ORIGINAL RESEARCH

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Development of a double-fractionated *Perilla frutescens* leaf extract and its possible use in functional dyspepsia

Giulia Nannoni[1], Alessandro Mattarocci[2], Giulia Volterrani[2], Alessandro Alì[3], Francesco Di Pierro[4]

Correspondence to: Giulia Nannoni, g.nannoni@labiotre.com

ABSTRACT

Functional dyspepsia (FD), a gastrointestinal disorder characterized by pain or a burning sensation in the epigastrium, postprandial fullness, early satiety, bloating and nausea, used to be treated with prokinetics such as metoclopramide, cisapride and domperidone. Unfortunately, these drugs have severe side effects, including cardiac and sudden death, and consequently are now rarely used or have been withdrawn from the market. However, botanicals could be a source of natural prokinetics which do not have these adverse effects. *Perilla frutescens* leaf extract is a possible candidate, with some data indicating a clinical role in FD. As with any botanical, perilla extract normally consists of a complex mixture of active ingredients characterized by different chemical and physical properties, including varying degrees of solubility in water and/or alcohol. Consequently, selection of the correct solvents is very important for the preparation of standardized botanical extracts. We have therefore evaluated which solvents, and in which order, should be used. Our findings show that a first extraction step performed in ethanol (80%) followed by a second extraction step using warm water results in standardized quantities of both lipophilic and hydrophilic perilla leaf actives such as rosmarinic acid, vicenin-2, perilla ketone, apigenin, luteolin, and apigenin and luteolin glycosides.

Keywords

Prokinetics

Perilla ketone

Vicenin-2

Rosmarinic acid

Multi-fractionated extract

Introduction

Functional dyspepsia (FD) is a common gastrointestinal disorder characterized by pain or a burning sensation in the epigastrium, postprandial fullness, early satiety, bloating and nausea [1]. The global prevalence of dyspepsia in adults is estimated to be 20.8%, but this figure varies depending on geographical location and disease definition [2]. Although the pathological mechanisms underlying FD have not yet been fully elucidated, research indicates that it has a multifactorial aetiology which includes delayed gastric emptying, impaired gastric fundus accommodation, visceral hypersensitivity and various psychosocial factors [3]. Prokinetic drugs such as metoclopramide (MCP) and domperidone used to be important for the treatment of FD. However, their use was questioned when the prokinetic drug cisapride, formerly a standard treatment, was withdrawn from the market in 2000 due to rare cardiac side effects. In 2014, new restrictions were announced by the European Medicines Agency (EMA) and numerous national regulatory authorities have issued black box warnings restricting the use of MCP because of the risk of rare extrapyramidal side effects. MCP is no longer authorized by the European Union for the treatment of chronic conditions such as FD and gastroesophageal reflux. The same applies to domperidone, due to rare cardiac side effects [4]. Domperidone is a peripherally selective dopamine D2 receptor antagonist used as an anti-emetic, gastroprokinetic agent and galactagogue [5], but its use is associated with an increased risk of sudden cardiac death, most likely through its prolongation of the cardiac QT interval and initiation of ventricular arrhythmias. The cause is thought to be blockade of hERG voltage-gated potassium channels. The risks are dose-dependent and appear to be greatest with high doses, in the elderly, and when administered with drugs that interact with domperidone and increase its circulating concentrations, namely CYP3A4 inhibitors [6]. As the potential side effects of prokinetics have raised concern, there is a need to develop new actives with a better safety profile for use in patients with contraindications. *Perilla frutescens* (L.) Britton is an annual edible herbaceous

