



Effects of *in vitro* gastrointestinal digestion and colonic fermentation on a rosemary (*Rosmarinus officinalis* L.) extract rich in rosmarinic acid



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ARTICLE INFO

Chemical compounds studied in this article:
 Rosmarinic acid (PubChem CID: 5281792)
 Yunnaneic Acid F (PubChem CID: 10651175)
 Luteolin 7-O-glucuronide (PubChem CID: 40490600)
 Sagerinic acid (PubChem CID: 23760102)

Keywords:

Antioxidant activity
 Antiproliferative potential
 Bactericidal effect
 Phytochemicals stability
 Rosmarinic acid-enriched extracts

ABSTRACT

The potential phytochemical losses occurring throughout the sequential steps of *in-vitro* gastrointestinal digestion and colonic fermentation of a rosemary aqueous extract were investigated. Crude (CE), digested (DE) and fermented (FE) extracts were characterized in terms of their phenolic profile and biological activities. Rosmarinic acid was the phytochemical that underwent the most significant transformation during digestion and fermentation, which amounted to 60% compared to the 26% degradation of the total phenolics. Overall, the simulated digestion step decreased the antioxidant activity estimated by DPPH, ABTS, FRAP, ORAC and TBARS assays. Both CE and DE did not present antiproliferative potential, however, FE exhibited a pronounced cytotoxic activity (GI₅₀ = 116 µg/mL) against HeLa cells. CE and DE showed to be moderate inhibitors of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), *S. aureus*, *Listeria monocytogenes*, whilst the FE acted as a moderate inhibitor of MRSA and MSSA.

1. Introduction

Rosmarinus officinalis L. is a plant belonging to the Lamiaceae family, original from the temperate countries of the Mediterranean region and popularly known as rosemary (Andrade et al., 2018). Beyond its worldwide use as a culinary delicacy due to its pleasant characteristic aroma, this plant is also vastly employed for therapeutic purposes since antiquity (Gonçalves et al., 2018).

Although most research on the rosemary herb has contemplated its biologically active essential oil, in the past decade *R. officinalis* aqueous extracts as well as their corresponding water-soluble isolated compounds have been extensively investigated. In addition to expanding knowledge about known medicinal properties of rosemary extracts, which includes antiproliferative and anti-cancer (Petiwala & Johnson, 2015; Shrestha, Song, Kim, Lee, & Cho, 2016; Tai, Cheung, Wu, & Hasman, 2012), anti-inflammatory (Gonçalves et al., 2018; Lucarini et al., 2013), analgesic (Lucarini et al., 2013), neurodegenerative (Rasoul, Maryam, Taghi, & Taghi, 2016), anti-infective (Andrade et al.,

2018) and antioxidant (Wollinger et al., 2016; Gonçalves et al., 2018) effects, other biological activities have been reported. A rosemary water-soluble extract, for instance, showed a great potential as anti-matrix metalloproteinase-1 agent in human dermal fibroblasts and reconstructed skin, thus figuring a promising ingredient for the prevention of skin photo damage (Martin et al., 2008). Furthermore, the synthesis of silver nanoparticles by using rosemary aqueous extracts was successfully performed with the obtainment of synthesized particles with antibacterial potential against human pathogens (Ghaedi, Yousefinejad, Safarpour, Khafri & Purkait, 2015). Moreover, phenolic diterpenes and phenolic acids (mainly carnosic acid, carnosol and rosmarinic acid) isolated from rosemary extracts have presented, both *in vitro* and *in vivo*, anti-obesity and anti-diabetic actions (Sedighi, Zhao, Yerke, & Sang, 2015). Finally, yet importantly, rosemary water extracts showed anti-hypertensive (Alu'datt, 2017) and hepatoprotective (Ramadan, Khalil, Danial, Alnahdi, & Ayaz, 2013) potentials *in vivo*. This great amount of evidence endorses the use of non-volatile rosemary extracts as functional ingredients and adjuvants in the

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<https://doi.org/10.1016/j.foodchem.2018.07.132>

Received 17 May 2018; Received in revised form 14 July 2018; Accepted 19 July 2018

Available online 19 July 2018

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treatment of several chronic diseases.

Currently, rosemary extracts are being applied as natural additives in food products, improving the shelf life of perishable foods such as ghee (Rahila et al., 2018) and increasing the stability of vegetable oils (Wang et al., 2018). Based on these and similar observations the European Union has approved the *R. officinalis* extract as a secure and efficient natural food preservative, rosmarinic acid being one of its main constituents (EFSA, 2008). Rosmarinic acid-rich extracts not only offer proven health benefits such as anti-inflammatory, antioxidant and hepatoprotective effects (Hasanein & Seifi, 2017; Lin et al., 2017; Villalva et al., 2018), but also inhibit oxidation in food systems without compromising their sensorial acceptance, what endorses the use of those preparations as functional food ingredients (Bakota, Winkler-Moser, Berhow, Eller, & Vaughn, 2015). In spite of all these studies revealing the multiple health benefits of rosemary, scientific evidence regarding the stability and bioavailability of their constituent bioactive compounds remains very scarce (del Pilar Sánchez-Camargo & Herrero, 2017).

