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Stability of Rosmarinic Acid in Aqueous Extracts from Different Lamiaceae Species after *in vitro* Digestion with Human Gastrointestinal Enzymes

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Summary

The present study compares the gastrointestinal stability of rosmarinic acid in aqueous extracts of thyme, winter savory and lemon balm with the stability of pure rosmarinic acid. The stability of rosmarinic acid was detected after two-phase *in vitro* digestion process (gastric and duodenal) with human gastrointestinal enzymes. The concentration of rosmarinic acid in undigested and digested samples was detected using HPLC-DAD. Results showed that gastrointestinal stability of pure rosmarinic acid was significantly higher than that of rosmarinic acid from plant extracts after both gastric and intestinal phases of digestion. Among plant extracts, rosmarinic acid was the most stable in lemon balm after gastric (14.10 %) and intestinal digestion phases (6.5 %). The temperature (37 °C) and slightly alkaline medium (pH=7.5) did not affect the stability of rosmarinic acid, while acid medium (pH=2.5) significantly decreased its stability (≥50 %). In addition, the stability rate of rosmarinic acid is influenced by the concentration of human gastrointestinal juices.

Key words: rosmarinic acid, *in vitro* digestion, stability, human gastrointestinal enzymes, Lamiaceae species

Introduction

Research on biologically active compounds, such as plant phenolics, represents a big scientific challenge due to a great number of identified substances, their chemical diversity as well as their huge biological potential, important for the maintenance of healthy balance in our body. Undoubtedly, rosmarinic acid is one of the most studied phenolic compounds, exhibiting different biological activities: antioxidant, anti-inflammatory, antimutagenic, antibacterial, antiviral, cytotoxic on human breast cancer cells, neuroprotective in human neurons, and nephroprotective (*1–8*).

In plants, rosmarinic acid is formed from amino acids phenylalanine (caffeic acid part of rosmarinic acid) and tyrosine (3,4-dihydroxyphenyllactic acid part) (*9*). It was isolated for the first time from *Rosmarinus officinalis* and its structure was characterized as an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (*10,11*). According to some authors (*12,13*), rosmarinic acid cannot be used as chemotaxonomic marker for differentiation of plant families due to its occurrence in various plant families such as Lamiaceae, Boraginaceae, Blechnaceae and Asteraceae. However, Lamiaceae species such as *Satureja montana* L., *Thymus vulgaris* L. and *Origanum majorana* L. are known as rich sources of rosmarinic acid (*14–16*).

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The majority of published studies on rosmarinic acid are focused on methods of its detection in various plant extracts (*17,18*), its seasonal variations in selected plants (*19*) as well as its specific biological activity in different models (cell cultures, rats, *in vitro* methods) (*5,8,20,21*). Therefore, there is a lack of studies on the stability of rosmarinic acid under gastrointestinal conditions using human gastrointestinal enzymes and on the influence of plant matrix on its stability rate. In that sense, *in vitro* models that mimic human physiology are recognized as simple, inexpensive and reproducible tools to study digestive stability of compounds (*22*). In addition, *in vitro* digestion methods are generally based on the use of commercial digestive enzymes, while studies with human gastrointestinal enzymes are still very rare.

Thus, the aim of this study is to determine the influence of digestion phase (gastric and duodenal), temperature and pH on the stability of rosmarinic acid. In this study human gastrointestinal juices (gastric and duodenal) collected from healthy donors are used instead of commercial gastrointestinal enzymes. The stability rate of rosmarinic acid is measured by HPLC-DAD technique.

Materials and Methods

Plant material

Plant materials used in this study were dry leaves of *Thymus vulgaris* L. (thyme), *Satureja montana* L. (winter savory), and *Origanum majorana* L. (lemon balm). The above-mentioned samples are commercially available, in form of tea-like preparations, and were purchased from a local herbal pharmacy (Suban d.o.o., Strmec Samoborski, Croatia). Pretreatment of the plant material included its homogenization using high speed grinder for 1 min.

Preparation of aqueous extracts

Pulverized plant materials (2.5 g) were extracted with distilled water (25 mL). To obtain better extraction yield, instead of conventional procedure, ultrasonic bath-assisted extraction was used. Flasks with suspensions were immersed in ultrasonic water bath heated to 50 °C. The extraction time was 1 h. After cooling, the samples were filtered and centrifuged for 5 min at 1800×*g*. The extraction of each sample was performed in triplicate. The obtained extracts of the same plant species were combined into the final extract that was used in further experiments.

