Identification by HPLC−DAD and HPLC−MS Analyses and Quantification of Constituents of Fennel Teas and Decoctions

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Qualitative and quantitative differences among the constituents in various fennel (*Foeniculum vulgare* Mill., family Apiaceae) teas prepared by classical infusion, microwave decoction, and dissolution are reported. Different commercial starting materials, such as fruit (unbroken and crushed), four herbal teas, and two instant herbal teas were evaluated. Chlorogenic acid (**1**), quercetin-3-O-β-D-glucuronide (**2**), *p*-anisaldehyde (**3**), and *trans*-anethole (**4**) were identified by HPLC−DAD and HPLC−MS as constituents of fennel teas. No coumarins, which are characteristic constituents of plants of Apiaceae family, were found. *Trans*-anethole (**4**), the main constituent of the essential oil, was present in all teas. In addition *p*-anisaldehyde (**3**), a degradation product of *trans*-anethole, was also identified in all teas with the exception of two samples. Chlorogenic acid (**1**) and quercetin-3-O-β-D-glucuronide (**2**) were also present in all teas. In addition, minor unidentified flavonol constituents were found in two teas. Quality, activity, and safety of the content of the investigated preparations are also discussed.

Keywords: *HPLC−DAD and HPLC−MS; fennel; herbal teas and instant herbal teas; infusion and microwave decoction; essential oil; polyphenols*

INTRODUCTION

Many herbal drugs whose efficacy has been attributed to the essential oil content are used as teas, i.e., chamomile, peppermint leaf, aniseed, and fennel (Czygan, 1989). However, very little is known about the qualitative and quantitative composition of the essential oil andother constituents present in the teas. Furthermore, children and newborns are frequent consumers of these teas, so that knowledge of their constituents represents a very important step for the evaluation of their safety and efficacy.

In this work the constituents of teas from fennel were evaluated. This drug is described in the 2nd edition of the European Pharmacopoeia both as "Sweet Fennel" and "Bitter Fennel", which consist of the dry, whole cremocarps and mericarps of *Foeniculum vulgare* sub. vulgare, var. dulce (Miller) Thellung and *Foeniculum vulgare* sub. vulgare, var. vulgare Miller, respectively. The sweet variety is mainly marketed in Italy, so it was employed for our investigation. Fennel and its herbal drug preparations are used for dyspeptic complaints such as mild, spasmodic gastric-intestinal complaints, bloating, and flatulence. It is also used for the catarrh of the upper respiratory tract (Czygan, 1989; Madaus, 1976; Merkes, 1980; Forster et al., 1980; Forster, 1983; Weib, 1991; Reynolds, 1993). According to the 9th edition of the German Pharmacopoeia, the essential oil is currently being considered as responsible for the pharmacological properties of fennel but it cannot be given as such to children and newborns because of its risk of laringospasms, dysponeas, and states of agitation (Dorsch et al., 1993). In this study, teas from commercial fruit were obtained from both crushed fruits, according to the published recommendations (Czygan, 1989), and from unbroken fruits. In addition, teas obtained from prepackaged teabags and from freeze-dried products of fennel have also been investigated. Prepackaged teabags marketed in Italy contain unbroken and/or crushed fruit or powdered drug. However, the use of unbroken fruit to prepare infusions is incorrect. Indeed some difficulties exist in extracting the essential oil by infusion due to its intracellular localization. However, crushed or powered fruit gradually lose their essential oil content during aging (Czygan,1989). Finally, the qualitative and quantitative contents of the teas prepared using prepackaged teabags 7 and 30 days after opening were also evaluated to investigate the possible loss of volatile constituents over time. According to the 2nd edition of the European Pharmacopoeia monograph, sweet fennel contains not less than 2.0% v/m of essential oil, calculated with reference to the anhydrous drug. The essential oil is constituted mainly by anethole (80%), and it contains not more than 10% estragole and not more than 7.5% fenchone (Brand, 1993). Other minor constituents may be present including α-pinene, limonene, β-pinene, β-myrcene, and *p*-cymene (Brand, 1993; Toth, 1967; Trenkle, 1972). Furthermore, sweet fennel contains other nonvolatile constituents such as flavonoids and coumarins (Kunzemann andHerrmann, 1977; Murray et al., 1982) which have not received much attention with regard to pharmacological properties. However, their presence in the teas could contribute to the pharmacological activity of such herbal drug preparations.