The aim of the present work was to investigate the potential phytochemical losses occurring throughout the sequential steps of *in-vitro* gastrointestinal digestion and colonic fermentation of a rosemary aqueous extract, with especial interest in assessing the stability of rosmarinic acid. For this purpose, the crude, digested and colonically fermented rosemary extracts were comparatively characterized in terms of their phenolic composition and also antioxidant, antibacterial and antiproliferative potentials. A rodent model was used for colonic fermentation mainly because most investigations on the biological effects of the rosemary aqueous extract were also done in rodents. The results should allow to correlate possible alterations in the phytochemical profiles of the extracts with their biological activities.

2. Materials and methods

2.1. Rosemary

Dried leaves of *Rosmarinus officinalis* (rosemary) were purchased at a health food store (Maringá, PR, Brazil) specialized in natural and organic products. The producer certifies that no agrochemicals were used in the cultivation of *R. officinalis*. This material constituted the matrix used to obtain three different extracts, according to the process shown in the diagram of Fig. 1.

2.2. Crude extract preparation

The crude extract was obtained according to the procedure previously described (Gonçalves et al., 2018), in which 20 g of grinded rosemary leaves were suspended in 100 mL of distilled water. The suspension was stirred with the aid of a magnetic stirrer for 1 h at room temperature (23 °C). This mixture was centrifuged at 10,000g for 10 min at 4 °C and the supernatant was filtered through a qualitative filter. The filtrate was lyophilized and stored in freezer until use.

2.3. *In vitro* digestion

The *in vitro* gastrointestinal digestion was simulated according to methodology described previously (Correa et al., 2017). Briefly, 13 g of the lyophilized rosemary crude extract was mixed with 39 mL of artificial saliva solution (2.38 g Na₂HPO₄, 0.19 g KH₂PO₄, 8 g NaCl in 1 L of distilled water). The pH was regulated to 6.75, at the temperature of 37 °C and α -amylase (200 U) was added. This blend was shaken at 150 rpm during 10 min. In sequence, the pH was adjusted to 1.2 and 39 mL of artificial gastric fluid (0.32 g pepsin in 100 mL of 0.03 M NaCl, pH 1.2) was included. The mixture was then incubated on a shaker at 37 °C for 120 min, under agitation of 150 rpm. Lastly, the pH was adjusted back to 6.0 following the addition of 6.5 mL of NaCl (120 mM), 6.5 mL of KCl (5 mM) and 39 mL of artificial intestinal fluid (0.15 g of

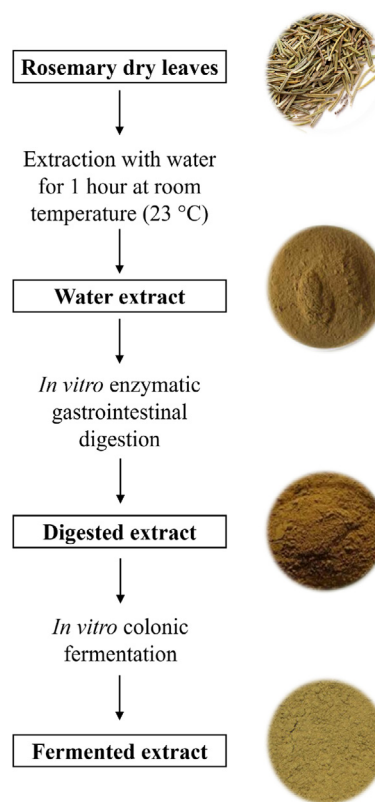


Fig. 1. Diagram of the obtaining of the rosemary extracts, which were submitted to HPLC-DAD-ESI/MS analysis, antioxidant, cytotoxicity and antibacterial assays.

pancreatin and 0.9g of bile extract in 100 mL of 0.1 M NaHCO₃). The mixture was incubated at 37 °C for 60 min, at 150 rpm. Thereon the obtained digested extract was freeze-dried and stored at –20 °C.

2.4. *In vitro* colonic fermentation

The fermentation medium, prepared as previously described by Karpinen, Liukkonen, Aura, Forssell, and Poutanen (2000), was a carbonate-phosphate buffer. The mineral medium was regulated to pH 7.0 and glucose was added to a final concentration of 0.8%. The mixture was purged with nitrogen until the anaerobic indicator (methylene blue) turned colorless. The inoculum was obtained from fresh feces collected from the entire large intestines of male Wistar rats (75-days old animals, average 250 g) immediately after euthanasia by decapitation under deep anesthesia (sodium thiopental 50 mg/kg). Handling of the rats was done in compliance with relevant laws and institutional guidelines for animal experimentation and previously approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol no. 4762290915/2015-CEUA-UEM). A fecal pool of 5 animals was made. Immediately after collecting, the material was homogenized with the culture medium at a ratio of 1:10 (w/v). The bottles were bubbled over again with nitrogen and closed airtight. Afterwards, the bottles were incubated at 37 °C for 24 h under agitation of 50 rpm, in order to simulate the condition in the colonic lumen. The initial pH was 7.0 and the final pH was around 5.0. A control with the culture medium and inoculum was prepared. In order to verify the absence of phenolics in the diet, the control sample was submitted to the Folin-Ciocalteu assay, with negative results. Subsequently, the material was ultra-centrifuged at 31,000 rpm during 30 min, sterilized by filtration (0.42 μ m), and freeze-dried. As phenolic compounds and antioxidant activity were not detected in the control, it was not considered for the antioxidant and bioactive assays.