[1]Research & Development, Labiotre, Tavarnelle Val di Pesa, Florence, Italy

[2]Quality Control, Labiotre, Tavarnelle Val di Pesa, Florence, Italy

[3]Consultant at Labiotre, Tavarnelle Val di Pesa, Florence, Italy

[4]Scientific Department, Velleja Research, Milan, Italy

plant native to Asia. Its common names include shiso, Japanese melissa and Japanese basil. Perilla belongs to the mint family, *Lamiaceae*. Green perilla leaves are used as a tea, as a food or as a spice [7]. In vitro, ex vivo and clinical studies have clearly shown that *P. frutescens* water extract exhibits anti-spasmodic and anti-inflammatory effects [8–10]. Apigenin, luteolin and rosmarinic acid (Fig. 1) seem to be responsible for its anti-inflammatory activity [11], and vicenin-2 for its anti-spasmodic effect (Fig. 1) [8]. While the prokinetic activity of the water extract of perilla is weak and has only a moderate impact on gastric motility, the methanol extract has a stronger intestinal pharmacological effect, likely due to the monoterpenoid perilla ketone (Fig. 1). Pharmacological studies on the mechanism of action of perilla ketone suggest that it stimulates the circular muscles of the intestine, thus promoting intestinal propulsion [12]. *P. frutescens* leaves contain different types of actives which vary in their solubility in water and in alcohol. The aim of our study was to develop a new *P. frutescens* leaf extract containing both water-soluble and alcohol-soluble actives for the formulation of new products to treat FD in patients at risk of side effects from traditional prokinetic drugs and, particularly, sudden cardiac in the elderly administered domperidone.

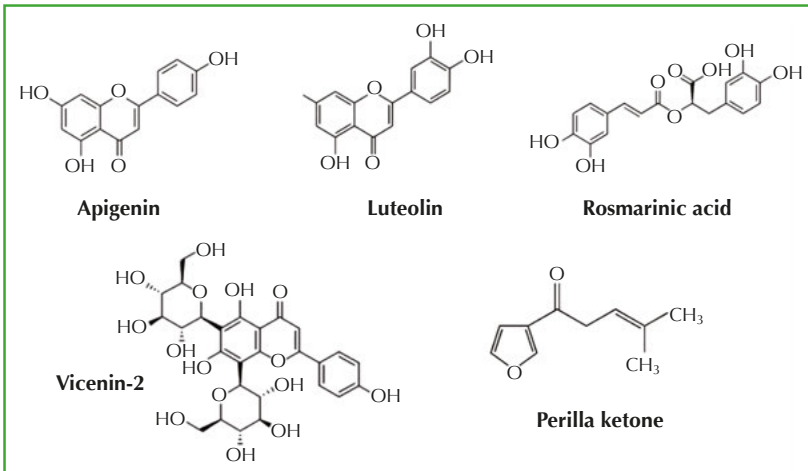

Figure 1 - Presumed actives present in *Perilla frutescens* leaves

Materials and methods

Samples of fresh and dried perilla leaves were obtained from different European producers and are all currently available in Italy. The results obtained from two samples ('X' and 'Y') are reported in this paper. The standard for perilla ketone was supplied by TLC PharmaChem, Canada, while standards for luteolin, rosmarinic acid, apigenin and vicenin-2 were supplied by Phytolab, Germany. Acetonitrile (HPLC grade) and ethanol were purchased from Sigma Aldrich Chemicals (USA). Samples of perilla leaves or extracts were sonicated in an ultrasonic bath for 30 min at room temperature. To quantify perilla ketone, 1 g of extract/leaves was extracted with two 50 ml portions of 80% ethanol, the solution was introduced into a 100 ml volumetric flask and ethanol was added to a final volume of 100 ml. The solution was then passed through a 0.45 µm filter and injected into an HPLC analyzer. To quantify rosmarinic acid, vicenin-2, luteolin, apigenin and the derivatives of luteolin and apigenin, 1 g of extract/leaves was extracted with two 50 ml portions of distilled water, the solution was introduced into a 100 ml volumetric flask and distilled water was added to reach a final volume of 100 ml. The solution was then passed through a 0.45 µm filter and injected into the HPLC analyzer. An Agilent model 1260 Infinity system equipped with a DAD detector and a Zorbax Rapid Resolution SB C18 250×4.6 mm, 0.5 µm column with a precolumn Zorbax Reliance Cartridge was used. Detection was set at 340 nm and 214 nm and flow was set at 0.8 ml/min. The eluents were 0.2% phosphoric acid in water (A) and acetonitrile (B). Gradients were: A/B 87/13 to 75/25 in 35 min; A/B 75/25 to 40/60 in 10 min. For calibration, all standards were dissolved in methanol (HPLC grade) purchased from Sigma Aldrich Chemicals. The calibration parameters are given in Table 1.