EXPERIMENTAL PROCEDURES

Solvents. Acetonitrile was HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Standards. Indena Research Laboratories (Settala, Milan, Italy) kindly provided rutin (batch n. K12408717, standard

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Table 1. Investigated Herbal Teas and Instant Herbal Teas

test product	type of preparation	recommended dosages (g)	lot	content
A	teabags	2.3	08141	brown powder
B	teabags	2.0	11H	unbroken and crushed fruits
C	teabags	2.0	309	finely ground plant material
D	teabags	2.25	31	unbroken fruits
E	powder	1.0		freeze-dried extract
F	powder	7.0		freeze-dried extract

purity 88.17% considering the content of residual solvents, moisture, and amount of impurities). Chlorogenic acid was purchased from Extrasynthese (Genay, France). *Trans*-anethole and *p*-anisaldehyde were purchased from Sigma Chemicals (Milan, Italy). Quercetin-3-O-β-D-glucuronide was isolated and characterized by the authors. The purity value of these standards was calculated or given by the suppliers (89.3, 99.0, 98.0 and 90.5%, respectively).

Samples. Aboca S.p.A. (Sansepolcro, Arezzo, Italy) kindly offered commercial samples of fruits of *Foeniculum vulgare*, subsp. *vulgare* var. dulcis (Miller) Thellung. The drug (lot 59710, 11/97) contained 27.7 mL/kg of essential oil determined according to the 2nd edition of European Pharmacopoeia assay. Herbal teas and instant herbal teas listed in Table 1 were purchased from local pharmacies and grocery stores and named as products A−F.

Sample preparations. Traditional teas were prepared from fruits by infusion of 2.5 g of both unbroken and freshly crushed drug in 150 mL of incipient boiling water, leaving it for 20 min in a covered cup and occasionally stirring (Czygan, 1989; 9th edition of the German Pharmacopoeia). Then the suspensions were rapidly filtered through cotton wool. A 5-mL fraction of the filtrate was used as such for the HPLC analysis. Teas were also prepared by 2 min decoction from 2.5 g of both unbroken and crushed fruits in a covered cup containing 150 mL of water in a microwave (potency 600 W). The suspensions were rapidly filtered and processed as described for the traditional teas. In addition, traditional teas were obtained using teabags of products A−D and named Ac, Bc, Cc and Dc, respectively. These were processed as previously described for the other samples. Other teas (Am, Bm, Cm, and Dm) were prepared by decoction in microwave of a teabag of products A−D and after removing the teabags, were processed as already reported. In addition, teas also were obtained by dissolving two instant herbal teas (E and F) in warm (30 °C) water (150 mL) using 7 g and 1 g of powders respectively, according to the label suggestions and named Ed and Fd. These teas were processed in the same manner as the other samples.

HPLC−DAD Analysis Instrumentation. The HPLC system consisted of a HP 1090L instrument equipped with a diode array detector (DAD) and managed by a HP 9000 workstation (Hewlett-Packard, Palo Alto, CA). The column was a LiChrosorb RP18 (5μm, 250 × 4 mm i.d.) (Merck, Darmastadt, Germany) maintained at 26 °C and equipped with a precolumn LiChrosorb RP18 (5μm, 10 × 4 mm i.d.) (Merck, Darmastadt, Germany). The mobile phase was a four-step linear solvent gradient CH_3CN/H_2O (see Table 2) with HCOOH (pH = 3.2) during a 40-min period at a flow rate of 1 mL/min. The injected volume of sample was 10 μL of solution. UV−vis spectra were recorded in the range 190−450 nm, and chromatograms were acquired at 210, 254, 280, and 330 nm. Peaks were detected at 254 nm. A typical chromatogram is reported in Figure 1. The t_R values for chlorogenic acid (**1**), quercetin-3-O-β-D-glucuronide (**2**), *p*-anisaldehyde (**3**), and *trans*-anethole (**4**) were 8.07, 18.8, 25.56, and 35.93 min, respectively. Structures of compounds **1**−**4** are reported in Figure 2.