2.5. Phenolic compounds analysis

The phenolic profile was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), as previously described (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016). The extracts were re-dissolved at a concentration of 10 mg/mL in a methanol:water (80:20, v/v) mixture. Detection was performed using a DAD (280 and 370 nm as preferred wavelengths) and in a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, and working in negative mode. The identification of the phenolic compounds was performed using standard compounds, when available, and by comparing the obtained information with available data reported in the literature. Calibration curves for each available phenolic standard (Extrasynthèse, Genay, France) were constructed based on the UV signal, and the quantification was performed using the most suitable phenolic compound. The results were expressed as mg per g of extract.

2.6. Antioxidant activity evaluation

Five methods were applied to assess the antioxidant activity of the rosemary extracts: (1) reduction power of the ferric ion (FRAP); (2) oxygen radical absorbance radical (ORAC); (3) reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma-Aldrich, St. Louis, MO, USA); (4) reduction of the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate) cation (ABTS, Sigma-Aldrich, St. Louis, MO, USA) and (5) inhibition of the production of thiobarbituric acid reactive substances (TBARS). For evaluating the antioxidant capacity of samples successive dilutions of the stock solution were prepared. Extracts' concentrations (mg of lyophilized extract/mL) providing 50% of antioxidant activity were obtained from the graphs of antioxidant activity versus sample concentrations. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich, St. Louis, MO, USA) was employed as a positive control.

FRAP and ORAC assays were conducted as described in details by Koehnlein et al. (2016). Standard curves were constructed employing trolox ($r^2 = 0.99$), thus the results were expressed as mmol trolox equivalents (TE)/mg lyophilized extract.

DPPH and ABTS activities were evaluated according to methodology of Corrêa et al. (2015). In order to determine the percentage of DPPH and ABTS discoloration, the following equation was applied: $[(A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}}] \times 100$.

Inhibition of the generation of thiobarbituric acid reactive substances (TBARS) was assessed essentially as described by Corrêa et al. (2015), except for the substitution of the lipid source for rat brains (instead of porcine brains). The malondialdehyde-thiobarbituric acid (MDA-TBA) color intensity was measured at 532 nm. Results were calculated as inhibition ratio (%) through the equation $[(A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}}] \times 100$ and were expressed as IC₅₀ values.

2.7. Cytotoxic properties evaluation

The lyophilized samples were dissolved in water at 4 mg/mL and then submitted to further dilutions. Four human tumour cell lines were tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Sulforhodamine B assay (SFB, Sigma-Aldrich, St. Louis, MO, USA) was performed as previously described (Barros et al., 2013). For evaluation of the cytotoxicity in non-tumour cells, a cell culture (assigned as PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure previously described (Abreu et al., 2011). Ellipticine (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control. Results were expressed in GI₅₀ values (concentration that inhibited 50% of the net cell growth).

2.8. Antibacterial activity evaluation

The microorganisms used were clinical isolates divided in seven Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from urine and expectoration) and five Gram-positive bacteria (MRSA-methicillin-resistant *Staphylococcus aureus*, MSSA-methicillin-susceptible *Staphylococcus aureus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*). MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology previously described (Dias et al., 2016). MIC was defined as the lowest extract concentration that prevented this change and exhibited inhibition of bacterial growth.

2.9. Statistical analysis

All results were expressed as mean values and standard deviations (SD), as an outcome of the three repetitions of the samples and concentrations that were used in all the assays. One-way analysis of variance (ANOVA) followed by Tukey's HSD test ($p = 0.05$) were applied to analyse the chemical analytical data. The antioxidant activity results were analysed using one-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test ($p < 0.05$). Analyses were carried out using IBM SPSS Statistics, version 23.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Phenolic compounds of *R. officinalis* preparations

The phenolic compounds profiles of the three different preparations of *R. officinalis* are presented in Table 1 and the quantifications are presented in Table 2. All three preparations showed a very similar profile. A total of sixteen phenolic compounds were identified, such as ten phenolic acids (caffeic and rosmarinic acid derivatives) and six flavonoids (luteolin and quercetin derivatives). The phenolic profile of the aqueous extract was previously described (Gonçalves et al., 2018). As expected, rosmarinic acid was the major component found in all samples, followed by yunnaneic acid F, luteolin-O-glucuronide and sagerinic acid (Table 2).

