Journal of Traditional and Complementary Medicine 9 (2019) 383–392

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Antibacterial and antioxidant effects of *Rosmarinus officinalis* L. extract and its fractions

Guilherme Pires Amaral [a, *], Caren Rigon Mizdal [b], Silvio Terra Stefanello [a], Andreas Sebastian Loureiro Mendez [c], Robson Luiz Puntel [d], Marli Matiko Anraku de Campos [b], Félix Alexandre Antunes Soares [a], Roselei Fachinetto [a]

[a] *Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Campus UFSM., 97105-900, Santa Maria, RS, Brazil*
[b] *Departamento de Análises Clínicas Toxicológicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, CEP 97105-900, Santa Maria, RS, Brazil*
[c] *Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, CEP: 90610-000, Bairro Azenha, Porto Alegre, RS, Brazil*
[d] *UNIPAMPA - Campus Uruguaiana, Universidade Federal do Pampa, BR-472 Km 7, Uruguaiana, 97500-970, RS, Brazil*

ARTICLE INFO

Article history:
Received 23 July 2017
Received in revised form
28 September 2017
Accepted 22 October 2017
Available online 9 October 2018

Keywords:
Microorganisms
Medicine
Food
Plant
Free radicals

ABSTRACT

The production of reactive species over physiological levels associated to pathogenic bacteria could represent a high risk for many diseases. The *Rosmarinus officinalis* L. is used around the world due its pharmacological proprieties. So, in this study our aim is to test for the first time if *R. officinalis* L. extract (eeRo) and its fractions (DCM, EA, ButOH) could have better or similar antioxidant action to standars and among themselves *in vitro* or *ex vivo,* in brain, stomach and liver of rats. Moreover, we intend to clarify their possible effects on pathogenic bacteria. The eeRo was obtained from the dried leaves subjected to an alcoholic extraction and fractioned. The quantification of the constituents of eeRo and fractions were done by HPLC. The antioxidant proprieties of *R. officinalis* was analyzed by $DPPH^{\bullet-}$ radical scavenging, total antioxidant, dichlorofluorescein, lipid peroxidation and sodium nitroprusside -induced lipid peroxidation assays. The Minimum inhibitory concentrations of *R. officinalis* L. were tested with standard strains of danger bacteria. The eeRo, DCM, EA had significant total antioxidant and $DPPH^{\bullet-}$ radical scavenging activities. The DCM and eeRo got significant effects against basal levels of reactive species in liver, stomach and brain. The eeRo and DCM protected the liver and brain against lipid peroxidation. The eeRo, DCM, EA and ButOH had inhibitory effect in the Gram-positive and Gram-negative bacteria. In general way, the DCM and eeRo had the best antioxidant and antibacterial effects among all tested fractions.

1. Introduction

Pathogenic bacteria can release toxins capable of causing lesions in different organs and the oxidative stress is produced as a consequence of these bacterial lesions.[1] Thus, oxidative stress caused by bacterial infections represents a potential health risk to various organs such as the liver[2] and stomach[3] and brain.[4] To synthesize such a medicine in the laboratory would be neither cost- nor time-effective, whereas medicines derived from the constituents of plants benefit from having undergone nature's toughest test, natural selection.[5] Under normal conditions, levels of reactive oxygen spcies (ROS) are controlled by endogenous antioxidante systems.[6] However, elevated production of free radicals over physiological levels, either directly or indirectly by lipid peroxidation, is a serious component of many diseases, including gastric ulcers and carcinogenesis.[7]

The *R. officinalis* L. belongs to the Lamiaceae family. It is popularly known in Europe as rosemary and in Brazil as Alecrim.[8] In many parts of the world, this plant is used for flavoring food, drinks, and cosmetics.[7] Some studies have demonstrated pharmacological

* Corresponding author.
E-mail address: guipiresa@yahoo.com.br (G.P. Amaral).
Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2017.10.006

effects of *R. officinalis* L. against inflammatory processes,[9] hepatotoxicity,[7] atherosclerosis, ischaemic heart disease, respiratory disorders, gastric ulcers, some kinds of cancers.[7] The known pharmacological effects of this plant are attributable to its phenolic components, such as rosmarinic acid, carnosic acid and carnosol.[8] These phenolic compounds have naturally strong antioxidant effects; among them, carnosic acid and carnosol account for 90% of antioxidant activity of *R. officinalis* L.[8]

In this context, the phenolic compounds are the main plant biomolecules that can be used as antioxidant agents in humans or animals. They could play a significant role in the prevention of oxidative stress and a high number of ROS-associated pathologies.[10] The natural plant products can also act against pathogenic bacteria in humans. For example, *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa,* and *Bacillus cereus* are all associated with a high number of human pathologies. The *S. aureus* is a major human pathogen that is capable of causing persistent and skin infections.[11] The *staphylococcus epidermidis* is generally found on human skin and mucosal surfaces they are considered opportunistic microorganisms responsible for cardiovascular pathologies[12] and nosocomial infections. The *P. aeruginosa* is a pathogen that is hospital-acquired. It remains inside mucus and grows under anaerobic conditions and causes bronchiolitis and pneumonia.[13] The *B. cereus* is adapted to growth in the intestinal tract of mammals and can cause emesis, diarrhea, gastroenteritis, and food borne disease.[14] In the present study, the aim is evaluates the ability of fractions of *R. officinalis* L. extract to act as antioxidants and scavenge radicals more effectively than standard compounds and among themselves *in vitro* or *ex vivo* in the brain, stomach, and liver of rats. Additionally, we elucidated the possible action of these compounds on Gram-positive and Gram-negative bacteria.