HPLC−MS Analysis Instrumentation. The HPLC system previously described was interfaced with a HP 1100 MSD

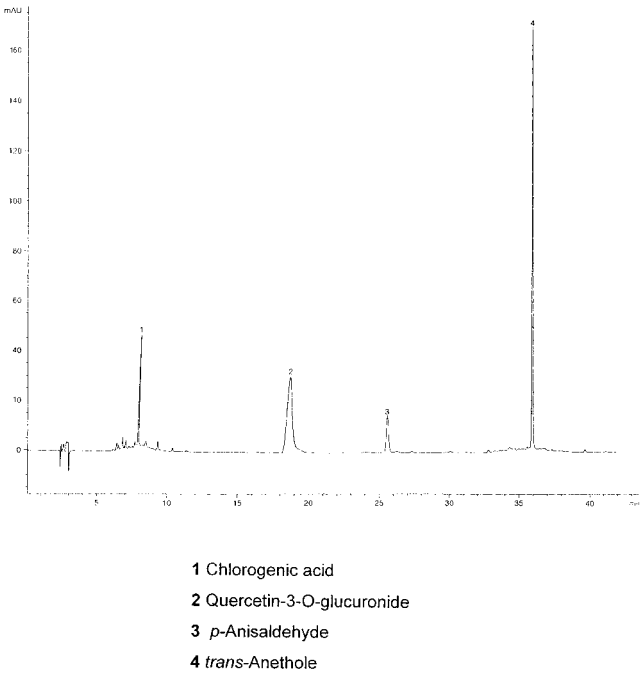

Figure 1. Chromatographic profile of the conventional tea of freshly crushed fruits with the HPLC−MS attributions of the components detected: **1**, chlorogenic acid; **2**, quercetin-3-O-glucuronide; **3**, *p*-anisaldehyde; **4**, *trans*-anethole.

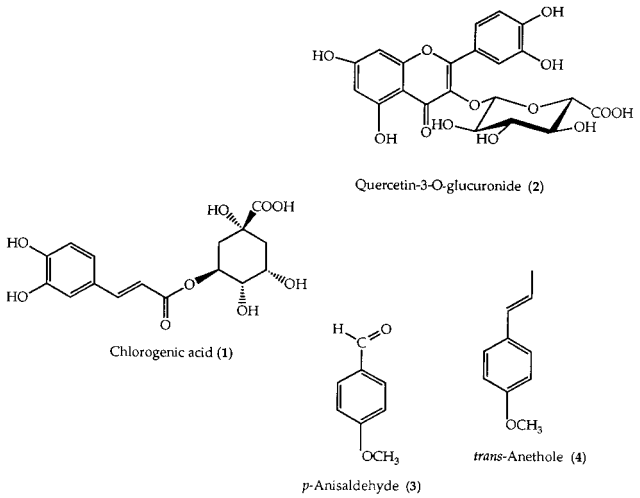

Figure 2. Chemical structures of compounds **1**−**4**.

Table 2. Mobile-Phase Composition Used for the HPLC−DAD Analysis

time (min)	% H_2O	% CH_3CN	flow (mL/min)
0.10	88.0	12.0	1.00
10.00	82.0	18.0	1.00
15.00	82.0	18.0	1.00
30.00	55.0	45.0	1.00
35.00	0.0	100.0	1.00
42.00	0.0	100.0	1.00
50.00	88.0	12.0	1.00

API-electrospray (Hewlett-Packard, Palo Alto, CA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of the HPLC−DAD analyses. The same column, mobile phase, time period, and flow rate were used. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values: gas temperature 350 °C at a flow rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from *m*/*z* 100 to 800 in the negative and

Table 3. List and Relative Amounts[a] of Constituents Detected in Teas from Fruits and in Instant Herbal Teas

teas	unbroken fruits (conventional)	unbroken fruits (microwave)	crushed fruits (conventional)	crushed fruits (microwave)	Ed[b]	Fd[b]
used dose (g)	2.5	2.5	2.5	2.5	1.0	7.0
chlorogenic acid (mg)	1.4 ± 0.11	1.6 ± 0.19	1.7 ± 0.21	2.0 ± 0.20	0.5±0.09	1.4±0.12
quercetin-3-O-β-D-glucuronide (mg)	6.9 ± 0.08	7.4 ± 0.08	8.6 ± 0.10	10.2 ± 0.11	0.3±0.08	2.4±0.27
other flavonols(mg)	Not found	Not found	Not found	Not found	Not found	Not found
anethole (mg)	2.6 ± 0.21	1.6 ± 0.19	10.0 ± 0.11	4.3 ± 0.39	5.7±0.44	9.1±0.81
anisaldehyde (mg)	1.3 ± 0.11	1.4 ± 0.13	1.1 ± 0.16	2.2 ± 0.20	Not found	0.3±0.09

[a] Means ± SD of three replicates. [b] Dissolved in warm water.

positive ion modes were obtained (scan time 1 s). The injected volume of sample solution was 10 μL.