Andrade et al. (2018), in their recent review on the *R. officinalis* phytochemistry, concluded that the most usual compounds present in rosemary extracts are rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid, carnosic acid and carnosol but also di- and triterpenes besides essential oils. Therefore, the phenolic profiles herein reported are corroborated by literature data. Alu'datt et al. (2017), using reversed phase HPLC, identified a distinct set of phenolic compounds in a water extract of *R. officinalis*. However, these authors also found caffeic acid (more than 73%), rosmarinic acid (almost 7%), luteolin and quercetin in different quantities.

Despite the fact that the optimal conditions for extracting rosemary triterpenoid and phenolic acids remain uncertain, it is known that rosmarinic acid is more soluble in aqueous solutions, normally the process at lower temperature ($< 50^\circ\text{C}$) being recommended in order to minimize both energy consumption and degradation of thermo-labile substances (Bernatoniene et al., 2016). However, in a previous work of our group, Ribeiro et al. (2016) characterized an aqueous extract of rosemary obtained by infusion with boiling water and found expressively greater contents of rosmarinic acid (68.5 mg/g), yunnaneic acid F (10.14 mg/g), lithospermic acid (9.9 mg/g), sagerinic acid (7 mg/g), luteolin (3.6 mg/g) and caffeic acid (1.1 mg/g), than those given in Table 2. Recently, Achour et al. (2018) characterized a hot water extract of *R. officinalis* (Tunisian rosemary tea) finding almost fifty phytochemicals, among which flavonoids, phenolic acids,

Table 1

Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and tentative identification of the phenolic compounds present in the crude extract, *in vitro* digestion preparation extract and colonic fermentation extract of *Rosmarinus officinalis*.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS ² (m/z)	Tentative identification
1	6.8	288	341	179(100),135(2)	Caffeic acid hexoside
2	8.9	318	387	369(26),207(100),163(47)	Caffeic acid acetylhexoside
3	10.4	297/sh323	179	135(100)	Caffeic acid
4	12.2	282/sh337	357	313(35),269(100),203(56),159(62),109(48)	Prolithospermic acid
5	13.9	280	555	493(69),359(18),313(32),295(100),197(23),179(28)	Lithospermic acid A isomer
6	16.2	275	597	359(18),295(18),197(63),179(58),135(98)	Yunnaneic acid F
7	19.01	345	461	285(100)	Luteolin-3-O-glucuronide
8	21.1	276	777	735(33),597(25),579(89),381(25),337(15),311(12),293(15),179(22),135(25)	Caffeic acid derivative (tetramer)
9	22.1	283	719	539(48),521(18),359(100),297(16),179(3),161(5)	Sagerinic acid isomer
10	22.7	326	655	609(53),301(100)	Quercetin-acetylrutinoside
11	22.8	328	359	197(95),179(92),161(100),135(55)	Rosmarinic acid
12	25.2	269/sh340	461	285(100)	Luteolin-O-glucuronide
13	27.4	324	493	359(100), 313(15), 295(65), 269(6)	Salvianolic acid A
14	29.15	330	503	285(100)	Acetyluteolin-O-glucuronide
15	29.6	337	503	285(100)	Acetyluteolin-O-glucuronide
16	31.4	337	503	285(100)	Acetyluteolin-O-glucuronide

terpenes, jasmonate, and lignans were identified. Rosmarinic acid (almost 160 $\mu\text{g/g}$ dried rosemary) was the major extracted constituent, whereas luteolin-7-O-rutinoside was the phytochemical with the lowest concentration in the same extract.

In general, the most pronounced phytochemical losses occurred throughout the simulated digestion step, after which a 26% reduction in the total phenolic (TP) content was observed. Correa, Gonçalves et al. (2017), when investigating the stability of yerba mate phenolic compounds throughout *in vitro* digestion and fermentation, reported reductions of 20–33% after the simulated digestion stage. Rosmarinic acid decreased by almost 61% after digestion, while a milder reduction for the other phenolic compounds was observed. Still, after the colonic fermentation process, our *R. officinalis* extract presented an additional TP content reduction of almost 21%, being that at this stage the rosmarinic acid underwent a new degradation of 13.7%. Sagerinic acid and luteolin were the compounds with the most pronounced alterations in the fermentation step, suffering depletions around 42% and 55%, respectively. Correa, Gonçalves et al. (2017) observed a more drastic decrease in the TP values for yerba mate tea (33%), whereas Corrêa, Haminiuk et al. (2017) reported minimal changes in the total

anthocyanin content of a grape pomace ethanolic extract submitted to *in vitro* fermentation posteriorly to simulated digestion.

Zorić et al. (2016) performed a comparative study on the stability of rosmarinic acid in water extracts of thyme, winter savory and lemon balm (all Lamiaceae species, such as rosemary) and the stability of pure rosmarinic acid after a two-phase *in vitro* digestion process using human gastric and duodenal juices. HPLC-DAD analysis showed that the stability of pure rosmarinic acid was notably superior to that of rosmarinic acid present in vegetal extracts, during both gastric and intestinal digestion phases. According to the authors, the stability of rosmarinic acid was not significantly impacted by temperature (37 °C) and slightly alkaline medium (pH = 7.5), although it presented a significant reduction ($\geq 50\%$) when in acid medium (pH = 2.5). Conclusively, human gastrointestinal juices concentrations directly affect rosmarinic acid stability.