Collection of human digestive juices

Human gastric and duodenal juices were collected from four donors (two male and two female) without known gastrointestinal pathology, and who were not taking acid secretion inhibitors or antibiotics. Gastric and duodenal juices were aspirated through the endoscope. Eight hours before the procedure, all liquid or food intake was ceased. For each patient, 3 mL of initially aspired juice were discarded and the remaining amount was collected in a sterile tube, which was centrifuged to remove mucus and cell debris. In order to reduce interindividual variations, batches of pooled gastric and intestinal juices were prepared and then stored at –20 °C until use. The approval for the collection of digestive juices was obtained from the Ethics Committee of the University Hospital Centre Split, Croatia.

Determination of enzymatic activity of collected juices

The procedure described by Almaas *et al.* (*23*) was used to determine enzymatic activity of the prepared pooled human gastric juice samples. Pepsin activity was measured using 2.5 % solution of bovine haemoglobin. The solution was prepared in 0.2 M phosphate buffer (pH=7.6) and then acidified (to pH=3) using H_2SO_4. In order to determine the human duodenal juice activity, casein solution (1 %) dissolved in 0.2 M phosphate buffer (pH=7.6) was used. A volume of 500 μL of prepared protein solutions was incubated with 5, 20 or 50 μL of gastrointestinal juice. The digestion reactions were stopped with the addition of 1 mL of 10 % trichloroacetic acid (TCA). Samples were measured spectrophotometrically at 280 nm. One unit (U) of enzyme activity is defined as the amount of enzyme that causes the absorbance change of 1 between the blank and the sample, after 20 min at 37 °C.

In vitro digestion process

A two-phase digestion procedure was performed according to the method described by Furlund *et al*. (*24*). Gastric and intestinal digestion phases were performed at 37 °C, in shaking bath (180 rpm). The volume of digestive juice corresponding to 1 U of enzymatic activity was 20 μL of human gastric juice and 25 μL of human duodenal juice. Before *in vitro* digestion procedure, the concentration of rosmarinic acid in the prepared aqueous extracts from selected plants was normalized (471.48 mg/L). For digestion, 4 mL of aqueous extracts were used. The pH of the samples was adjusted to pH=2.5 using 1 M HCl for gastric phase, and to pH=7.5 using 2 M NaOH for intestinal phase. The concentration of human juices used for this assay was 20 U per g of plant material for gastric and 62.4 U per g of plant material for intestinal phase. In order to determine the influence of different concentrations of juices on the stability of rosmarinic acid, following concentrations were used: 5, 10, and 20 U per g of plant material for gastric phase, and 15.6, 20, and 62.4 U per g of plant material for intestinal phase. The incubation period of gastric phase was 30 min, while aliquots of intestinal samples were collected after 60 and 120 min of intestinal phase. Enzymatic reactions were stopped on ice and the samples were stored at –20 °C until analyses. All digestion processes were run in duplicate. Stability rate of rosmarinic acid represents the ratio of its concentration before *in vitro* digestion and after gastric or intestinal digestion phases.

HPLC-DAD detection of rosmarinic acid

The rosmarinic acid was analyzed by a direct injection of the extracts, previously filtered through a 0.45-μm pore size membrane filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Chromatographic separation was performed using HPLC instrument with Agilent 1260 quaternary LC Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with diode array detector