Identification of Tea Constituents. Identification of constituents **1**-**4** was performed by HPLC−MS analysis and/or by comparing the retention time of the peaks with authentic samples. The purity of peaks was checked by comparing the UV spectra obtained with a DAD coupled to the HPLC system with those obtained by authentic reference samples and/or by examination of the MS spectra.

Linearity. Linearity range of responses was determined on five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from 0.10 to 2 μg ($r > 0.999$).

Repeatability. To evaluate the repeatability, six fennel fruit samples from the same batch were crushed and immediately used to prepare teas that were analyzed as such by RP−HPLC. Each constituent of such fennel teas was evaluated to calculate the relative standard deviation. The following data were obtained: chlorogenic acid 1.93%, rutin 1.85%, quercetin-3-O-β-D-glucuronide 1.96%, *p*-anisaldehyde 2.09%, and *trans*-anethole 2.27%.

Reproducibility. To evaluate the reproducibility of the injection integration, 10 μL of a standard solution of rutin (0.1 μg/1 μL) and of crushed fennel fruit sample of traditional tea preparation were injected six times and the relative standard deviation values were calculated. The following data were obtained: chlorogenic acid 1.32%, rutin 0.80%, quercetin-3-O-β-D-glucuronide 0.98%, *p*-anisaldehyde 1.12%, and *trans*-anethole 1.27%.

Quantitation. All the fennel teas were analyzed in triplicate and a calibration graph with six datapoints of external standard was used. The contents of constituents were calculated taking into account the mean of the response factor of rutin in the reference solutions, i.e., area/concentration (mg/mL) × purity/100, and the response factor of the considered constituent relative to rutin (RRF), as reported in the literature (Brolis et al., 1998). This value was determined by calculating the ratio between the average response factor of each compound and the average response factor of rutin at 254 nm.

RESULTS AND DISCUSSION

This is the first report of the analysis of the constituents of fennel teas using a simple, direct, rapid, and robust RP−HPLC method. Accuracy, reproducibility and speed were found acceptable. Fennel teas were prepared both by infusion with occasional stirring according to the 9th edition of German Pharmacopoeia, and by decoction in microwave as described in the Experimental Section. Commercial fruits of *Foeniculum vulgare*, subsp. *vulgare*, var. dulcis (Miller) Thellung containing 27.7 mL/Kg of essential oil, commercial teabags, and instant teas were used in this investigation.

Four major constituents were identified: chlorogenic acid (**1**), quercetin-3-O-β-D-glucuronide (**2**), *p*-anisaldehyde (**3**) and *trans*-anethole (**4**). A λ of 254 nm was used for HPLC quantitative evaluation of constituents, as all the constituents showed appreciable absorbance at this wavelength. Rutin was used as external standard. Good linearity of the calibration curves was achieved between 0.1 and 2 μg ($r > 0.999$); the repeatability and reproducibility of the method showed satisfactory results.

All compounds were identified by means of HPLC−DAD and HPLC−MS analyses. Peak **1** in the chromatogram of Figure 1 displays UV spectra with maxima at 236 and 327 nm and shows a quasi-molecular ion $[M+H]^+$ at *m*/*z* 355 and $[M+H-H_2O]^+$ ion at *m*/*z* 337. Peak **1** was identified as chlorogenic acid by comparison of t_R, UV spectra, and MS spectra with those of an authentic sample. Peak **2** displays UV spectra with maxima at 259 and 355 nm, typical of flavonols. Moreover, the presence in the ESI−MS spectrum of an ion at *m*/*z* 301 indicates that this compound is a quercetin derivative. A quasi-molecular ion $[M-H]^+$ at *m*/*z* 477 and the fragment ion at *m*/*z* 301 are evident due to the loss of glucuronic acid. Peak **2** was identified as quercetin-3-O-β-D-glucuronide by comparison of t_R, UV spectra, and MS spectra with those of an authentic sample. Peaks **3** and **4** were identified as *p*-anisaldehyde and *trans*-anethole by comparison of t_R and UV spectra (maxima at 281 and 257 nm, respectively) and MS spectra with those of authentic samples. *P*-anisaldehyde shows a quasi-molecular ion $[M+H]^+$ at *m*/*z* 137 and *trans*-anethole shows a quasi-molecular ion $[M+H]^+$ at *m*/*z* 149.