Although some studies indicate that rosmarinic acid is metabolized by the gut microbiota into caffeic acid and derivatives before its absorption, information on the microorganisms and enzymes involved in such biotransformation is limited (Bel-Rhliid et al., 2009; Lafay & Gil-Izquierdo, 2008). Gaps regarding absorption, metabolism and urinary

Table 2

Phenolic compounds quantification (mg/g) in the crude extract, *in vitro* digestion preparation extract and colonic fermentation extract of *Rosmarinus officinalis* (mean \pm SD).

Peak	Crude extract	<i>In vitro</i> digestion	Colonic fermentation
Caffeic acid hexoside ^(A)	0.024 \pm 0.001 ^b	0.0304 \pm 0.0001 ^a	tr
Caffeic acid acetylhexoside ^(A)	tr	tr	tr
Caffeic acid ^(A)	0.054 \pm 0.001 ^a	0.036 \pm 0.001 ^b	0.031 \pm 0.001 ^c
Prolithospermic acid ^(A)	1.43 \pm 0.05 ^a	1.20 \pm 0.02 ^b	1.182 \pm 0.005 ^c
Lithospermic acid A isomer ^(B)	1.50 \pm 0.01 ^a	0.93 \pm 0.02 ^c	0.99 \pm 0.01 ^b
Yunnaneic acid F ^(B)	5.6 \pm 0.2 ^a	5.1 \pm 0.2 ^b	4.49 \pm 0.11 ^c
Luteolin-3-O-glucuronide ^(C)	1.6 \pm 0.1 ^a	1.39 \pm 0.04 ^b	0.99 \pm 0.01 ^c
Caffeic acid derivative (tetramer) ^(A)	0.117 \pm 0.003 ^a	0.059 \pm 0.002 ^b	0.059 \pm 0.001 ^b
Sagerinic acid isomer ^(B)	2.44 \pm 0.02 ^a	2.09 \pm 0.01 ^b	1.22 \pm 0.05 ^c
Quercetin-acetylrutinoside ^(D)	tr	tr	tr
Rosmarinic acid ^(B)	6.9 \pm 0.2 ^a	2.7 \pm 0.1 ^b	2.33 \pm 0.04 ^c
Luteolin-O-glucuronide ^(C)	3.9 \pm 0.1 ^a	3.56 \pm 0.02 ^b	1.62 \pm 0.02 ^c
Salvianolic acid A ^(B)	1.8 \pm 0.1 ^a	1.21 \pm 0.01 ^b	1.2 \pm 0.1 ^b
Acetyluteolin-O-glucuronide ^(C)	1.6 \pm 0.01 ^a	1.44 \pm 0.01 ^b	1.33 \pm 0.01 ^c
Acetyluteolin-O-glucuronide ^(C)	1.6 \pm 0.1 ^a	1.4 \pm 0.1 ^b	1.08 \pm 0.02 ^c
Acetyluteolin-O-glucuronide ^(C)	1.6 \pm 0.1 ^a	1.06 \pm 0.01 ^b	1.061 \pm 0.001 ^b
Total phenolic acids	19.8 \pm 0.6^a	13.3 \pm 0.3^b	11.5 \pm 0.2^c
Total flavonoids	10.4 \pm 0.4^a	8.9 \pm 0.1^b	6.08 \pm 0.05^c
Total phenolic compounds	30 \pm 1^a	22.2 \pm 0.4^b	17.6 \pm 0.1^c

tr – traces; nd – not detected. Standard calibration curves: A – Caffeic acid ($y = 388345x + 406369$, $R^2 = 0.9939$); B – Rosmarinic acid ($y = 191291x - 652903$, $R^2 = 0.999$); C – luteolin-6-C-glucoside ($y = 4087.1x + 72589$, $R^2 = 0.999$); D Quercetin-3-O-rutinoside ($y = 13343x + 76751$, $R^2 = 0.9998$). In each row and for the different extraction procedures, different letters mean significant differences ($p < 0.05$).

excretion of rosmarinic acid in humans still need to be fulfilled, however, the available evidence suggests differences in its metabolism between humans and rats (Baba et al., 2005).

Bel-Rhlid et al. (2009) studied the *in vitro* hydrolysis of rosmarinic acid using diverse esterases and the probiotic microorganism *Lactobacillus johnsonii*. In addition, experiments with the gastrointestinal (GI) tract model (TIM-1), which consists of four connected compartments mimicking the stomach, duodenum, jejunum, and ileum, were also performed. The aim was to verify if this hydrolysis happens under one of these circumstances: (1) chemically due to the environmental conditions (temperature, pH and bile salts) of the TIM-1 model; (2) catalyzed by secreted enzymatic activity, or (3) catalyzed via selected enzymes and microorganisms. No rosmarinic acid hydrolysis was detected under the physiological conditions of the TIM-1 model, neither was the transformation of this compound found to be catalyzed by the secreted lipase and pancreatic enzymes. However, the incorporation of *L. johnsonii* cells into the rosemary extract in the GI model promoted a hydrolysis of the interest compound of almost 100%. Therefore, the results of Bel-Rhlid et al. (2009) endorse the hypothesis that rosmarinic acid is degraded by the gut microbiota prior to absorption and metabolization.