2. Materials and methods

2.1. Chemical, apparatus and general procedures

All chemical were of analytical grade. Ethanol was obtained from local suppliers with purity 99%. Methanol, acetic acid, chlorogenic acid and caffeic acid purchased from Merck (Darmstadt, Germany). Rosmarinic acid, carnosic acid, quercetin, rutin, kaempferol, ascorbic acid and gallic acid were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Obtaining of ethanolic extract of R. officinalis L. (eeRo)

The eeRo was obtained from the dried leaves (40 °C) of this plant, which were collected in the Botanical garden of Federal University of Santa Maria, Brazil. The leaves were subjected to an alcoholic extraction (100% ethanol, 1.5 h, 60–70 °C) in the Soxhlet apparatus with some modifications in relation to original technique.[15] The voucher specimen was deposited in the herbarium of UFSM under the number of SMDB 15.050. The access to genetic patrimonial was approved by CNPq under the number 010757/2014-7.

2.3. Fractioning of eeRo

O eeRo was solubilized in a solution of ethanol and water 1:1, this solution was added separately to dichloromethane, ethyl acetate and buthanol, at different times of fractionation, in a separator funnel to get DCM, EA and ButOH fractions, respectively according to Kamdem et al., 2012 with some modifications.[10]

2.4. Quantification of constituents of eeRo and its fractions by HPLC–DAD

The chromatographic analyses were conducted using a prominence liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an SLC-10A controller, LC-20AD pump, SIL-10AF auto sampler and SPDM10A PDA detector. LC Solution V. 1.24 SP1 system software was used to control the equipment and to calculate data and responses from the LC system. A reversed phase ODS-Hypersil Thermo Scientific C18 column (250 × 4.6 mm i.d., 5-μm particle size) (Bellefonte, United States) was used. The mobile phase consisted of 2% acetic acid (solvent A) and methanol (solvent B) with a flow rate of 0.8 mL/min; DAD detection at 280 nm; injection volume of 20 and 50 μl for standers and tested compounds, respectively. A gradient elution was performed as follows: 5% of solvent B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% of solvent B at 10, 20, 30, 40, 50 and 60 min, respectively. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.070–0.140 mg/ml for the gallic, chlorogenic, caffeic, rosmarinic, carnosic acids, rutin, quercetin and kaempferol.

2.5. Antioxidant assays without tissue

2.5.1. $DPPH^{\bullet}$ radical scavenging method

The radical scavenging activities of eeRo and its fractions were determined as previously described by Brand-Williams et al., 1995 with modifications.[16] This Kinetic method determine the antiradical activities of antioxidants based in reduction of free radical 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). The reduction of radical $DPPH^{\bullet}$ is observed with the decrease of absorbance at 518 nm at 0 min, 1 min and every 15 min until the reaction reached a plateau. Each fraction and the ascorbic acid were tested at concentrations of 1–300 μg/ml. $DPPH^{\bullet}$ was added to final concentration of 0.3 mM and allowed to react at room temperature for 30 min in dark conditions. The absorbance was measured using Spectra Max Plate Reader® M_2 (Molecular Devices), Sunnyvale, California, USA.

2.5.2. Total antioxidant capacity assay

The total antioxidant potential of eeRo and its fractions were evaluated by the phosphomolybdenum method as previously described.[17] This is a spectrophotometric method used to quantitative determination of antioxidant capacity of plant extracts and vitamins. This assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequente formation of a green phosphate/Mo(V) complex at acidic pH. The compounds were tested at concentration of 1–300 μg/ml with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate, 3 ml) in a water bath at 95 °C for 90 min. After cooling the mixture to room temperature, the absorbance was measured at 695 nm against a blank control.

2.6. Animals

Male adult Wistar rats (250–300 g), from our own breeding colony were used. The animals were maintained on a 12 h light: 12 h dark cycle, at a room temperature of 22 ± 2 °C with free access to food and water. The animals were treated according to the standard guidelines of the Committee on the Ethics of Animal Experiments of the Federal University of Santa Maria, Brazil. The protocol was approved by the same Committee (044/2012).

2.7. Tissue preparation

The rats were sacrificed by decapitation, and the liver, brain and

stomach were quickly removed, placed on ice, and homogenized in 10 volumes of cold tris buffer (10 mM, pH 7.4). The homogenates were centrifuged at 4000 g at 4 °C for 10 min to yield a low-speed supernatant fraction (S1) for each tissue that was used for SNP-induced lipid peroxidation and H2DCF-DA assays. The liver and brain samples were prepared in a Potter manual homogenizer containing a straight glass rod and a 10 ml glass tube of local provider. The stomach samples were prepared in T10 Basic Ultra Turrax homogenizer 230V, made in China by Ika. The tubes containing the liver, brain and stomach samples were immersed in ice during processing and the temperature was maintained at around 4 °C.

2.8. Antioxidant assays with tissue homogenates

2.8.1. Sodium nitroprusside (SNP)-induced lipid peroxidation assay

The antioxidant effect of the eeRo and its fractions (1–300 μg/ml) were evaluated against SNP (10 μM)-induced thiobarbituric acid reactive substances (TBARS). The S_1 was pre-incubated for 1 h at 37 °C in a buffered medium with the compounds in the presence or absence of SNP. Lipid peroxidation formation was determined spectrophotometrically at 532 nm, using malondialdehyde (MDA) as a standard, according to Ohkawa et al, 1979.[18]

2.8.2. H_2DCF-DA assay

2′-7′-Dichlorofluorescein (DCF) levels was used to evaluate the basal cellular formation of reactive oxygen species (ROS).[19] Aliquots (20 μl) of S_1 supernatants in tris–HCl buffer (10 mM; pH 7.4) were incubated with gallic acid or eeRo and its fractions (1–300 μg/ml) and 2′-7′-dichlorofluorescein diacetate DCFH-DA (1 mM). After DCFH-DA addition, the medium was maintained in the dark for 60 min until fluorescence was measured (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and the results were analyzed as a percentage value in relation to the control group.

