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Original Article

Extraction of Flavonoids and Quantification of Rutin from waste Tobacco Leaves

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Abstract

The potential of tobacco waste as a viable source of medicinally important flavonoids like rutin has been investigated. Three flavonoids, apigenin, quercetin and rutin, have been isolated from waste tobacco leaves, and their identities have been confirmed by UV-visible, ^{1}H-NMR and ^{13}C-NMR spectroscopy. By using analytical HPLC, the amount of rutin present in the tobacco leaves, before and after fermentation, and also in waste tobacco leaves, has been determined as 1.5, 0.5 and 0.6%, respectively.

Keywords: Waste tobacco leaves; Flavonoid; Rutin; HPLC; NMR.

Introduction

Tobacco is used for a variety of purposes throughout the world. Men and women of all levels of society have used smoking tobacco and smokeless tobacco. Despite its harmful effects on health including lung cancer, pulmonary diseases, bad breath, discoloured teeth, excessive tooth surface wear, decreased ability to taste and smell, gingival recession, advanced periodontal soft and hard tissue destruction, tooth loss, oral leukoplakia and increased risk of cancer in mouth and gums (1), tobacco is still a plant of immense medicinal value. It is a rich source of medicinally useful alkaloids and flavonoids. Rutin, a well-known natural antioxidant, is one of the medicinally important flavonoids found in tobacco. It makes up to 1% of the whole dried tobacco plant (2). Rutin can reduce capillary fragility, swelling and bruising and has been used in the treatment of venous insufficiency (varicose veins, haemorrhoids, diabetic vascular disease, and diabetic retinopathy), and for improving micro-vascular blood flow (pain, tired legs, night cramps, and restless legs) (3, 4). It induces cAMP synthesis (5), inhibits phospholipase (6) and superoxidase (7), inhibits secretion and aggregation of platelets (5), mild vasoconstrictor and increasing the resistance of small blood vessels through inhibition of COMT (8), anti-tumour (9) and analgesic with non-opiod mechanism (10). Rutin has also been used as a colouring agent, food additive in various food preparations and drinks, and for various purposes in cosmetics. Major commercial sources of rutin include Sophora japonica, Eucalyptus spp, *Fagopyrum sculentum* (11) and *Ruta graveolens* (12). However, the necessity of identifying a better and/or cheaper commercial source for rutin and other related flavonoids is still valid. Tobacco is a rich source of medicinally important flavonoids including rutin. A large amount of tobacco waste is produced in tobacco factories. We have investigated the potential of tobacco waste for being used as an excellent source of flavonoids.

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We now report on the isolation and identification of three flavonoids from waste tobacco leaves, and quantification of rutin in this waste.

Experimental

General

NMR 200 MHz (Bruker spectrospin 200), HPLC (Shimadzu, LC-8A), UV-Visible spectrophotometer (Shimadzu, 2100). All chemicals and solvents such as silica gel plate (GF254), methanol, petroleum ether, chloroform, ethyl acetate, n-butanol and rutin standard were purchased from Merck chemical company. The NMR spectra were measured in DMSO-d6 and recorded at 200.13 MHz for ^{1}HNMR and 50.32 MHz for ^{13}C NMR spectra. Chemical shifts were given in δ values relative to TMS. UV-Visible spectrum of each compound was determined in methanol and after addition of different shift reagents such as $AlCl_3$, $ALCl_3$/HCl, MeOH, AcONa and AcONa/H_3BO_4 at 190-500 nm (13).

Plant material

Three samples of leaves including dried fermented, unfermented and the wastes of tobacco were obtained from Khoi (Northeast City in East Azerbaijan Province) tobacco factory. They were ground and examined.

Extraction of flavonoids from waste tobacco

Dried and ground waste tobacco leaves (100g) were percolated using 70% methanol. This procedure went on until negative cyanidin test. Then the methanol of the extract was evaporated under vacuum at 50°C by rotary evaporator to get an aqueous extract containing flavonoids.

Isolation of flavonoids

The resulted aqueous extract was partitioned using petroleum ether, chloroform, ethyl acetate and n-butanol to obtain respective fractions, which were analysed by TLC, and a cyanidin test was carried out. The ethyl acetate fraction, which contained the highest amounts of flavonoids, was subjected to column chromatography using various mobile phases: ethyl acetate (48 fractions), ethyl acetate - methanol-water (70-15-5) (96 fractions) and methanol-water (85-25) (48 fractions). Flow rate of mobile phase was maintained at 6 drops/min. TLC of CC fractions was carried out on silica gel plates using EtOAC- MeOH- H_2O (65-10-15) as a mobile phase. Flavonoid spots were visualised under UV lamp and also using ammonia vapour. Each group of fractions, FI (20-50), FII (55-70) and FIII (83-100) had at least one flavonoid. Main flavonoids of each fraction group were further purified by preparative TLC on silica gel [mobile phases: EtOAC-MeOH (95-5), EtOAC -MeOH (90-10) and EtOAC-MeOH-H_2O (55-15-4)], which resulted in isolation of three flavonoids **1-3**, respectively. Their structures were elucidated by spectroscopic means.