Concentration (mg/ml)	Area	Calibration
Perilla ketone		
0.054	3219.81763	P = 0.00002
0.027	1670.34814	I = –0.00293
0.0054	480.41721	R = 0.99993
Rosmarinic acid		
0.960	28058.36602	P = 0.00003
0.480	15169.56292	I = –0.01221
0.096	143.14367	R = 0.99878
Luteolin		
0.940	3614.79395	P = 0.00003
0.470	1796.07703	I = 0.00064
0.0094	338.744	R = 1
Apigenin		
0.0568	2751.93555	P = 0.00003
0.0284	1370.54663	I = 0.00023
0.00568	265.02667	R = 1
Vicenin-2		
0.0384	663.81714	P = 0.00006
0.0192	352.65927	I = –0.00057
0.00384	69.13033	R = 0.99931

Table 1 - Calibration parameters

Results and discussion

The aim of our study was to produce a *P. frutescens* leaf extract with a richer mixture of active ingredients bearing in mind their varying solubility in water and in alcohol. Previous observations have suggested that apigenin, luteolin, rosmarinic acid and vicenin-2 are present in the water-soluble fraction of perilla extract and that perilla ketone is present in the lipophilic fraction.

We compared the amount of actives obtained by water extraction and by ethanol extraction from crop-cultivated dry perilla leaves ('X' and 'Y') acquired from two different suppliers. The HPLC-DAD chromatograms of the ethanol and water extracts evaluated at 214 and 340 nm are shown in Figs. 2 and 3. As seen in Table 2, analysis of the different chromatogram peaks in Figs. 2 and 3 reveals that better extraction of perilla ketone, apigenin and luteolin is obtained with ethanol, rosmarinic acid is extracted similarly by both ethanol and water, and vicenin-2 is visible only in the water extract. Moreover, in the chromatograms of the water extract, peaks corresponding to luteolin glycosides (RT 14.1–14.3 and 23.1–23.3) and to apigenin glycosides (RT 18.9–19.1 and 30.2–30.4) are also visible between 13 and 23 min. Material X contains more active principles than material Y. On the basis of these results, we examined whether, on material X, it was better to extract the raw material using ethanol first and then water on the residue, or better to extract using water first and then ethanol on the residue. Table 2 gives the results of extraction using ethanol first and then water. As shown in Figs. 4 and 5 and in Table 3, perilla ketone is better extracted using ethanol first. There is a higher, though low, concentration of apigenin and lu-

