except mandarin) of *Citrus* fruits peels better than the reference drug used (by 68.3%). All the isolated pectin, from the four cultivars, has significant effect on the three parameters. The comparative HPLC rapid quantification of nobiletin in the different by-product citrus varieties hexane fractions revealed that nobiletin is present in higher concentration in mandarin (10.14%) than the other species. Nobiletin and 4′,5,7,8-tetramethoxy flavone were isolated from mandarin peels hexane fraction by chromatographic fractionation. This is the first report of the comparative HPLC quantification of nobiletin and biological studies of different citrus peels species as agro-waste products. Based on these results, we suggest the possibility that *Citrus* fruits peels may be considered as an antidiabetic and hypocholesterolemic nutraceutical product.

Introduction

One of the most important popular threat factors for coronary heart disease, heart attack and stroke is the high cholesterol (AHA, 2014). Large amount of fruit peels are pitched as waste from fruit processing industry in spite of the well declared biological activities of these peels compared to other discarded portions. Among the fruits, *Citrus* fruits which its yield is approximated as 80 million tons per year are regarded as precious healthy diet since its nutrients boost haleness and guard against chronic disease (Gnanasaraswathi et al., 2014). Huge amounts of scraps are produced yearly, following juice manufacturing, from about one third of *Citrus* fruits (Li et al., 2006). Citrus peels, which constitute the main residue, contain more bioactive compounds than do juices (Bocco et al., 1998; Gorinstein et al., 2001) and are a suitable provenance of pectin (Sakai and Okushima, 1980). While citrus peels exhibit potent antioxidant, antimicrobial, anti-inflammatory activities (Murakami et al., 2000; Lin et al., 2011; Dhanavade et al., 2011) and have a reverse relationship with the coronary heart disease incidence by its potency in decreasing plasma cholesterol level (Bok et al., 1999; Wilcox et al., 2001; Whitman et al., 2005; Lee et al., 2011; Assini et al., 2013), pectin is useful in medical purpose, in which it aids in decreasing serum cholesterol level, dislodging heavy metal ions from the body, equilibrating blood pressure and assisting in weight reduction (Tang et al., 2011).

A plentiful source of polyhydroxyl flavonoids, such as hesperidin, neohesperidin and naringin, are the citrus peels which are

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also the unique source of polymethoxyflavones with high content such as nobiletin, tangeretin and sinesetin (Li et al., 2006; Londoño et al., 2010). In previously *in vivo* studies, lower doses of citrus polymethoxylated flavones (PMF) can decrease plasma cholesterol level more than that of flavanones (Morin et al., 2008) and modulated lipid metabolism in cells and animals (Lee et al., 2011). Two polymethoxylated citrus flavonoids, tangeretin and nobiletin, moderately inhibited both cholesterol (CH) and triacylglyceride (TG) synthesis, while weaker effects were reported by other PMF (*e.g.*, sinensetin) and non-PMF (*e.g.*, hesperetin and naringenin). Hence, attention should be paid for their proper extraction as potential compounds and check their suitability as therapeutics. This will increase the aggregate value of the industrial waste.

Our study was carried out on four agro-waste *Citrus* peels species cultivated in Egypt, after previously exploring their peels oil benefits (Abd-Elwahab et al., 2016), [mandarin (*Citrus reticulata* Blanco cv. Egyptian), sweet orange (*Citrus sinensis* (L.) Osbeck cv. Olinda Valencia), white grapefruit (*C. paradisi* Macfad. cv. Duncan) and lime (*C. paradisi aurantiifolia* (Christm.) Swingle cv. Mexican)], Rutaceae, to evaluate and compare their hypocholesterolemic and anti-diabetic activities as agro-waste products, to isolate the main flavonoids in the active fractions and to quantify them by HPLC method for nutraceutical purposes to facilitate the conversion of this waste into high value-added products, thus allowing it to be a recycled component of functional food material.

Material and methods

Plant material

Samples of the fresh fully mature ripe citrus fruit peels [mandarin (*Citrus reticulata* Blanco cv. Egyptian), sweet orange (*C. sinensis* (L.) Osbeck cv. Olinda Valencia), white grapefruit (*C. paradisi* Macfad. cv. Duncan) and lime (*C. aurantiifolia* Swingle cv. Mexican)], Rutaceae, were identified by Citrus department, Horticultural Institute, Ministry of Agriculture, Giza, Egypt and voucher specimens numbers 14-7-2016-I, 14-7-2016-II, 14-7-2016-IV and 14-7-2016-III, respectively, were deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt. The material were collected in February 2011 (for sweet orange), September 2011 (for lime), 2nd half of December 2011 (for grapefruit) and 2nd half of January 2012 (for mandarin), from the private orchard of El-Mazloom company for horticulture production at 78 km Cairo-Ismailia road.