High-performance liquid chromatography

HPLC analysis was performed on a Model LC-8 instrument (Shimadzu, Japan). The analytical column was Shim-Pack CLC-CN (6 x 150 mm ID) packed with 7µm particles. The column was operated at ambient temperature. In the chromatography of waste leaves of tobacco flavonoids, phosphate buffer (PH=2.6)- MeOH-Dioxan (100-6-6, v/v/v) was selected as the eluent. The flow – rate and volume injection were 1.5 mL/min and 20 µL respectively. Detection was performed at 359 nm.

Standard preparation

Rutin (**3**) was dissolved in the mobile phase to obtain a concentration of 100 µg mL^{-1} and was filtered through a membrane filter (0.45 µm pore size) prior to injection.

Sample preparation

Ground samples (60 mesh, 1g) were suspended in the mobile phase (30 mL) and extracted at ambient temperature for 12 h. This extraction was repeated 3 times. After filtration, the filtrates were transferred to a 100 mL volumetric flask and diluted with the mobile phase to volume. The resulting solution was filtered through a membrane filter (0.45 µm pore size) prior to injection.

Result

UV-visible spectroscopy

UV-visible and shift reagent data of three flavonoids (**1-3**) are presented in table 1.

NMR Spectroscopy

The ^{1}H-NMR ^{13}C-NMR data of flavonoids (**1-3**) were assigned as follows:

Apigenin (1): ^{1}H NMR (chemical shift δ in ppm, coupling constant *J* in Hz) 6.81(1H, s C2-H), 6.21(1H, d, *J*=2.1, C6-H),6.50 (1H, d, *J*=2.1,C8-H), 7.95 (2H, d, *J*=8.8,C2', 6'-H), 6.94 (2H, d, *J*=8.8, C3',5'-H),10.39 (1H, s, C4'-OH), 12.94 (1H, s, C5-OH), 10.80 (1H, s, C7-OH); ^{13}C NMR (chemical shift δ in ppm) 165.0 (C-2),103.7 (C-3), 182.6 (C-4), 158.2 (C-5), 99.7 (C-6), 164.6 (C-7), 94.8 (C-8), 162.3 (C-9),104.6 (C-10), 122.0 (C-1'), 129.4 (C-2'), 116.8 (C-3'), 162.0 (C-4'), 116.8 (C5'), 129.4 (C-6').

*Quercetin (**2**):* ^{1}H NMR (chemical shift δ in ppm, coupling constant J in Hz) 6.20 (1H, d, J=2 Hz, C6-H), 6.42 (1H, d, J=2 Hz, C8-H),7.69 (1H, d, J=2.2 Hz,C2'-H), 6.90 (1H, d, J=8.5 Hz,C5'-H),7.56 (1H, dd, J=8.5,2.2 Hz, C6'-H),9.41(1H, s, C4'-OH), 9.35 (1H, s, C3'-OH), 9.63 (1H, s, C3-OH), 12.52 (1H, s, C5-OH), 10.82 (1H, s, C7-OH); 13C NMR (chemical shift δ in ppm) 147.6 (C-2),136.6 (C-3), 176.7 (C-4), 157.0 (C-5), 99.0 (C-6), 164.7 (C-7), 94.2 (C-8), 161.6 (C-9), 103.9 (C-10), 122.8 (C-1'), 116.5 (C-2'), 145.9 (C-3'), 148.6 (C-4'), 115.9 (C-5'), 120.8 (C-6').