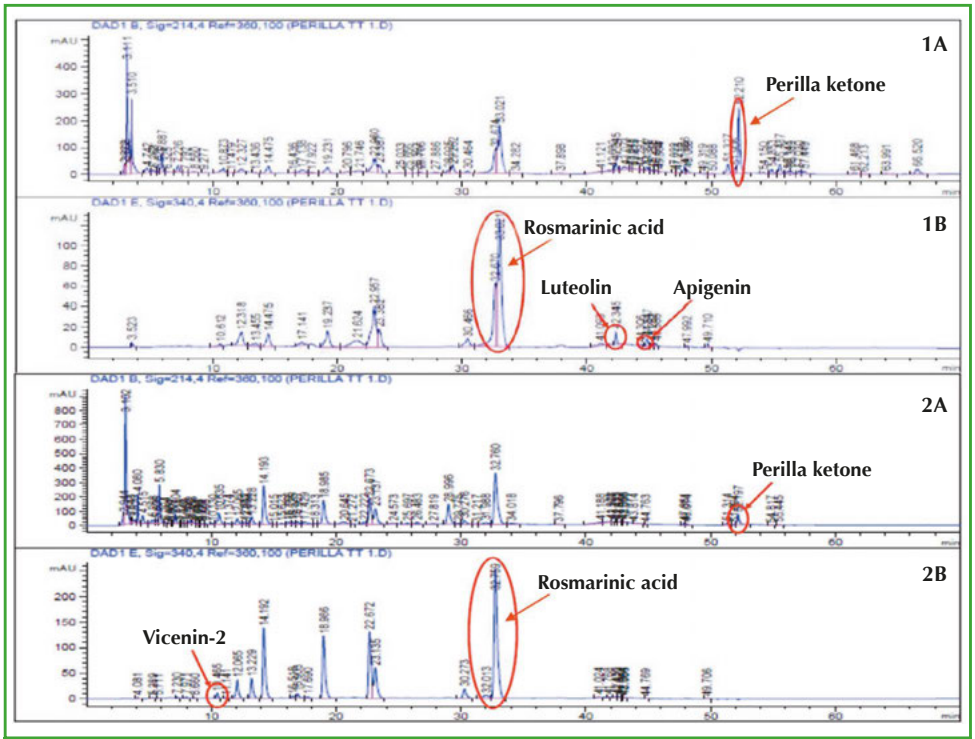

Figure 2 - HPLC-DAD chromatograms of raw material X extracted using ethanol (80%) and evaluated at 214 nm (**1A**) and 340 nm (**1B**), and extracted using water and evaluated at 214 nm (**2A**) and 340 nm (**2B**)

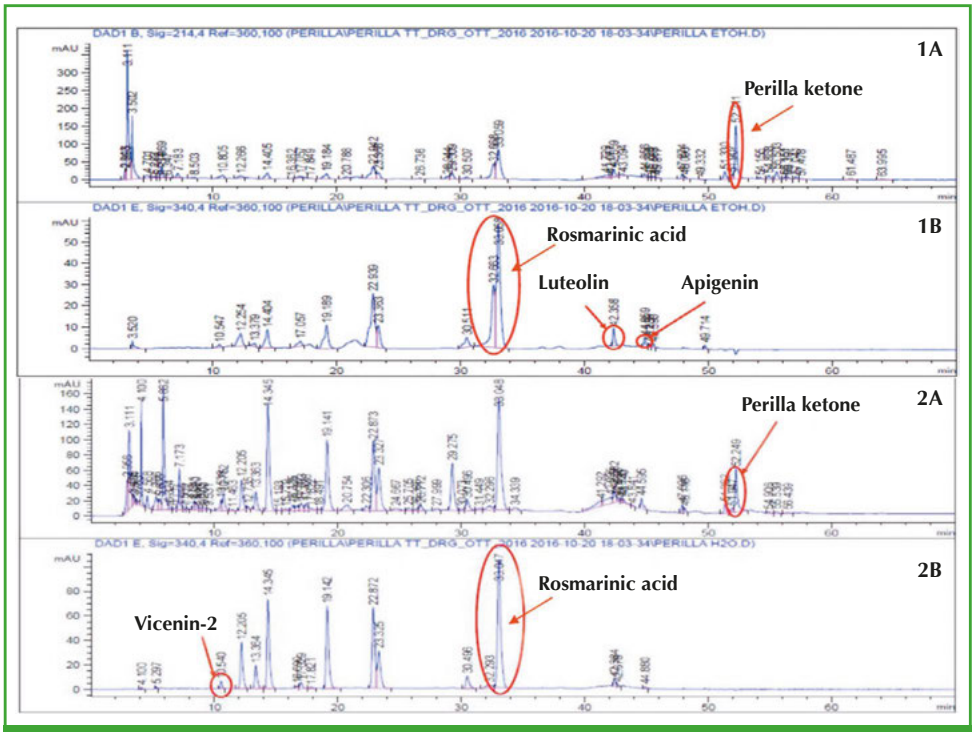

Figure 3 - HPLC-DAD chromatograms of raw material Y extracted using ethanol (80%) and evaluated at 214 nm (**1A**) and 340 nm (**1B**), and extracted using water and evaluated at 214 nm (**2A**) and 340 nm (**2B**)