Controversially, when compared to the data obtained by Bel-Rhlid et al. (2009), in our experiments the major rosmarinic acid degradation was observed during the *in vitro* digestion step, although a significant additional decrease occurred in the fermentation process using rat feces. The main cause for the increased loss of rosmarinic acid in our experiments could be the use of a pancreatic extract with a higher content in esterases. Although this seems a plausible explanation for the different observations, this interpretation cannot be regarded as a definitive one in as much as no corresponding increments in the contents of caffeic acid and derivatives were observed after this process. For this equally puzzling observation the cause may also be rather simple, although its confirmation depends on additional experimental activity. One cannot exclude that caffeic acid may also have undergone transformation so that the detected contents correspond actually to the net difference between production and transformation reactions.

3.2. Evaluation of bioactive properties

The *in vitro* antioxidant, antibacterial and antiproliferative properties, besides hepatotoxicity, of the three rosemary preparations were assessed and the results are compiled in Tables 3–5.

3.2.1. Antioxidant activity

Five distinct antioxidant tests were used to evaluate the antioxidant capabilities of the rosemary preparations. The use of more than two methods for assessing antioxidant capacity of plant extracts is important, considering that antioxidant compounds act by distinct mechanisms, each having its specific target within the reaction matrix (Corrêa, Haminiuk, Sora, Bergamasco & Vieira, 2014). Thus,

Table 3

Antioxidant activity of *Rosmarinus officinalis* preparations assessed by five distinct antioxidant methods.

Antioxidant assay	Crude extract	<i>In vitro</i> digestion	Colonic fermentation
DPPH EC ₅₀ (µg/mL)	14.93 ± 0.54a	20.11 ± 0.47b	19.99 ± 0.15b
ABTS EC ₅₀ (µg/mL)	6.46 ± 0.31a	7.89 ± 0.32b	8.02 ± 0.40b
FRAP (mM TE/mg material)	2.46 ± 0.25a	1.69 ± 0.14b	1.76 ± 0.22b
ORAC (mM TE/mg material)	9.06 ± 0.53a	8.19 ± 0.61a	5.54 ± 0.04b
TBARS EC ₅₀ (µg/mL)	260.36 ± 6.72a	369.41 ± 27.26b	554.02 ± 16.42c

The results are presented as mean ± SD. In each line different letters mean significant statistical differences ($p < 0.05$). EC₅₀ values correspond to the sample concentration providing 50% of antioxidant activity.

Table 4

Cytotoxic properties (µg/mL) of the crude extract, *in vitro* digestion preparation extract and colonic fermentation extract of *Rosmarinus officinalis*. (mean ± SD).

	Crude extract	<i>In vitro</i> digestion	Colonic fermentation	Ellipticine
Antiproliferative activity (GI₅₀ µg/mL values)				
MCF-7 (breast carcinoma)	> 400	> 400	> 400	0.91 ± 0.04
NCI-H460 (lung cancer)	> 400	> 400	> 400	1.03 ± 0.09
HeLa (cervical carcinoma)	> 400	> 400	116 ± 10	1.91 ± 0.06
HepG2 (hepatocellular carcinoma)	> 400	> 400	> 400	1.14 ± 0.21
Hepatotoxicity (GI₅₀ µg/mL values)				
PLP2	> 400	> 400	> 400	3.22 ± 0.67

Table 5

Antimicrobial activity (MIC values, mg/mL) of the crude extract, *in vitro* digestion preparation extract and colonic fermentation extract of *Rosmarinus officinalis*.

	Crude extract	<i>In vitro</i> digestion	Colonic fermentation
Gram negative bacteria			
<i>Acinetobacter baumannii</i>	2.5	5	20
<i>Escherichia coli</i> ESBL	5	5	5
<i>Escherichia coli</i>	5	5	5
<i>Klebsiella pneumoniae</i>	20	20	20
<i>Klebsiella pneumoniae</i> ESBL	10	10	10
<i>Morganella morganii</i>	2.5	2.5	2.5
<i>Pseudomonas aeruginosa</i>	20	20	> 20
Gram positive bacteria			
<i>Enterococcus faecalis</i>	5	10	10
<i>Listeria monocytogenes</i>	1.25	0.625	5
MRSA	0.625	1.25	1.25
MSSA	0.625	1.25	1.25
<i>Staphylococcus aureus</i>	1.25	1.25	2.5

MIC values correspond to the minimal sample concentration that inhibited the bacterial growth.

heterogeneous chemical reactivities imply in different degrees of antioxidant activity in the various chemical assays (Correa et al., 2017). To our best knowledge, this is the first report on the study of the antioxidant potential of an aqueous extract of *R. officinalis* using such a set of techniques.

