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Flavonoids and fatty acids of *Camellia japonica* leaves extract

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Abstract: The ethanol extract from the leaves of *Camellia japonica* L., Theaceae, showed antiradical potential in the DPPH test using TLC plates (SiO_2). Aiming the isolation of active compounds, this extract was partitioned between BuOH:H_2O (1:1) and the two obtained phases were also evaluated to detection of antiradical activity. The active BuOH phase was fractionated in Sephadex LH-20 and silica (normal or reverse phase) to afford three aglycone flavonoids (quercetin, kaempferol and apigenin), which have been described in the *C. japonica* at first time, two glycosilated flavonoids (rutin and quercetrin), and a mixture of saturated fatty acids. The structures of isolated compounds were defined by NMR and GC/MS analyses.

Short Communication

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Introduction

Camellia japonica L., Theaceae, is a tree belonging to Theaceae family and has been used in folk medicine in Japan, China, and Korea to the treatment of stomachic illness and as anti-inflammatory (Yoshikawa et al., 1996). Chemically, this species showed to be constituted by triterpenes (Itokawa et al., 1981; Akihisa et al., 1997; Thao et al., 2010), saponins (Yoshikawa et al., 1996; Ito et al., 1967; Itokawa et al., 1969; Yoshikawa et al., 1994), glycosylated flavonoids (Onodera et al., 2006), and tannins (Hatano et al., 1991; Han et al., 1994; Hatano et al., 1995a; Hatano et al., 1995b; Yoshida et al., 1989; Park et al., 2002). Several of these derivatives showed biological activity including antioxidant (Onodera et al., 2006), antifungal (Nagata et al., 1985), inhibitory effect on human immunodeficiency virus-1 protease (Hatano et al., 1991; Han et al., 1994; Hatano et al., 1995; Yoshida et al., 1989; Park et al., 2002), and cytotoxic potential (Thao et al., 2010).

As part of our ongoing effort to isolate antiradical compounds from vegetal species using TLC/DPPH assay (Ceruks et al., 2007), in this paper we describe the isolation of three free (quercetin, apinenin, and kampferol) and two glycosilated (rutin and quercetrin) flavonoids from BuOH phase from EtOH extract of *C. japonica* leaves. Furthermore, a mixture composed by fatty acids was identified by GC/MS after methylation procedure.

Material and Methods

General experimental procedures

^{1}H NMR spectra were recorded at 200 and 300 MHz and ^{13}C NMR at 50 and 75 MHz on Bruker AC-200 and DPX-300 spectrometers, respectively. $CDCl_3$, DMSO-d_6, and methanol-d_4 (Tedia Brazil) were used as solvent and as internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (*J*) in Hz. GC/LREIMS were measured in a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass spectrometer. Temperature programming was performed from 150-300 °C at 15 °C/min, then isothermal at 300 °C for 5 min. The injector and detector temperatures were 150 and 320 °C, respectively, and helium was used as the carrier gas. Sephadex LH-20 (Amersham Biosciences) was used for column chromatographic separation. Silica gel 60 PF254 (Merck) was used for analytical TLC (0.25 mm) and reverse-phase silica gel (RP18 F254 S, Merck) was used for preparative TLC (1.0 mm).

Plant material

Camellia japonica L., Theaceae, leaves were collected in March, 2006 in São Paulo/SP, Brazil. The plant was identified by Dr. Oriana A. Fávero by comparison with a voucher specimen deposited at the Herbarium of Instituto de Botânica de São Paulo, São Paulo, SP, Brazil.

Extraction and isolation of the constituents

Dried and powdered leaves of *C. japonica* (252 g) were deffated n-hexane (three times at room temperature) and then exhaustively extracted by maceration with EtOH at room temperature. Evaporation of the solvent under reduced pressure afforded the crude ethanol extract (4.7 g), which was partitioned between BuOH:H_2O (1:1). The organic phase, after dried over anhydrous Na_2SO_4, was concentrated under reduced pressure yielding 3.4 g of a residue. Part of this material (2.9 g) was purified using Sephadex LH-20 column chromatography and eluted with MeOH to afford 71 fractions (50 mL) which were pooled together in seven groups after TLC analysis. Group 2 (690 mg) was subjected to Sephadex LH-20 column chromatography and eluted with $CHCl_3$:MeOH (1:1) to afford thirteen fractions (10 mL each). After TLC analysis, these fractions were combined into three sub-groups (I-III). Sub-group II (35 mg) was composed by pure **4** while sub-group III (42 mg) by pure **5**. Group **4** (65 mg) was purified by reverse phase preparative TLC (MeOH:H_2O 1:1) to afford 36 mg of a mixture of fatty acids and 14 mg of **1**. Fraction 6 (40 mg) was purified by reverse phase preparative TLC (MeOH:H_2O 1:1) to afford pure **3** (4 mg) and **2** (9 mg).