*Rutin (**3**)*: ^{1}H NMR (chemical shift δ in ppm, coupling constant J in Hz) 6.21(1H, d, J=2, C6-H), 6.40 (1H, d, J=2, C8-H),7.55 (1H, d, J=2.1,C2'-H),6.86 (1H, d J=9,C5'-H),7.56 (1H, dd, J=9,2.1, C6'-H),9.71 (1H, s, C4'-OH), 9.21 (1H, s, C3'-OH), 12.62 (1H, s, C5-OH), 10.86 (1H, s, C7-OH), 5.35 (1H, d, J=7.4, H1-G),5.12 (1H, d, J=1.9, H1-R), 1.00 (3H, d, J=6.1,CH3-R); ^{13}C NMR (chemical shift δ in ppm) 157.3 (C-2),134.1 (C-3), 178.2 (C-4), 157.5 (C-5), 99.5 (C-6), 164.9 (C-7), 94.5 (C-8), 162.1 (C-9), 104.8 (C-10), 122.5 (C-1'), 116.1(C-2'), 145.6 (C-3'), 149.3 (C-4'), 117.1 (C-5'), 122.0 (C-6'),101.6 (C1-G), 74.9 (C2-G), 77.3 (C3-G), 72.7 (C4-G), 76.7 (C5-G), 67.9 (C6-G), 102.2 (C1-R), 70.8 (C2-R), 71.2 (C3-R), 71.4 (C4-R), 69.1(C5-R), 18.6 (C6-R) [R and G represent signals from rhamnose and glucose moieties, respectively].

HPLC quantification of rutin (3) in samples

The HPLC conditions described in the experimental section allowed good separation for the main flavonoid, rutin (**3**), of waste material tobacco (Figure 1). As the results obtained for **3** were in good agreement with published data for rutin, separation and quantitative determination of rutin in three samples were performed by HPLC. The amount of rutin in the tobacco leaves before and after fermentation, and also in waste material tobacco were 1.5, 0.5 and 0.6%, respectively.

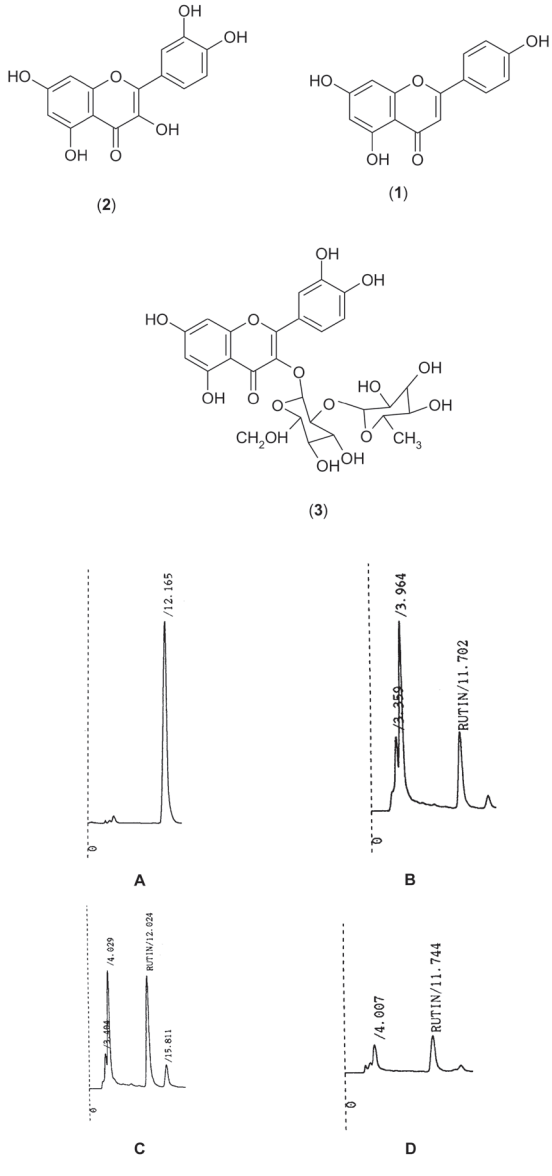

Figure 1. HPLC chromatograms. (A) Rutin; (B) tobacco leaves before fermentation; (C) tobacco leaves after fermentation; (D) waste tobacco leaves. Mobile phase: buffer (PH=2.6) – MeOH - Dioxan (100-6-6, v/v/v).

Table 1. UV-visible absorption peaks of flavonoids **(1-3)** in MeOH and their shifts in different solvents

Compounds	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/ H_3BO_3
1	335 267.4 298(sh)	392 274.8 325.6(sh)	381.6 346.4 301.6 274.8	381 345 299 274.5	381.8 274.6 299.4(sh)	337 268 301(sh)
2	371 301(sh) 268(sh) 255	326(dec) 247.2 (sh)	455 332 270	426 362 302(sh) 265	389(dec) 327 290 262	386 301 260
3	359 299(sh) 267(sh) 257	411 328 272	430 303(sh) 275	400 360(sh) 300 271	387 322 273	378 292 260

Discussion

UV spectrum of methanolic solution of apigenin (1) supported the flavone structure (table 1). Bathochromic shift of band I in presence of MeOH and its stability after ten minutes relate to 4'-hydroxy, and bathochromic shift of band II about 7 nm is an indication for 7-hydroxyl. Bathochromic shift with $AlCl_3$ and its stability in the presence of HCl relate to 5-hydroxyl (Mabry et al. 1970). The UV, ^{1}H-NMR and ^{13}C-NMR data for 1 were in good agreement with that of apigenin (14). This is the first report on the presence of apigenin in the waste leaves of tobacco.