As shown in Table 3, our crude rosemary extract showed promising values of antioxidant capacity in all trials. El-Naggar, Abdel-Farid, Germoush, Elgebaly, and Alm-Eldeen (2016) and Kontogianni et al. (2013) found less expressive results for, respectively, methanolic (IC₅₀ = 55 µg/mL) and ethyl acetate (IC₅₀ = 40.6 µg/mL) extracts of rosemary measured via DPPH assay.

The simulated digestion significantly impacted the antioxidant activity estimated by all methods (except for ORAC), causing reductions of 34.7%, 22.1% and 41.7% in the DPPH, ABTS and TBARS assays, respectively, but an increase of 31.3% in the FRAP assay. Although the colonic fermentation step did not cause significant changes detectable by most methods, it promoted a 32.4% increase in the antioxidant activity assessed by the ORAC method and an additional decrease in the antioxidant capacity measured by the TBARS assay, this time of 33.3%. Correa, Gonçalves et al. (2017), when assessing the antioxidant activity of mate beverages using the same set of techniques, reported that the *in vitro* digestion and fermentation processes caused a decrease in the antioxidant capabilities of their samples, except for the ABTS assay. Corrêa, Haminiuk et al. (2017), on their turn, found that the digestion step did not cause such impacts on a grape pomace hydroalcoholic

extract, although the fermentation step caused quite significant reductions in the antioxidant capacity evaluated by the DPPH scavenging activity and TBARS inhibition.

In order to prevent the degradation of the bioactive components of rosemary extracts (mainly rosmarinic acid) and therefore to ensure their functionality as food ingredients, the use of microencapsulation techniques constitute a very interesting and effective strategy. Ribeiro et al. (2016), in a previous study of our group, showed the feasibility of applying rosemary aqueous extracts to functionalize cottage cheeses. With the aim at preserving extracts' antioxidant activity, the authors microencapsulated a lyophilized rosemary aqueous extract by using an atomization/coagulation technique and calcium alginate as coating material. The process, which had an encapsulation efficiency of almost 100%, did not interfere with the nutritional value of the products. Most important, cheese samples incorporated with microencapsulated extracts presented superior antioxidant properties throughout their shelf life when compared with the control.

3.2.2. Cytotoxic properties

Data regarding the antiproliferative effects of the *R. officinalis* extracts on four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) are displayed in Table 4, expressed as concentrations promoting 50% of cell growth inhibition (GI_{50}). Both the crude and digested rosemary extracts did not present antiproliferative effects against any of the tested tumor cell lines. Interestingly, the fermented rosemary extract exhibited a considerable cytotoxic activity ($GI_{50} = 116 \mu\text{g/mL}$) against one of the cancer cell lines, namely the HeLa (cervical carcinoma). Corrêa, Haminiuk et al. (2017), when analysing grape pomace preparations, reported a similar trend: the crude and digested extracts presented no antiproliferative effects against the same cell lines herein assessed. The corresponding grape pomace fermented extract displayed antiproliferative activity against all tumor cells, with a GI_{50} value of $251 \mu\text{g/mL}$ against HeLa cells. Likewise, Correa, Gonçalves et al. (2017) found that the colonic fermentation process improved the cytotoxicity of a mate tea extract against MCF-7, NCI-H460 and HeLa cell lines.

In the past years, three diterpenes isolated from rosemary extracts, namely carnosic acid, carnosol and rosmanol, have received great attention of the scientific community due to their promising anti-cancer effects, verified both in *in vitro* and *in vivo* studies (Petiwala & Johnson, 2015). Dilas et al. (2012), which assessed the antiproliferative effects of three *R. officinalis* commercial oil-soluble formulations (with carnosic acid as their main active component), found average IC_{50} values of $10.59 \mu\text{g/mL}$ and $12.37 \mu\text{g/mL}$ against HeLa and MCF-7 tumor cell lines, respectively. Tai et al. (2012) showed that a *R. officinalis* hydroethanolic extract inhibited the proliferation of ovarian cancer cell lines and synergistically improved the effects of the chemotherapeutic cisplatin, activity attributed to extract's phenolic components with molecular weight inferior to 1000 Da. In addition, sageone, an abietane-type phenolic diterpene isolated from a rosemary aqueous methanol extract, displayed a significant cytotoxic action against SNU-1 human gastric cancer cells (Shrestha et al., 2016). More recently, Amar et al. (2017) reported that a *R. officinalis* ethyl acetate fraction (rich in phenolic diterpenes carnosol, carnosic acid and methyl carnosate) exerted pronounced antiproliferative action against U937 and CaCo-2 cells, with IC_{50} values of $14.8 \mu\text{g/mL}$ and $14.95 \mu\text{g/mL}$, respectively.

Considering that the rosemary components herein identified underwent significant reductions throughout the digestion and fermentation steps (Table 2), the phytochemicals involved in our rosemary extract's antiproliferative activity against HeLa tumor cells are probably not those shown in Table 1. Thus, other components of our rosemary aqueous preparation, including non-phenolic compounds, may indeed be responsible for its antiproliferative effects. Finally, we consider the hypothesis that the fecal microbiota that transformed the extract during the *in vitro* fermentation process could play a role in its bioactivity.