Methylation procedure

The 36 mg of free fatty acids were accurately weighed into a 50 mL flask and dissolved in 5 mL of MeOH. After addition of 5 mL of HCl (1.5 mol/L), the solution was refluxed during 2 h, extracted with 2 x 20 mL of EtOAc and successively with H_2O until pH 7. The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of solvent was obtained 28 mg of methylated derivatives, which were immediately analyzed by GC/LREIMS.

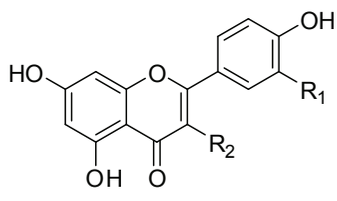
Assay to detection of antiradical potential

Compounds **1-5** (1.0 mg/mL in methanol) were applied to TLC plates, eluted with appropriate solvent systems and sprayed with a solution 2.0 mg/mL of DPPH in methanol. After evaporation of the solvent (about 5 min), the antiradical potential, comparatively with positive control α-tocopherol (1.0 mg/mL in $CHCl_3$), was observed by the appearance of yellow spots on violet background, according to that described in the literature (Ceruks et al., 2007).

Results and Discussion

Fractionation of the BuOH phase from EtOH extract of the leaves *Camellia japonica* L., Theaceae, yielded the isolation of five flavonoids (**1-5**) whose structures were identified based the analysis of data obtained from NMR spectra. Additionally, was obtained a mixture composed by saturated fatty acids which was characterized by GC/LREIMS analysis after methylation procedure.

The ^{1}H NMR spectra of compounds **1-5** showed the signals of H-6 and H-8 of flavonoids due the doublets at range δ 6.1-6.4 (J ~ 2.0 Hz) and at 6.2-6.4 (J ~ 2.0 Hz), indicating a same substitution profile to ring A. Similarly, the three peaks at 7.2-7.6 (d, J ~ 2.0 Hz, H-2′), 6.8 (d, J ~ 8.5 Hz, H-5′) and 7.2-7.5 (dd, J ~ 8.5 and 2.0 Hz, H-6′), observed in the spectra of compounds **1, 4**, and **5**, were indicative of 1,3,4-trissubtituted aromatic ring B. The spectra of compounds **2** and **3** showed doublets at δ 6.9 (J=8.5 Hz, 2H) and 7.7-8.0 (J=8.5 Hz, 2H), assigned to hydrogens H-3′ and H-2′, respectively, from aromatic ring B. Based in the above evidences, associated to the presence of additional signal at δ 6.43 (s, H-3) to compound 2, the structures of free flavonoids were defined as quercetin (**1**), apigenin (**2**) and kaempferol (**3**), whose occurrence has been described at first time to *C. japonica*. Comparison of the ^{13}C NMR data of these compounds with those reported in the literature (Agrawal, 1989), confirmed the structural proposition. ^{1}H NMR spectra of **4** and **5** showed similar signals of those observed to 1 besides of additional peaks at δ 5.30 (d, J=7.0 Hz)/4.40 (br s) and at δ 5.23 (d, J=1.0 Hz), which could be assigned to annomeric hydrogens from rutinose and rhamnose units (Lee et al., 2004). Finally, after comparison of ^{13}C NMR data with those reported in the literature (Agrawal, 1989), the structures of these glycosylated flavonoids were defined as rutin (**4**) and quercetrin (**5**).



1 R_1 = OH; R_2 = OH
2 R_1 = R_2 = H
3 R_1 = H; R_2 = OH
4 R_1 = OH; R_2 = OGlu-Rham
5 R_1 = OH; R_2 = ORham

The ^{1}H NMR spectrum to the mixture of fatty acids showed one deformed triplet at δ 0.89 (t, *J*=7.1 Hz), one intense singlet at δ 1.20 and one triplet at δ 2.30 (*J*=7.5 Hz). The absence of signals between δ 4.5-6.0 indicated the occurrence of saturated chain (Moreira et al., 2007). Aiming the characterization of these derivatives, the fraction was methylated and than analyzed by GC/MS allowing the identification of six esters (methyl tridecanoate, methyl tetradecanoate, methyl pentadecanoate, methyl hexadecanoate, methyl heptadecanoate, and methyl octadecanoate), as showed in Table 1.

Table 1. Relative amount of methyl derivatives from fatty acids identified in the leaves of *C. japonica.*

Compound	Relative amount / %
Methyl tridecanoate	2.2
Methyl tetradecanoate	2.8
Methyl pentadecanoate	17.6
Methyl hexadecanoate	8.8
Methyl heptadecanoate	5.1
Methyl octadecanoate	37.0
Total	72.5

All compounds isolated from the BuOH phase (**1-5**) were subjected to evaluation of antiradical potential by spraying a solution of 2.0 mg/mL of DPPH in methanol in a TLC plate (SiO_2). After 5 minutes, were observed strong yellow spots on violet background, suggesting that compounds **1-5** are responsible for antiradical potential observed in the crude extract/phases, as previously reported (Coutinho et al., 2010; Ceruks et al., 2007; Rosa et al., 2010).

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