Ethanol	mg/ml	%	Water	mg/ml	%	Ethanol	mg/ml	%	Water	mg/ml	%
Material X						*Material Y*					
RA	0.1953	0.892	RA	0.1984	0.890	RA	0.0609	0.610	RA	0.0648	0.650
Ap	0.0025	0.011	Ap	0.0000	0.000	Ap	0.0010	0.010	Ap	0.0000	0.000
Lu	0.0057	0.026	Lu	0.0000	0.000	Lu	0.0026	0.026	Lu	0.0018	0.018
V-2	0.0000	0.000	V-2	0.0082	0.035	V-2	0.0000	0.000	V-2	0.0036	0.036
P-k	0.0838	0.386	P-k	0.0396	0.137	P-k	0.0346	0.345	P-k	0.0141	0.143

Table 2 - Concentration (%) of rosmarinic acid (RA), apigenin (Ap), luteolin (Lu), vicenin-2 (V-2) and perilla ketone (P-k) in the ethanol and water extracts of leaves of material X (left) and material Y (right)

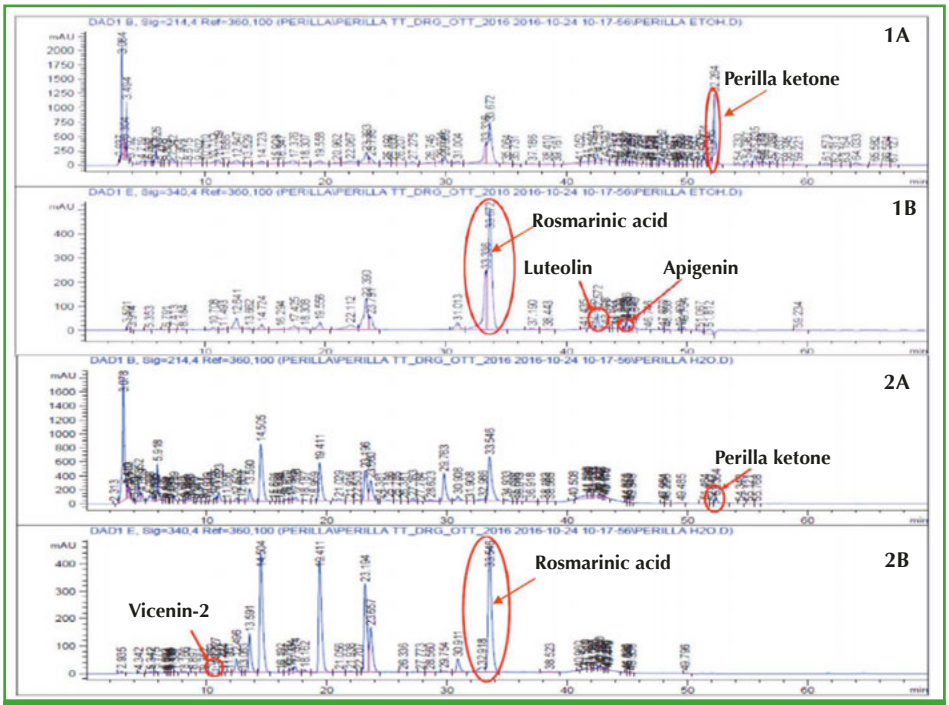

Figure 4 - HPLC-DAD chromatograms of raw material X extracted using ethanol (80%) and evaluated at 214 nm (**1A**) and 340 nm (**1B**), and then extracted using water and evaluated at 214 nm (**2A**) and 340 nm (**2B**)