The UV spectrum of methanolic solution of quercetin (2) exhibited two major absorption bands at 371 nm and 255 nm (table 1), which confirmed the flavonol structure. Degradation of 2 in presence of MeONa and hypsochromic shifts with $AlCl_3$/HCl and AcONa/H_3BO_4 supported the presence of 3, 3, 4' trihydroxy system. Bathochromic shifts with AcONa were related to 7-hydroxyl and the bathochromic shift with AlCl3/HCl to 5-hydroxyl. On the basis of UV, ^{1}H-NMR and ^{13}C-NMR data, compound 2 was identified as quercetin (5).

The UV spectrum of compound 3 showed two major absorption bands at 359 nm and 257 nm, which indicated the presence of flavonol structure. Bathochromic shift with MeONa supported the presence of 4'-hydroxyl and with AcONa indicated the 7- hydroxyl functions. The $AlCl_3$ and $AlCl_3$/HCl spectrum of 3 showed 5-hydroxyl and ortho dihydroxy in ring B. This fact indicated that the 3-hydroxyl was absent or substituted (table 1). The ^{1}H-NMR and ^{13}C-NMR of 3 revealed the chemical shifts of protons and carbons essentially identical with those reported in the literature for rutin (14).

The results of quantitative determination by HPLC of rutin in various samples indicated that the rutin content of waste leaves of tobacco (0.6%) was less than that of unfermented leaves of tobacco (1.5%). However, it can be used as an economical source of rutin. On the other hand, the waste leaves of tobacco can be a cheaper source of rutin extraction in pharmaceutical industry. The amount of rutin in fermented leaves (0.5%) was less than that of unfermented leaves (1.5%) which indicated that rutin was probably metabolised in the fermentation process by enzymes such as ß–glucosidase and flavonol 2,4-dioxygenase (15).

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References

(1) DerMarderosian A. *The Review of Natural Products*. 2nd ed. Facts and Comparisons, Missouri (2001) 554-555

(2) Miller LP. *Phytochemistry Organic Metabolites*.

Van Nostrand and Reinbold Company, New York (1973) 2:382-384

(3) Grinberg LN, Rachmilewitz EA and Newmark H. Protective effects of rutin against hemoglobin oxidation. *Biochem. Pharmacol.* (1994) 48: 643-649

(4) Beretz A and Cazenave J. The effect of flavonoids on blood vessel wall interactions. In: Cody V, Middleton E, Harborne JB and Beretz A. (eds.) *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties*. Alan R Liss, New York (1988) 187-200

(5) Harborne JB. The *Flavonoids*. Chapman and Hall, London (1988) 622-645

(6) Mors WB, Nascimento MC, Pereira BMR and Pereira NA. Plant natural products active against snake bite - The molecular approach. *Phytochem*. (2000) 55: 627-642

(7) Robak J and Gryglewski RJ. Flavonoids are scavenger of superoxide anions. *Biochem. Pharmacol.* (1988) 37: 837-4

(8) Bruneton J. *Elements de Phytochimie et de Pharmacognosie.* Technique et Documentation, Lavoisier (1987) 161-163

(9) Carlo GD, Mascolo N, Izzo AA and Capasso F. Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sciences* (1999) 65: 337-353

(10) Sambantham P. Analgesic activity of certain bioflavonoids. *Indian J. Pharm. Sci.* (1985) 47: 230-231

(11) Evans WC. *Trease and Evans' Pharmacognosy.* 14th ed. WB Saunders Company Ltd, London (1996) 251

(12) Afshar J and Delazar A. Rutin from *Ruta graveolens* L., J. of School of Pharmacy (Medical Sciences University of Tehran) (1994) 4:1-12

(13) Mabry TJ, Markham KR and Thomas MB. T*he Systematic Identification of Flavonoids* Springer-Verlag, New York (1970) 35-40

(14) Breitmaier E and Voelter W. *Carbon- 13 NMR Spectroscopy.* VCH Verlagsgesellschaft mbH, New York (1989) 450-457

(15) Hund HK, Breuer J, Lingens F, Huttermann J, Kappl R and Fetzner S. Flavonol 2, 4-dioxygenase from *Aspergillus niger* DSM 821, a type 2, cuII-containing glycoprotein. *Eur. J. Biochem*. (1999) 263: 871-8

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