Trans-anethole (**4**), the main constituent of essential oil, was present in all teas. In addition *p*-anisaldehyde (**3**), a degradation product of *trans*-anethole, not present in fennel essential oil obtained by steam distillation, was also identified in all teas with the exception of those from products A and E. Chlorogenic acid (**1**) and quercetin-3-O-β-D-glucuronide (**2**) were also present in all teas in addition to minor unidentified flavonol constituents found in teas obtained from products A and C. For all the investigated teas the content of the essential oil per 150 mL of preparation was calculated and the amounts of constituents **1**−**4**, using the response factor relative to rutin (RRF), were also reported. The results are given in Tables 3 and 4.

The qualitative and quantitative composition of volatile constituents in the various teas was quite different. In traditional teas of freshly crushed fruits 88% of the volatile constituents consisted of *trans*-anethole (**4**), which was present in an amount about 8-fold more than *p*-anisaldehyde (**3**). However, there was a higher content of *p*-anisaldehyde in the teas obtained by microwave decoction, probably due to the different extraction conditions and/or to degradation of **4** to **3**. If unbroken fruits were used, the content of *p*-anisaldehyde was higher, as well. A tea obtained by dissolution (Fd) showed high content of *trans*-anethole and a small amount of *p*-anisaldehyde, and their percentages were similar to those of traditional teas of freshly crushed

fruits. The other sample obtained by dissolution (Ed) showed only *trans*-anethole. Also, in conventional teas obtained by the infusion of teabags *trans*-anethole content was higher than that obtained by microwave decoction. This value slightly decreased in teas prepared using teabags 7 or 30 days after package opening (see Table 4). Both teas (Cc and Cm) obtained by product C showed the presence of only *p*-anisaldehyde.

Concerning the other substances found in the teas, chlorogenic acid (**1**) is a very common metabolite of higher plants, and quercetin-3-O-β-D-glucuronide (**2**) is a quite unusual glycoside of quercetin which, however, is a known constituent of fennel fruit (Kunzemann and Herrmann, 1977). Chromatograms of Ac, Am, Cc, and Cm teas showed traces of other flavonoids. Their maxima in the UV spectra were typical of flavonol derivatives, probably glycosides of quercetin and/or kaempferol. These compounds, however, are already reported as minor constituents of aerial parts of *Foeniculum vulgare* (Kunzemann and Herrmann, 1977). In all teas they were detected in very low concentrations and it was not possible to fully identify them. Their isolation and identification is presently in progress.

The contents of chlorogenic acid (**1**) and quercetin-3-O-β-D-glucuronide (**2**) were always higher in the teas obtained by microwave decoctions than those obtained by infusion, probably due to the extraction methodology. The content of **1** per cup in traditional teas of freshly crushed fruits was 1.7 mg, which increased to 2.0 mg in teas from microwave decoction of freshly crushed fruits. The content of **2** was 8.6 mg per cup in traditional teas of freshly crushed fruits and 10.2 mg in teas obtained by decoction using a microwave. The content of these polyphenols was slightly lower in teas obtained from unbroken fruits (see Table 3). Tea Ed showed a very low content of these substances (0.5 mg of **1** and 0.3 mg of **2**), whereas Fd showed a higher content (1.4 mg of **1** and 2.4 mg of **2**). The amount of **1** in Fd was generally similar to that in all teas obtained from the other products investigated (A−D). All teas obtained from products A−D showed a content of **2** similar to that obtained in the tea from dissolution of product F, or more (values between 1.2 mg and 6.3 mg). However, in every case these values were lower than those found in traditional teas of freshly crushed fruits. In general, the considerable amount, per cup, of these two polyphenols could suggest their contribution in the activity of these herbal drug preparations.