2.9. Protein quantification

Protein concentration was estimated by the Bradford method using with bovine serum albumin as standard.[20] **This method describes a protein determination which involves the binding of Coomassie Brilliant Blue to protein causing a shift in the maximum absorption from 465 to 595 nm which is measured. This process is completed around 2 min, it is stable for 1 h and there are litlle or no interference from cations and carbohydrates. Aliquots (10** μl) **diluted 1:100 from** S1 supernatants in tris–HCl buffer (10 mM; pH 7.4) **or** bovine serum albumin **(10, 20, 30, 40** μl **from 1 mg/ml solution) were added to final volume 1 ml of Coomassie Brilliant Blue. This solution was** determined spectrophotometrically at **595 nm.** The results were expressed as protein mg/ml.

2.10. Assays with bacteria

2.10.1. Microorganisms

We used the standard strain of *Staphylococcus aureus* (ATCC®25923TM), *Staphylococcus epidermidis* (ATCC®12228TM), *Pseudomonas aeruginosa* (ATCC®27853TM) and *Bacillus cereus* (ATCC®14579TM). For the experiments the inoculum was standardized according to the McFarland 0.5 scale by optical density on spectrophotometer (0.08 e 0.1 of absorbance at 625 nm).

2.10.2. Minimum inhibitory concentration (MIC)

To verify whether the eeRo and its fractions have influence on bacteria growth, we assessed the MIC. Bacteria was seeded on plates with Mueller Hinton agar and allowed to grow for 24 h at 37 °C. A total of 50 μL of the standardized microorganism suspension diluted 1:1000 in Mueller Hinton broth was placed in each test well of a 96-well microtiter plate, along with an equal volume of eeRo and its fractions to be tested at different concentrations (8, 16, 32, 64, 128, 256, and 512 μg/mL). The plates were incubated for 24 h at 37 °C. The MIC was considered as the lowest concentration of the test product able to inhibit the growth of microorganisms evidenced by the use of 2, 3, 5 triphenyltetrazolium chloride 1%.[21]

2.11. Statistical analysis

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OSX, GraphPad Software, San Diego, CA). Significance was assessed by one-way ANOVA analysis of variance, followed by Newman–Keuls test for post hoc comparison for all assays. Values of $p < 0.05$ were considered statistically significant. Results are expressed as mean of three to six determinations.

3. Results

3.1. HPLC analysis

HPLC fingerprinting of a crude extract of *R. officinalis* L. and its fractions revealed presence of gallic acid (tR = 6 min) in eeRo (0.0044 mg/g), EA (tR = 6 min; 0.41 mg/g); chlorogenic acid (tR = 15 min) in eeRo (0.93 mg/g), ButOH (5.35 mg/g); caffeic acid (tR = 16 min) in eeRo (0.28 mg/g), DCM (0.01 mg/g), EA (1,75 mg/g); rosmarinic acid (tR = 20.5 min) in eeRo (12.381 mg/g), DCM (267 mg/g), EA (115 mg/g), ButOH (7.47); quercetin (tR = 26 min), in eeRo (7.9 mg/g), EA (27.7 mg/g); carnosic acid (tR = 65 min) in eeRo (89.83 mg/g), DCM (23.5 mg/g), EA (9.6 mg/g), ButOH (5.07 mg/g) (Table 1).

3.2. Total antioxidant capacity (TAC) assay

The ascorbic acid at 30 μg/ml was used as the control (Fig. 1). The eeRo, EA, and DCM at 100 μg/ml and the ButOH at 300 μg/ml had similar TAC to control. Moreover, the eeRo, EA, and DCM at 300 μg/ml had TAC significantly higher than control (Fig. 1). In this way, the eeRo at 1–300 μg/ml was not significantly different from the DCM and EA. The DCM, at all concentrations tested, was not significantly different from the EA. On the other hand, the eeRo, DCM, and EA at 100 and 300 μg/ml had better antioxidant activity than ButOH at the same concentrations (Fig. 1).

3.3. DPPH$^{\bullet}$ radical scavenging assay

Additionally, the eeRo at 10–100 μg/ml exerted a greater

Table 1
The quantification of phenolics and flavonoids composition of ethanolic extract of Rosmarinus officinalis L. (eeRo) and its fractions: DCM (from dichloromethane), EA (from ethyl acetate), ButOH (from buthanol). Data are reported as means of three determinations.

Compounds	eeRo mg/g	DCM mg/g	EA mg/g	ButOH mg/g
gallic acid	0.0044	–	0.41	–
chlorogenic acid	0.93	–	–	5.35
caffeic acid	0.28	0.01	1.75	–
rutin	–	–	–	–
rosmarinic acid	12.381	267	115	7.47
quercetin	7.9	–	27.7	–
kaempferol	–	–	–	–
carnosic acid	89.83	23.5	9.6	5.07