None of the rosemary preparations presented toxicity against the

liver primary culture PLP2, once their GI_{50} values were all higher than the highest concentration tested ($400 \mu\text{g/mL}$) (Table 5). Actually, El-Naggar et al. (2016) confirmed the effectiveness of a rosemary hydro-methanolic extract against the cyclophosphamide-induced hepatotoxicity in a mice model due to its high antioxidant activity. The herein verified absence of cytotoxicity against liver cells, on its turn in a great *in vitro* model for assessing human cytotoxicity, is a crucial issue considering the potential application of the tested rosemary preparation in food systems (Corrêa et al., 2015).

3.2.3. Antibacterial activity

R. officinalis extracts' minimum inhibitory concentration (MIC) values for seven Gram-negative and five Gram-positive bacteria are presented in Table 5. All the three rosemary preparations were more active against Gram-positive bacteria than against Gram-negative bacteria. The same observation was made by Sacco et al. (2015) when studying the antimicrobial potential of rosemary non-volatile phenolic fractions. Neither the simulated digestion nor the fermentation stage affected the antimicrobial activity of our rosemary extracts against the tested Gram-negative bacteria, except for *A. baumannii*. Our crude extract was more efficient against MRSA and MSSA bacteria, with a significant decrease of 2-fold in its inhibitory activity against Gram-positive and Gram-negative bacteria after the digestion step, although no activity alterations were observed after the fermentation process. Several authors have demonstrated a synergic action of rosemary isolated compounds (especially rosmarinic and carnosic acids) with antibiotics against MRSA clinical isolates, with promising increases in antibacterial efficiency (Ekambaram et al., 2016; Vázquez, Fiorilli, Guido, & Moreno 2016). Our crude extract presented activity against *L. monocytogenes*, which increased by 2-fold after the *in vitro* digestion, and posteriorly reduced by 8-fold after colonic fermentation (Table 5).

Sacco et al. (2015) assessed the antimicrobial effects of three rosemary ethanolic extracts (with distinct phenolic compositions) against *E. coli*, *P. aeruginosa* and *S. aureus* strains using a broth dilution method. According to the authors, all tested extracts were more active against *E. coli* (MBC < 0.07 mg/mL), while less effective against *P. aeruginosa* (MBC values around 0.20 mg/mL), being the extract rich in terpenoid derivatives, such as carnosic acid, which is known to present the best antibacterial activity. Our data revealed a similar tendency, as our MIC values for *E. coli* and *P. aeruginosa* were of 5 mg/mL and 20 mg/mL , respectively. Klančnik, Guzej, Kolar, Abramovič, and Možina (2009), when evaluating the activity of rosemary commercial extracts against *S. aureus* ATCC 25923 (clinical isolate) via microdilution tests, reported MIC values similar to ours ($0.156\text{--}5 \text{ mg/mL}$).

Pursuant to their MIC values, natural extracts can be classified as strong (MIC below 0.5 mg/mL), moderate (MIC between 0.6 and 1.5 mg/mL) or weak (MIC above 1.6 mg/mL) inhibitors (Corrêa et al., 2018). Thereby, our crude and digested rosemary extracts are moderate inhibitors of *L. monocytogenes*, MRSA, MSSA and *S. aureus*, whilst our fermented extract is a moderate inhibitor of MRSA and MSSA. However, the three rosemary preparations are classified as weak inhibitors against all the other bacteria herein tested. Notwithstanding, it is worth to mention that the bacteria strains employed in our antibacterial assay are clinical isolated multiresistant strains, which present an antibiotic profile resistance considerably superior to the ATCC bacterial strains (Dias et al., 2016).

4. Conclusion

In view of the relevant bioactivity exhibited by the *R. officinalis* aqueous extract rich in rosmarinic acid, the outcomes herein reported indicate its potential use as a food additive, either as a preservative and/or as a functional ingredient. However, the use of rosmarinic acid rich preparations as functional food ingredients should take into account the stability and bioavailability of this compound, which showed to undergo an expressive degradation throughout the *in vitro*

gastrointestinal digestion and colonic fermentation processes. In order to avoid phytochemical losses that certainly affect the functionality of rosemary extracts, the use of microencapsulation techniques to ensure the delivery of these bioactive compounds could be performed and investigated in future studies. Finally, further *in vivo* studies (such as dietary intervention), are required with the purpose of unravelling and confirming the bioactivities found in the present study.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

G.A. Gonçalves and V.G. Correa thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support provided for their post-graduate studies in the State University of Maringá. R.C.G. Corrêa thanks Conselho Nacional de Desenvolvimento Científico e Tecnologia (CNPq) for financing her postdoctoral research at State University of Maringá (Process number 167378/2017-1). R.M. Peralta (Project number 307944/2015-8) and A. Bracht (Project number 304090/2016-6) are CNPq research grant recipients. The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2013), L. Barros and R. Calhelha contracts; and also thank to FEDER-Interreg España-Portugal programme for financial support through the project 0377_iberphenol_6_E.



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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.07.132>.

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