Coumarins, which are characteristic metabolites of fennel and in general of the Apiaceae family (Murray et al., 1982), were found in none of the teas investigated. This fact was very important for drug use safety.

Concerning fennel anti-spasmodic effects, it is reported that in vivo studies of 2−3 g of traditional teas of freshly crushed fruits/kg body weight in cats inhibited in situ ileum spasm (approximately 150 s, 50%) induced by acethylcoline and histamine (Schuster, 1971). Therefore, only Fd, which shows a content of volatile constituents in this range, may be considered active for this therapeutic indication. Concerning fennel secretolytic and expectorant effects, in vivo studies on rats refer only to anethole. Inhalation of anethole (3 mg/kg) augmented the volume output of respiratory tract fluid (Boyd and Sheppard, 1971). If the necessary dosage for an adult human of 70 kg was considered, the expected anethole dosage would be 210 mg of anethole corresponding to about 235 mg of essential oil. Since it is not possible to

Table 4. List and Relative Amounts[a] of Constituents Detected in Herbal Teas

teas	Ac			Am			Bc			Bm			Cc			Cm			Dc			Dm		
used dose (g)	2.3			2.3			2.0			2.0			2.0			2.0			2.25			2.25		
opened days	0	7	30	0	7	30	0	7	30	0	7	30	0	7	30	0	7	30	0	7	30	0	7	30
chlorogenic acid (mg)	1.0 ±0.11	1.0 ±0.33	1.0 ±0.24	1.4 ±0.11	1.3 ±0.20	1.3 ±0.28	0.9 ±0.09	0.9 ±0.14	0.9 ±0.261.1 ±0.13	1.2 ±0.18	1.1 ±0.221.3 ±0.21	1.3 ±0.36	1.3 ±0.291.5 ±0.32	1.5 ±0.28	1.5 ±0.381.0 ±0.08	1.0 ±0.14	1.0 ±0.211.0 ±0.18	1.0 ±0.24	1.0 ±0.28					
quercetin-3-O-β-D-glucuronide (mg)	1.2 ±0.15	1.2 ±0.18	1.2 ±0.20	1.5 ±0.09	1.5 ±0.18	1.5 ±0.26	4.8 ±0.56	4.8 ±0.67	4.9 ±0.70	5.0 ±0.54	5.1 ±0.59	5.0 ±0.64	3.9 ±0.29	3.9 ±0.32	3.9 ±0.37	4.2 ±0.32	4.2 ±0.48	4.2 ±0.50	5.8 ±0.54	5.8 ±0.50	5.8 ±0.54	6.3 ±0.58	6.3 ±0.50	6.3 ±0.58
other flavonols (mg)	traces	traces	traces	traces	traces	traces	not found	not found	not found	not found	not found	not found	traces	traces	traces	traces	traces	traces	not found	not found	not found	not found	not found	not found
anethole (mg)	1.2 ±0.11	1.2 ±0.22	1.0 ±0.32	0.7 ±0.10	0.7 ±0.19	0.5 ±0.21	1.7 ±0.20	1.7 ±0.26	1.3 ±0.20	1.6 ±0.24	1.5 ±0.21	1.2 ±0.17	not found	not found	not found	not found	not found	not found	1.7 ±0.12	1.6 ±0.16	1.4 ±0.14	1.2 ±0.13	1.1 ±0.09	1.0 ±0.14
anisaldehyde (mg)	not found	not found	not found	not found	not found	not found	0.8 ±0.09	0.9 ±0.14	1.1 ±0.17	0.7 ±0.07	0.8 ±0.08	0.9 ±0.09	1.0 ±0.08	1.0 ±0.11	0.9 ±0.13	0.9 ±0.15	0.9 ±0.16	0.8 ±0.10	0.7 ±0.09	0.8 ±0.11	1.0 ±0.12	0.7 ±0.09	0.7 ±0.11	0.8 ±0.11

[a] Means ± SD of three replicates.

obtain such an essential oil content in the infusions, our results lead to the conclusion that none of the tested teas contain such essential oil in an amount sufficient to explain the secretolytic and expectorant purposes.

However, because the putative pharmacological properties of the infusions have not yet been completely verified, experimental evidence of the pharmacological properties are needed for an appraisal of a possible effect of other constituents of the teas such as flavonoids and chlorogenic acid.

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