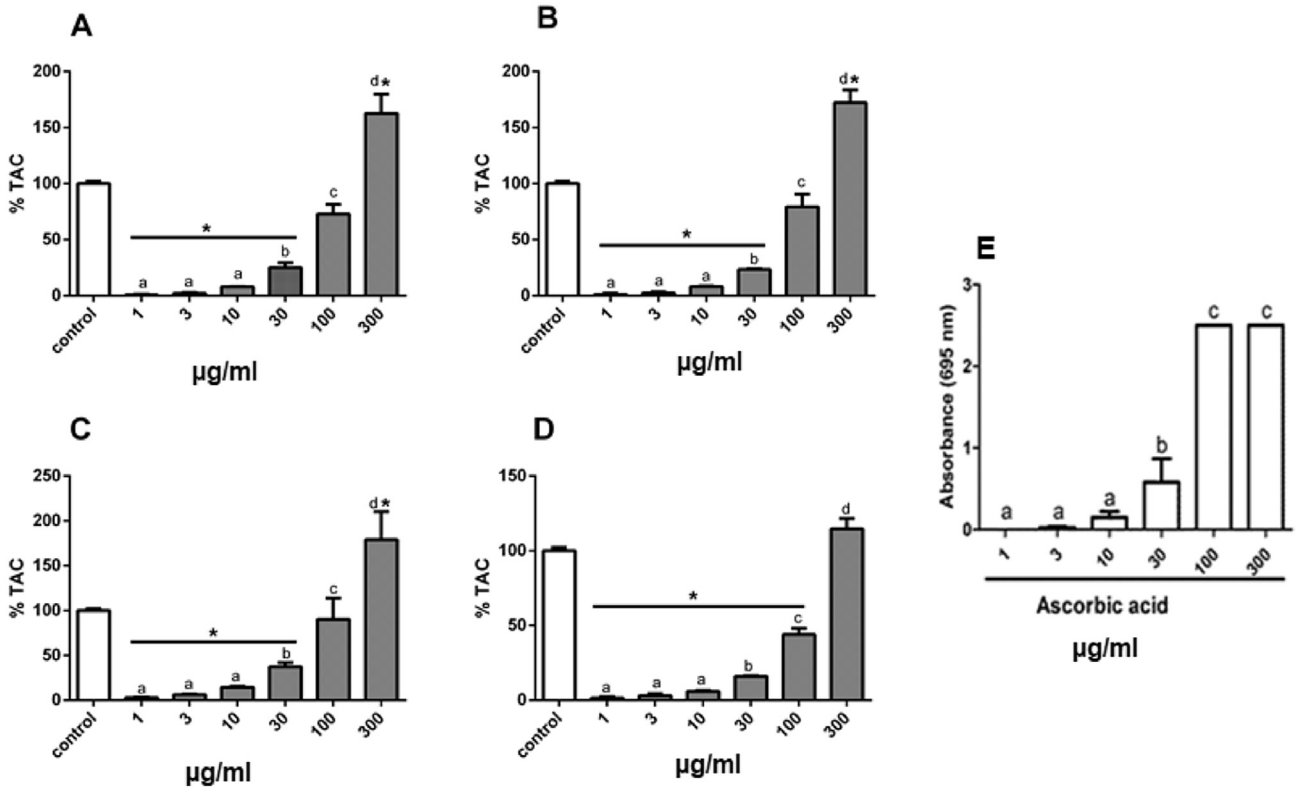

Fig. 1. Assay of **total antioxidant capacity assay (TAC)** of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), But OH (from buthanol) (D). Ascorbic acid (E). The ascorbic acid was used as control at 30 µg/ml. Data are reported as means ± SEM of three to six determinations. One-way ANOVA, followed by Newman–Keuls test posttest, respectively. *$p < 0.05$, significant difference when compared to control. $^{a,b,c,d}p < 0.05$ significant difference among groups with different letters.

radical-scavenging effect than EA and ButOH, respectively. The DCM at 10 µg/ml had a greater radical-scavenging effect compared to EA. The DCM and EA at 10–100 µg/ml had a greater protective effect than the ButOH (Fig. 2). However, the ButOH, eeRo and DCM, and EA, respectively at 3–100, 3–30, and 3–10 µg/ml had worst radical scavenging action than ascorbic acid (Fig. 2).

3.4. The H_2DCF-DA assay

In liver, the eeRo at 300 µg/ml decreased basal levels of ROS (Fig. 3-A) similarly to gallic acid (Fig. 3E). The eeRo and DCM at 300 µg/ml exerted a greater protective effect in liver than gallic acid at the same concentrations tested. The DCM and EA at 100–300 µg/ml decreased basal levels of ROS in relation to the control. Moreover, the DCM at 300 µg/ml had greater effects than it did at 100 µg/ml. On the other hand, the ButOH was not significantly different to control. The eeRo was not significantly different from the DCM and EA. The DCM had not significantly different effect to EA (Fig. 3).

In stomach, the eeRo at 300 µg/ml reduced ROS levels when compared to control (Fig. 4-A). Similar to the antioxidant standard (Fig. 4E), the DCM at 100–300 µg/ml reduced ROS levels in comparison to control. However, the EA and ButOH had no significant differency in relation to control. There was no difference among gallic acid, eeRo, and the other fractions. The comparison between eeRo and DCM had no significant difference (Fig. 4).

In brain, there was no significant difference among eeRo and DCM or EA. The DCM at 30–300 µg/ml significantly reduces ROS levels when compared to the control. The DCM had better antioxidant effects at 100–300 µg/ml than at 30 µg/ml. The eeRo (Fig. 5A) and gallic acid (Fig. 5E) at 300 µg/ml had greater effect than they at 100 µg/ml against ROS. The eeRo, DCM, and EA fractions were not significantly different than gallic acid (Fig. 5E).

3.5. SNP-induced lipid peroxidation assay

In liver, the gallic acid (Fig. 6E) and the eeRo and DCM were able to protect the liver against lipid peroxidation induced by SNP (Fig. 6). The gallic acid at 1–300 µg/ml had a crescent protective effect (Fig. 6E). The EA fraction at 3–300 µg/ml and the ButOH fraction at 100–300 µg/ml significantly reduced lipid peroxidation levels induced by SNP. When the eeRo fraction was compared to gallic acid, there was no difference found at the same concentrations tested (Fig. 6).