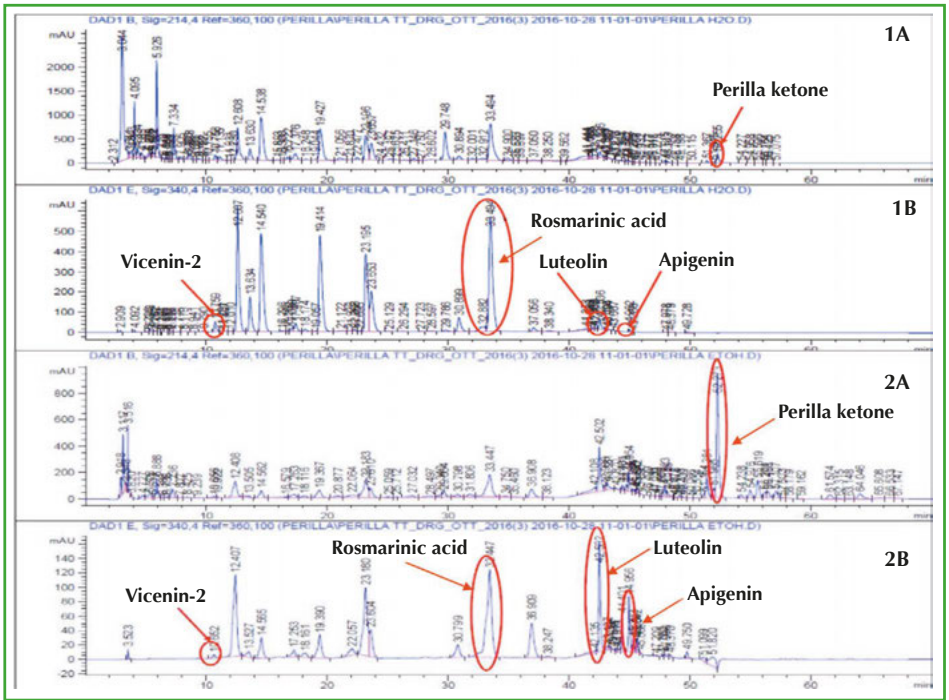

Figure 5 - HPLC-DAD chromatograms of raw material X extracted using water and evaluated at 214 nm (**1A**) and 340 nm (**1B**), and then extracted using ethanol (80%) and evaluated at 214 nm (**2A**) and 340 nm (**2B**)

teolin in the ethanol extract, while their glycoside derivatives show a higher concentration in the water extracts. Consequently, we decided to titre the glycoside derivatives in the water extract and expressed them as aglycones. The best concentrations of vicenin-2 and rosmarinic acid are obtained when extraction starts with water.

According to these results, we established that the best method is to first to extract the raw material using ethanol (80%) and then to extract the residue using water in a dynamic extractor at 60°C. After extraction, the two phases, which are still fluids due to their water or ethanol content, are centrifugated and concentrated under vacuum at a temperature below 45°C in a thin layer evaporator. The concentration process transforms the fluid extracts into soft extracts and increases the values of all actives by about 50%. The soft extracts are next pasteurized and dried in a fluid bed granulator using maltodextrins as support and then combined to form a unique product (Fig. 6). The active content of the dry extract is shown in Table 4. These values are similar to those of the soft extracts be-

	mg/ml	%	rel %		mg/ml	%	rel %
Ethanol				***(then) Water***			
RA	0.6309	0.808	65.910	RA	0.3807	0.418	34.090
Ap	0.0051	0.007	100.00	Ap	0.0000	0.000	0.0000
Ap-D	0.0133	0.017	9.369	Ap-D	0.1504	0.165	90.631
Lu	0.0179	0.023	100.00	Lu	0.0000	0.000	0.0000
Lu-D	0.0301	0.039	11.233	Lu-D	0.2776	0.305	88.767
V-2	0.0117	0.015	44.383	V-2	0.0171	0.019	55.617
P-k	0.2244	0.287	94.087	P-k	0.0164	0.018	5.913
Water				***(then) Ethanol***			
RA	0.4798	0.528	74.625	RA	0.1398	0.179	25.375
Ap	0.0020	0.002	12.217	Ap	0.0124	0.016	87.738
Ap-D	0.1874	0.206	95.345	Ap-D	0.0078	0.010	5.655
Lu	0.0150	0.016	24.775	Lu	0.0389	0.050	75.225
Lu-D	0.3355	0.369	90.097	Lu-D	0.0316	0.041	9.903
V-2	0.0337	0.037	86.314	V-2	0.0046	0.006	13.686
P-k	0.0363	0.040	15.257	P-k	0.1729	0.222	84.743