Preparation of extracts and isolation of pectin

Preparation of a citrus peels aqueous homogenates: the aqueous homogenates were prepared by mixing 1.5 g of fresh peel of each species with 100 ml distilled water in a blender, stored in bottle and kept in refrigerator (2–5 °C) until used.

Preparation of a citrus peels alcoholic extracts: the alcoholic extracts were prepared by percolating 200 g of fresh peel with 80% methanol (600 ml), filtered off and the resulting extracts were evaporated under reduced pressure.

Preparation of a *Citrus* peels hexane extracts: part of the previously prepared alcoholic extract residue (10 g) for each Citrus fruits peels were suspended separately in distilled water (30 ml), then extracted with *n*-hexane and the resulting hexane extracts were evaporated under reduced pressure.

Isolation of pectin from citrus peels: each species of fresh citrus peels (50 g) was boiled with known volume of distilled water (100 ml), decanted, cooled to room temperature (25 °C) and then four volumes of absolute ethanol (400 ml) was added to precipitate the pectin, kept in refrigerator (2–5 °C) for two days and then filtered to obtain pectin which is freeze dried and stored in desiccators until used.

Citrus peels alcoholic and hexane extracts residues and the isolated pectins (1.5 g of each) were mixed with 100 ml distilled water (1.5%), stored in bottle and kept in refrigerator (2–5 °C) until used.

Materials

For biological study

Pure cholesterol powder analytical grade (C75209) and bile salts powder (48305) were purchased from Sigma Chemical Co., St. Louis, USA. Metformin (Cidophage®): Chemical Industries Development Co. (CID Co.), Giza, Egypt, as an anti-diabetic reference drug. Atorvastatin 5 mg (Lipitor®): Pfizer Company, Cairo, Egypt, as a hypolipidemic reference drug.

For chromatographic study

Silica gel 60 (89943 Fluka) for column chromatography and precoated silica gel plates 60 F254 for TLC were obtained from Sigma-Aldrich Co. LLC and Merck Millipore Corporation, Germany, respectively. Acetonitrile (34860), methanol (34860) and phosphoric acid (79606) of HPLC grade were purchased from Sigma Chemical Co., St. Louis, USA. ^{1}H-(300 MHz) NMR spectra were recorded on Varian Mercury apparatus at 25 °C using TMS as an internal standard and chemical shifts were given in δ values.

Isolation of polymethoxyflavones

The air dried powdered mandarin peels (850 g) were macerated in 75% alcohol (2× 3 l) at room temperature and the alcohol extract was evaporated under vacuum, to obtain a yellowish brown residue (233 g). This residue was suspended in distilled water (500 ml) and then fractionated with hexane (250× 3 ml), to yield 5 g residue after evaporation under reduced pressure. The latter was chromatographed on silica gel column; gradient elution was carried out using hexane containing 10% stepwise increments of acetone till 100% acetone. Fractions, 100 ml each, were collected to yield 40 fractions and subjected to TLC on pre-coated silica gel plates, using solvent systems chloroform: methanol (98:2). Fractions (17–20) eluted with hexane:acetone (6:4) showed a major spot that appears yellow in UV at $\lambda_{365\,nm}$ and gave a yellow color with *p*-anisaldehyde spray reagent, ammonia and aluminum chloride. These fractions were pooled together and the solvent was evaporated under reduced pressure to yield compound **1** as a yellow powder (1.5 g). Fractions (29–32) eluted with hexane: acetone (3:7) showed a major spot that appears yellow in UV at $\lambda_{365\,nm}$ and gave a yellow color with *p*-anisaldehyde spray reagent, ammonia and aluminum chloride. These fractions were pooled together and the solvent was evaporated under reduced pressure to yield compound **2** as a yellow powder (0.75 g).

HPLC analysis

HPLC analyses were performed using Agient Technologies 1200 series; consisting of G1322A Degasser (serial No. JP94172767), G1311A Quat pump (serial No. DE62972789), G1314B VWD (serial No. DE71365992) and G1328B Man. inj. (serial No. DE60561522). The wavelength used for the quantification of the flavanones glycosides with the UV detector was 325 nm. The chromatographic separation was carried out on LiChrosher(R) 100 RP-18 endcapped (5 μm) column. LiChroCART(R) 250-4 HPLC-Cartridge, Agilent Technologies, Part No. 79925ODE-584, Cartridge No. 031399 and Sorbent Lot No. L010077333.

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