In brain, the gallic acid (Fig. 7E) and the eeRo, DCM, and EA significantly protected brain against the effects of lipid peroxidation induced by SNP (Fig. 7). Furthermore, gallic acid (Fig. 7E) and the DCM were found to have greater effects at 3–300 µg/ml than at 1 µg/ml. The EA at 1–10 µg/ml had a crescent protective effect against lipid peroxidation with higher effect at 10–300 µg/ml. The ButOH at 30–300 µg/ml significantly protected brain against lipid peroxidation caused by SNP. However, ButOH had greater effects at 100–300 µg/ml than at 30 µg/ml (Fig. 7).

3.6. Effects of eeRo, DCM, EA and ButOH on bacteria

The crude extract and its fractions isolated from *R. officinalis* L. had a significant antimicrobial effect (Table 2). The order of approximate decrease in efficiency for bacteria were as follow as:

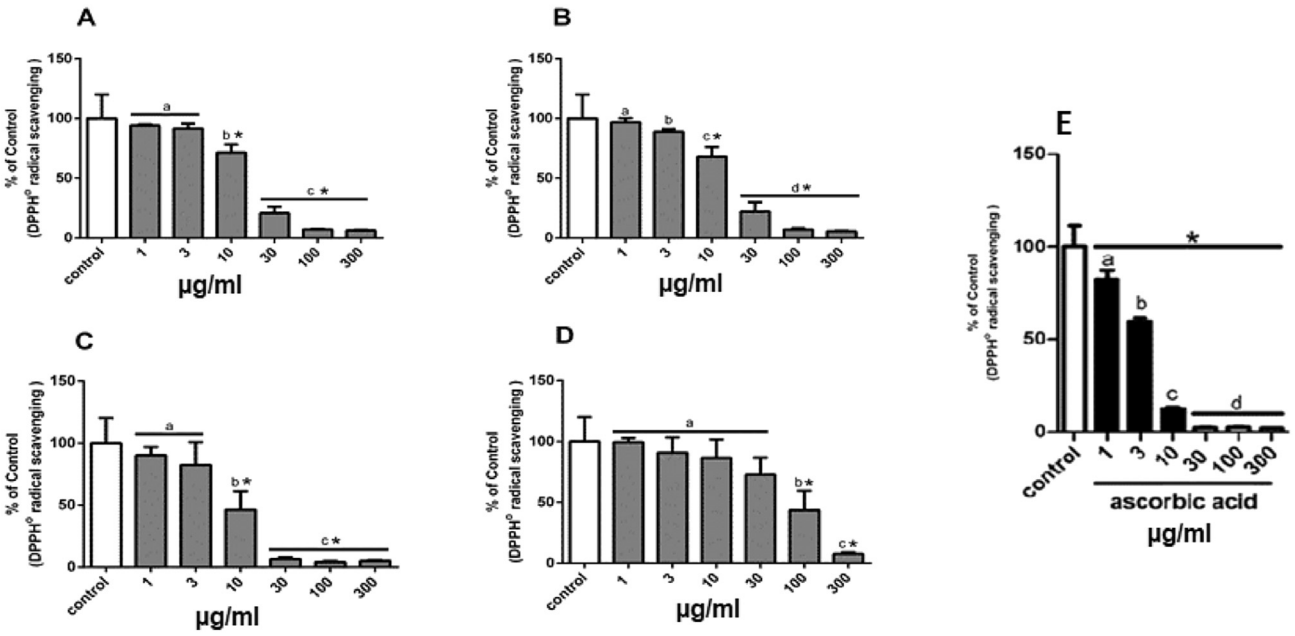

Fig. 2. Analyze of **DPPH$^{\bullet}$ radical scavenging capacity** of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D), ascorbic acid (E). Data are reported as means ± SEM of three to six determinations. One-way ANOVA followed by Newman–Keuls test. *$p < 0.05$, significant difference when compared to control. $^{a,b,c,d}p < 0.05$ significant difference among groups with different letters.