Table 3 - Relative concentration (rel %) of rosmarinic acid (RA), apigenin (Ap), apigenin derivatives (Ap-D), luteolin (Lu), luteolin derivatives (Lu-D), vicenin-2 (V-2) and perilla ketone (P-k) in the ethanol and then water extract (top) and in the water and then ethanol extract (bottom)

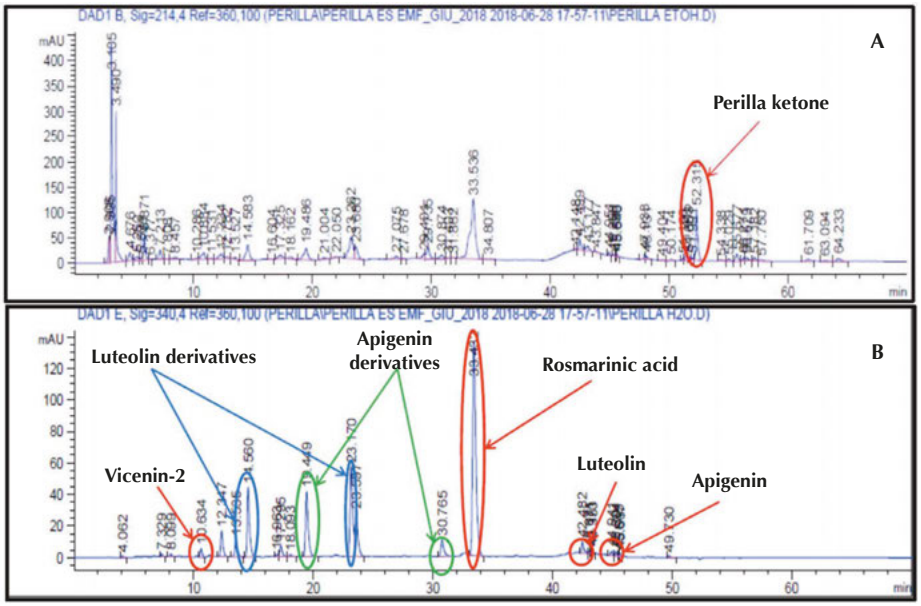

Figure 6 - HPLC chromatograms of the dry multi-fractionated extract of *Perilla frutescens* leaf evaluated at 214 nm (**A**) and 340 nm (**B**)

Active	Concentration (g/kg)
Rosmarinic acid	1.338 ± 0.125
Apigenin	0.002 ± 0.005
Luteolin	0.002 ± 0.005
Vicenin-2	0.058 ± 0.010
Perilla ketone	0.510 ± 0.135

Table 4 - Final concentrations in the dry multi-fractioned *Perilla frutescens* leaf extract

cause the water (about 40%) is replaced by the same volume of maltodextrins. Soft extracts are difficult to handle and formulate, so it is necessary to turn them into dry extracts for producing nutraceuticals; however, the drying temperature must be kept below 45°C to preserve all actives in the correct proportions.

In conclusion, the results of our study demonstrate that this two-step process guarantees a relatively high concentration of each active in the dried granulated product. In our opinion, this double-fractionated *P. frutescens* leaf extract is a new botanical endowed with prokinetic (gastric and intestinal), anti-spasmodic and anti-inflammatory properties which should be clinically tested in subjects with FD.

Conflict of interest

Giuia Nannoni, Alessandro Mattarocci, Giulia Volterrani and Alessandro Alì work for Labiotrè. Francesco Di Pierro declares no conflict of interest. As he is current Editor-in-Chief of *Nutrafoods*, Francesco Di Pierro has appointed Dr. Alexander Bertuccioli as Editor-in-Chief responsible for reviewing this paper.

Human and animal rights

This paper does not contain any studies performed on animals or on humans.

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