(DAD), an automatic injector and ChemStation software. The separation of compounds was performed on a Nucleosil 100-5C18 (250 mm×4.6 mm, i.d. 5 μm) column (Macherey-Nagel GmbH & Co. KG). The solvent composition and the used gradient conditions were described previously by Generalić Mekinić *et al*. (*13*). For gradient elution, mobile phase A contained 2 % of acetic acid (T.T.T., Sveta Nedelja, Croatia) in water, while solution B contained 2 % of acetic acid in acetonitrile (BDH Prolabo, VWR International, Lutterworth, UK). The used elution program was as follows: from 0 to 3 min 2 % B, from 3 to 25 min 30 % B, from 25 to 35 min 80 % B, and finally for the last 5 min again 0 % B. The flow rate was 1.0 mL/min and the injection volume was 24 μL. Detection was performed with UV/Vis–photo diode array detector (Agilent Technologies) by scanning from 250 to 300 nm. Identification of rosmarinic was carried out by comparing retention times and spectral data with those of the authentic standards at 280 nm. The quantifications of rosmarinic acid were made by the external standard method. Working rosmarinic acid standard solutions were prepared by diluting the stock solution to yield five concentrations in a range from 175 to 700 mg/L. Quantitative determination was carried out using the calibration curves of the standard ($y=31.834x$, $R^2=0.99$). Quantitative determination was based on peak area from HPLC analyses and from the mass concentration of the compound. The results were expressed in mg per mL of extract, as mean value±standard deviation (N=2 replicates).

Statistical analysis

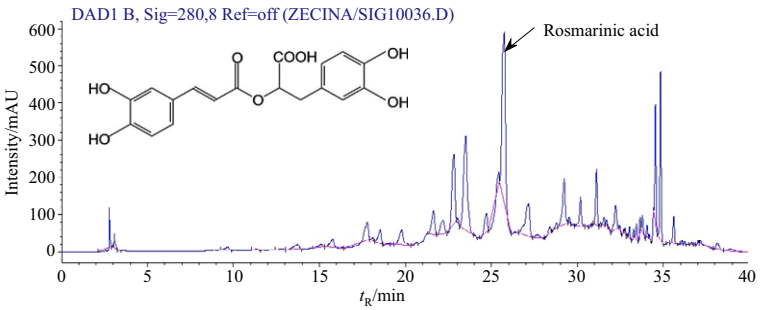
Statistical analysis was performed using GraphPad InStat3 software (GraphPad Software Inc., San Diego, CA, USA). The relationship between the obtained parameters was described using Pearson's correlation coefficient r. Differences at $p<0.05$ were considered to be statistically significant.

Results and Discussion

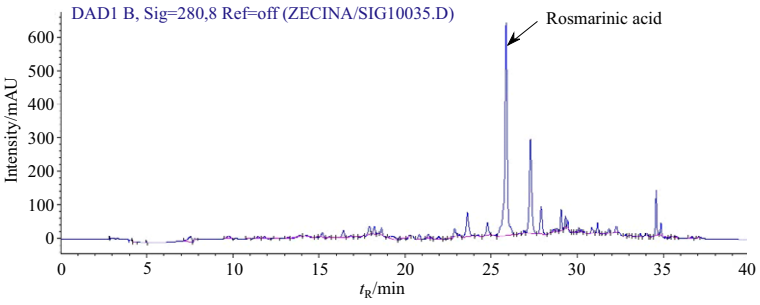
Lamiaceae species are known as a rich source of rosmarinic acid (*14–16*). In this study, three different plants from the Lamiaceae family were used: *Thymus vulgaris* L., *Origanum majorana* L. and *Satureja montana* L. Prepared aqueous extracts contained different concentrations of rosmarinic acid, measured by HPLC-DAD (Fig. 1). Therefore, before the two-step *in vitro* digestion, the concentration of rosmarinic acid was normalized in all prepared aqueous extracts so that the concentration measured in thyme (471.48 mg/L) was taken as primary concentration before *in vitro* digestion. Table 1 shows the concentration and the stability rate of rosmarinic acid in plant extracts in comparison with pure rosmarinic acid after the gastric digestion phase (pH=2.5). The duration of gastric phase was 30 min because liquids with a low protein concentration are considered to have very short transit time in the stomach (*25*). Rosmarinic acid was the most stable in *Origanum majorana* L. extract (14.10 %) in comparison with *Satureja montana* L. extract (5.8 %) and *Thymus vulgaris* L. extract (0.8 %) after digestion phase. However, the stability rate of pure rosmarinic acid was significantly higher (30.77 %) than in rosmarinic acid from plant material. Incubation temperature (37 °C) did not affect the stability of rosmarinic acid, which is in accordance with the results of other authors (*26,27*). On the other hand, the incubation period of 30 min in the acidic medium (pH=2.5) decreased its concentration by more than 50 % (Table 1). The obtained results are not in accordance with those presented by Dinis *et al.* (*28*), who concluded that rosmarinic acid in *Mentha* species was stable at low pH under simulated gastrointestinal conditions using commercial enzymes (pepsin and pancreatin). Contrary to commercial enzymes, human digestive juices consist of a variety of enzymes, inhibitors and bile salts that collectively contribute to the digestion of food sample (*29*). In human gastric juices the following components have been detected: pepsin, trypsin, gastricsin, bile, small peptides and protein fragments (*30*). Duodenal juice contains pancreatic and intestinal enzymes such as proteolytic enzymes, intestinal lipases, enterokinase, trypsinogen, chymotrypsinogen, and amylase (*31*).