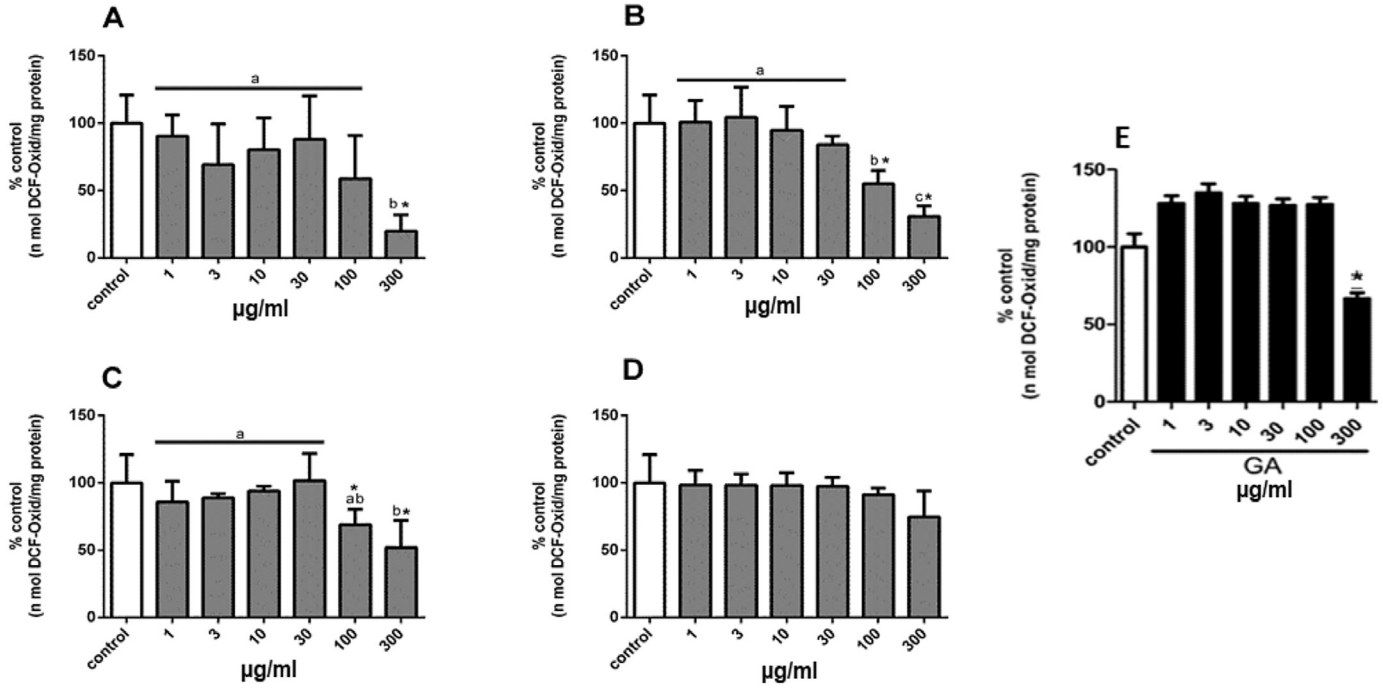

Fig. 3. Test of protective effect of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) and the gallic acid (GA) (E) **on basal formation of reactive oxygen species (ROS) in the liver**. Data are reported as means ± SEM of three to six determinations. One-way ANOVA followed by Newman–Keuls test for post-hoc. *$p < 0.05$, significant difference when compared to control. $^{a,b,c,d}p < 0.05$, significant difference among groups with different letters.

a) The S. aureus: DCM (better efficiency) > eeRo > EA > ButOH (anyone effect);
b) The S. epidermidis: DCM (better efficiency) = eeRo > EA > ButOH (worst efficiency);
c) The P. aeruginosa: DCM (better efficiency) = eeRo > EA = ButOH (worst efficiency);
d) The B. cereus: DCM (better efficiency) = eeRo > EA > ButOH (anyone effect).

4. Discussion

Various plants have been used for years in traditional medicine due to their advantageous effects.[7] After thorough analysis of the

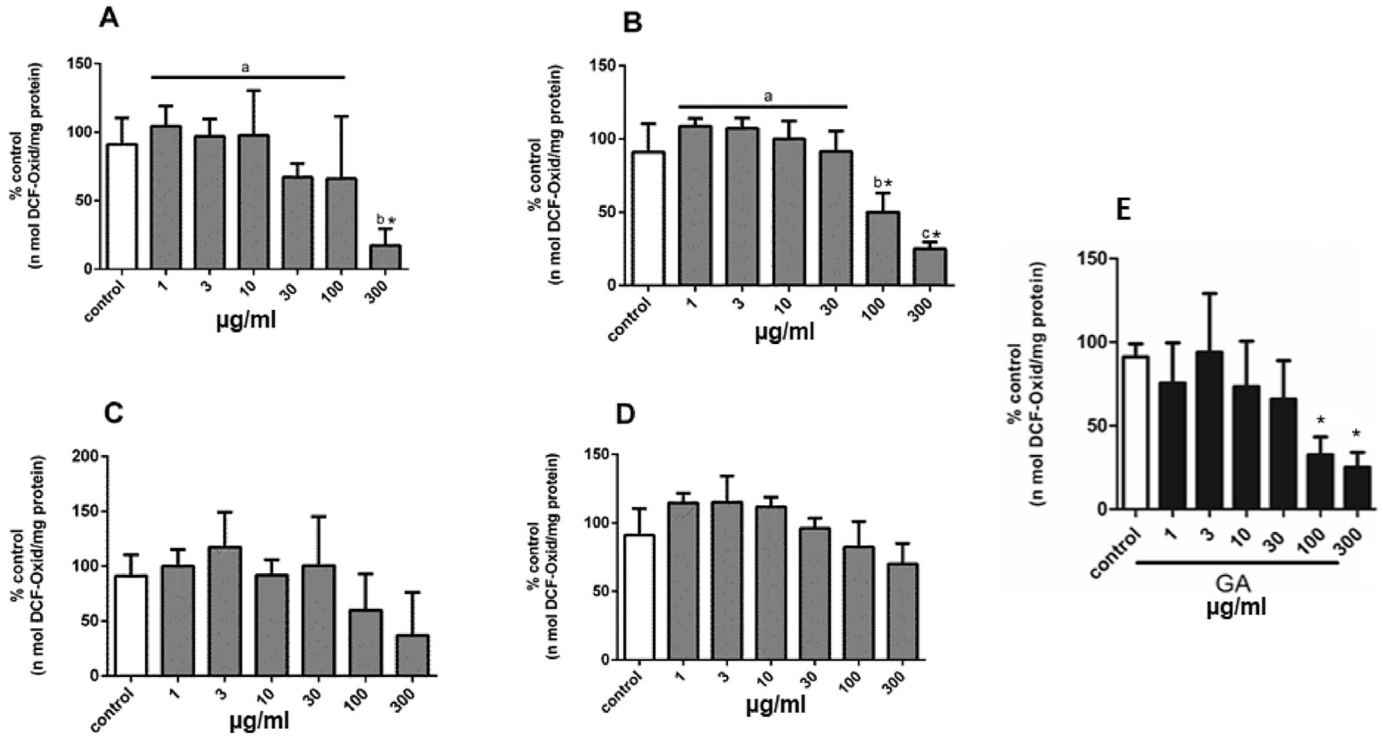

Fig. 4. Test of protective effect of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) and the gallic acid (GA) (E) **on basal formation of reactive oxygen species (ROS) in the stomach.** Data are reported as means ± SEM of three to six determinations. One-way ANOVA followed by Newman–Keuls test for post-hoc. *$p < 0.05$, significant difference when compared to control. $^{a,b,c,d}p < 0.05$, significant difference among groups with different letters.