Results of the stability determination of rosmarinic acid after intestinal digestion phase are shown in Table 1. Aliquots of digested samples were collected after 60 and 120 min of intestinal phase. Duration time of 120 min for intestinal phase was chosen according to *in vivo* results obtained by Troost *et al*. (*32*). The stability after intestinal digestion phase of pure rosmarinic acid differs completely from the stability of rosmarinic acid in plant extracts (Table 1). In comparison with its primary concentration in plant extracts, rosmarinic acid was almost completely degraded after intestinal digestion phase. After 120 min of intestinal digestion, the highest concentration was detected in *Origanum majorana* L. extract (30.8 mg/L). The obtained results are not in line with the results by Dinis *et al.* (*28*), who reported that pancreatin (commercial mixture of amylase, lipase and protease) did not provoke the degradation of rosmarinic acid in *Mentha* species. Also, Bel-Rhild *et al*. (*33*) did not observe any hydrolysis of rosmarinic acid after the passage of rosemary extract through the gastrointestinal tract model (using commercial digestive enzymes). Putative reasons for such discrepancies could be explained by two factors: the difference in *in vitro* digestion procedure between commercial digestive enzymes and human gastrointestinal enzymes, and the matrix effect – the rosmarinic acid can behave differently in *Mentha* species and/or *Rosmarinus officinalis* extract in comparison with other plant species containing it. In contrast to rosmarinic acid in aqueous plant extracts, pure rosmarinic acid showed very high resistance to digestion by intestinal human juices (its stability rate was approx. 78 % after 120 min of intestinal phase). In addition, according to the results presented in Table 1, the degradation of rosmarinic acid after intestinal phase is not influenced by the pH. At pH=7.5 the rosmarinic acid is almost completely stable. Table 2 shows the influence of different concentrations of human gastric and duodenal juices on the stability of rosmarinic acid in the prepared aqueous extract from *Satureja montana* L. In order to investigate the influence of the concentration of human gastrointestinal juices, three different concentrations were tested: 5, 10 and 20 U per g of plant material of gastric juices, and 15.6, 20 and 62.4 U per g of plant material of duodenal juices. The obtained results showed that rosmarinic acid stability

Thymus vulgaris L.



Satureja montana L.



Origanum majorana L.

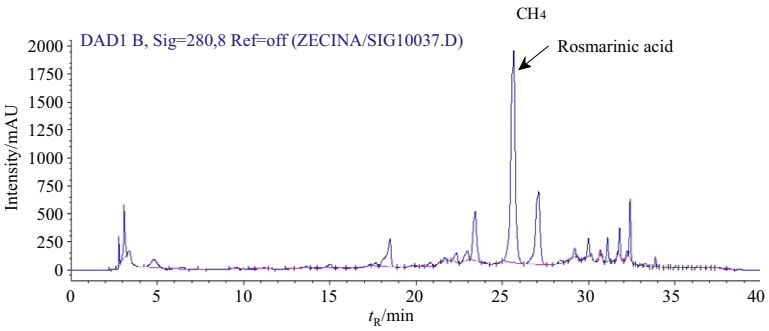


Fig. 1. The investigated Lamiaceae species and their HPLC-DAD chromatograms with the signed peak of the dominant compound (rosmarinic acid) and its structure. The rosmarinic acid concentration in *Satureja montana* L., *Origanum majorana* L. and *Thymus vulgaris* L. extracts was 1579.56, 821.06 and 471.48 mg/L, respectively. Photos taken from Wikimedia Commons database

Table 1. Concentrations and stability rates of rosmarinic acid in aqueous extracts of *Thymus vulgaris* L., *Satureja montana* L. and *Origanum majorana* L. after *in vitro* gastric and intestinal digestion