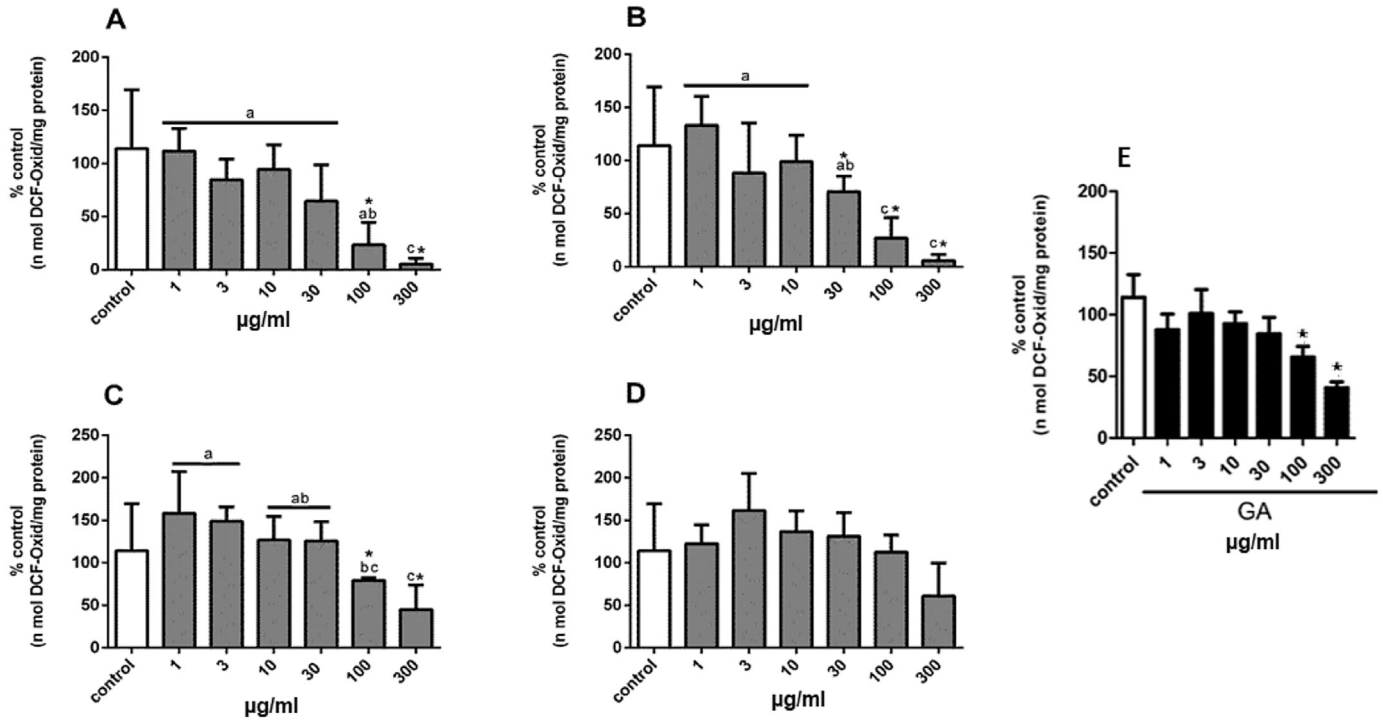

Fig. 5. Test of protective effect of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol (D) and gallic acid (GA) (E) **on basal formation of reactive oxygen species (ROS) in the brain**. Data are reported as means ± SEM of three to six determinations. One way ANOVA, followed by Newman–Keuls test for post-hoc. *$p < 0.05$, significant difference when compared to control. $^{a,b,c,d}p < 0.05$, significant difference among groups with different letters.