Sample	Gastric digestion		Intestinal digestion			
	t=30 min		*t*=60 min		*t*=120 min	
	γ/(mg/L)	Stability/%	γ/(mg/L)	Stability/%	γ/(mg/L)	Stability/%
Thymus vulgaris	3.99±0.03	0.8±0.0	20.4±1.8	4.3±0.3	2.32±0.07	0.49±0.01
Satureja montana	27.5±0.0	5.8±0.0	17.7±0.2	3.75±0.03	15.9±0.2	3.81±0.05
Origanum majorana	66.5±0.2	14.10±0.02	29.10±0.09	6.17±0.01	30.8±4.2	6.5±0.6
Rosmarinic acid (control)	145.1±0.4	30.77±0.06	463.3±0.1	98.26±0.03	370.8±0.2	78.64±0.03
Rosmarinic acid (undigested)	(206.6±28.1)*	(43.81±0.03)*	n.d.	n.d.	(430.9±1.3)**	(91.4±0.2)**

*at pH=2.5, **at pH=7.5; n.d.=not detected
Concentration of human gastric juice was 20 U per g of plant material and of human duodendal juice 62.4 U per g of plant material. Results are expressed as mean value±standard deviation. The initial concentration of rosmarinic acid was 471.48 mg/L in all samples (including control)

depends on the concentration of human gastrointestinal juices (the lowest the concentration of digestive juice, the highest the stability of rosmarinic acid; Table 2).

Higher stability rate of rosmarinic acid after *in vitro* intestinal digestive phase may be also related to its ability to react with pancreatic enzymes that inhibit their activity

Table 2. Concentrations and stability rates of rosmarinic acid in aqueous extract of *Satureja montana* L. after *in vitro* gastric and intestinal digestion in relation to different concentrations (U per g of plant material) of human gastric juices (HGJ) and human intestinal duodenal juices (HDJ)

HGJ/(U/g)	*t*=30 min	
	γ/(mg/L)	Stability/%
5	96.9±1.6	20.6±0.2
10	80.1±0.2	16.99±0.03
20	27.5±0.0	5.8±0.0

HDJ/(U/g)	*t*=60 min		*t*=120 min	
	γ/(mg/L)	Stability/%	γ/(mg/L)	Stability/%
15.6	48.3±0.5	10.24±0.07	81.47±0.05	17.3±0.0
20.0	25.8±0.2	5.46±0.03	36.65±0.05	7.61±0.01
62.4	17.7±0.2	3.75±0.03	34.5±0.2	3.81±0.05

The concentration of rosmarinic acid in undigested aqueous extract was 471.48 mg/L. Results are expressed as mean value±standard deviation

(*34*). It was reported that rosmarinic acid-derived quinones react with amino acid side chains and free thiol groups of the enzyme (*35*). In addition, the interaction between phenolic acids and flavonoids has been reported and the presence of some flavonoids such as luteolin and apigenin enhances the stability rate of rosmarinic acid (*36*,*37*). According to the available data, the highest content of luteolin and apigenin among the three selected Lamiaceae plants was detected in *Origanum majorana* aqueous extracts, in which the rosmarinic acid stability was the highest after both, gastric and intestinal phases (*38*).

Conclusions

There is a lack of information about the gastrointestinal stability of rosmarinic acid. Results of this study showed several interesting observations on digestive stability of rosmarinic acid using *in vitro* digestion with human gastrointestinal enzymes. The huge difference was found in the stability of pure rosmarinic acid and rosmarinic acid in aqueous extracts from different plant material belonging to Lamiaceae family. Pure rosmarinic acid was more stable after duodenal phase (78.64 %) than during gastric phase (30.77 %). Acid medium greatly reduced the stability of rosmarinic acid (>50 %), while the effect of the incubation temperature and slightly alkaline medium were not significant. There were differences in the stability of rosmarinic acid among different plant extracts. In our study, rosmarinic acid showed the highest stability after both digestive phases in *Origanum majorana* aqueous extract. In addition, the concentration of human gastrointestinal juices affected the stability of rosmarinic acid in both digestive phases. Finally, the obtained results showed lower gastrointestinal stability of rosmarinic acid than in other studies using commercial digestive enzymes.

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