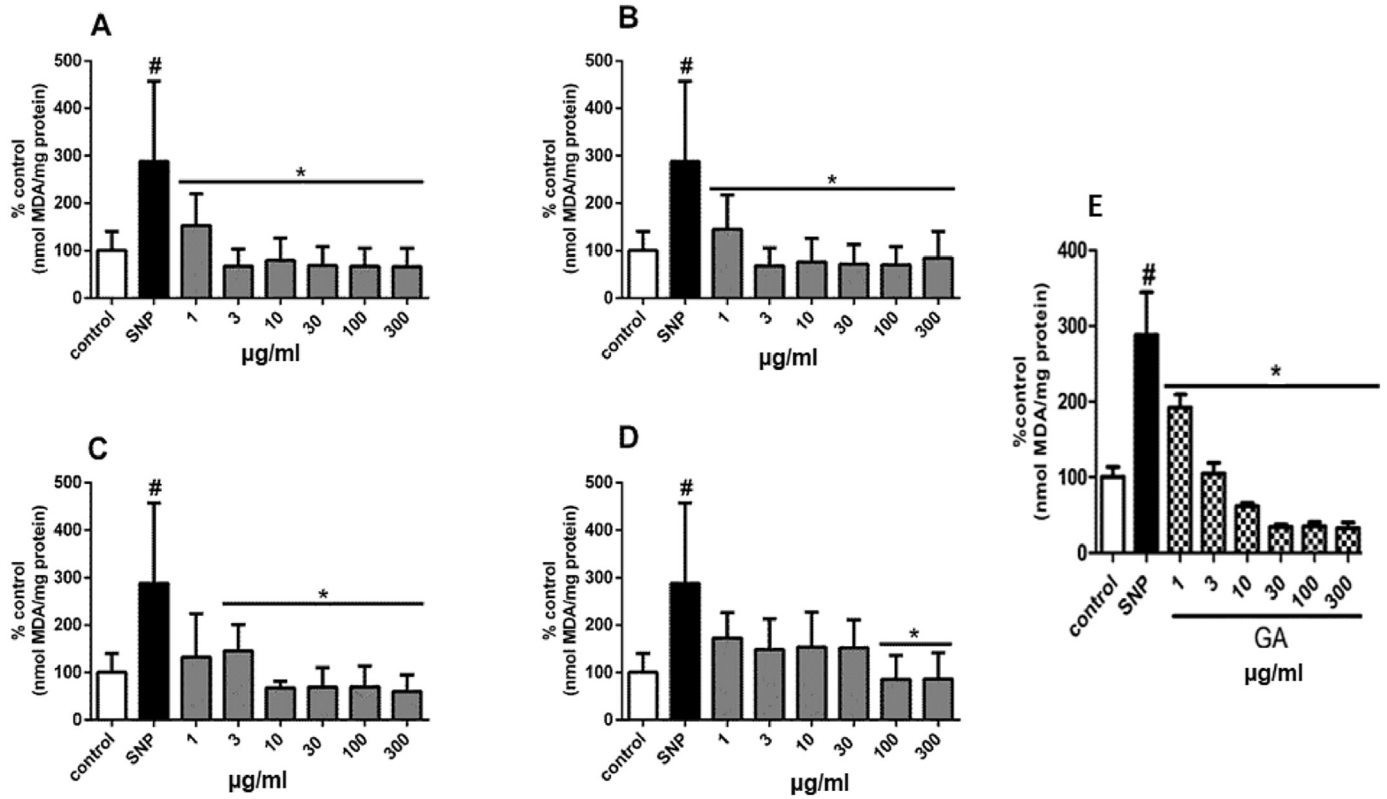

Fig. 6. Analysis of the protective action of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) and the gallic acid (GA) (E) **on lipid peroxidation induced by sodium nitroprusside (SNP)** at 10 µM in the **liver**. Data are reported as means ± SEM of three to six determinations. One-way ANOVA followed by Newman–Keuls test for post-hoc. $^{\#}p < 0.05$ and $^{*}p < 0.05$, significant difference when compared to control and SNP, respectively.

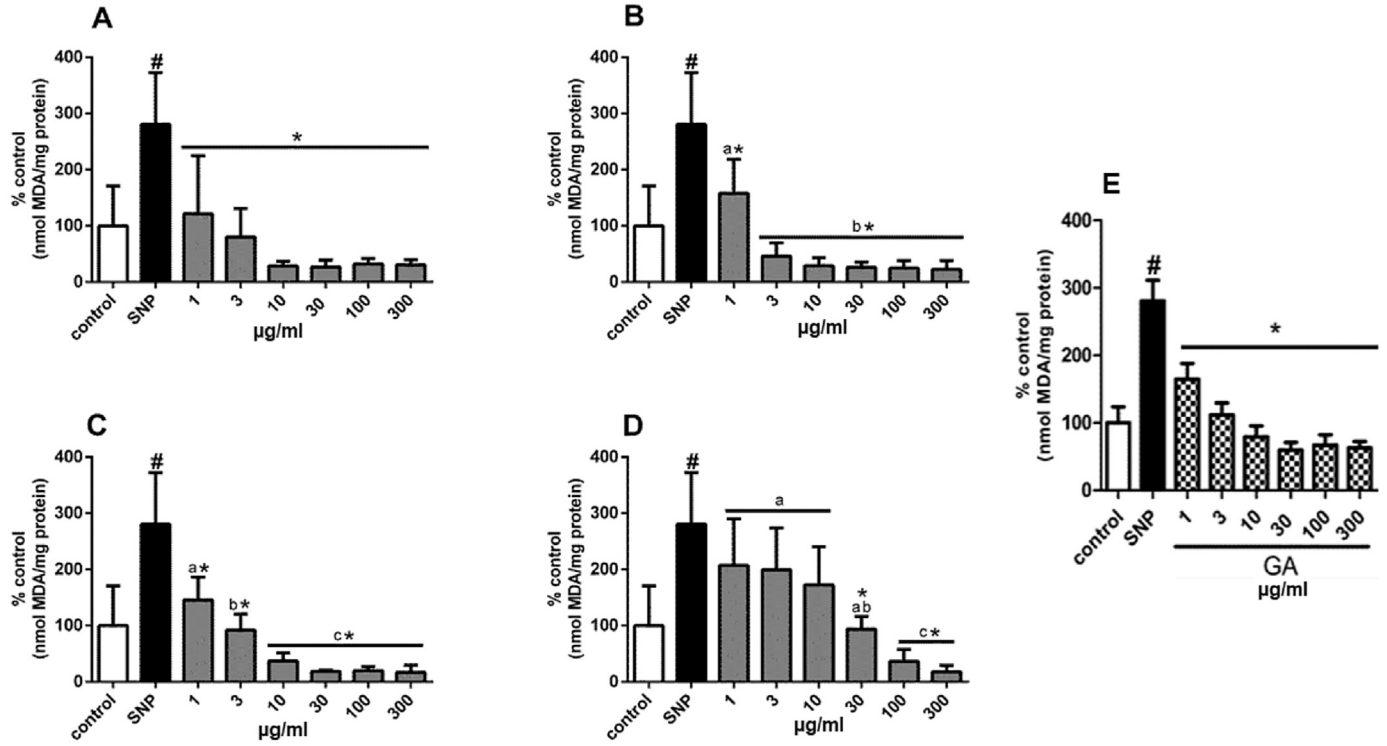

Fig. 7. Analysis of the protective action of ethanolic extract of *R. officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) and the gallic acid (GA) (E) on **lipid peroxidation induced by sodium nitroprusside (SNP)** at 10 µM in the **brain.** Data are reported as means ± SEM of three to six determinations. One-way ANOVA followed by Newman–Keuls test for post-hoc. $^{\#}p < 0.05$ and $^{*}p < 0.05$, significant difference when compared to control and SNP, respectively. $^{a,b,c,d}p < 0.05$, significant difference among groups with different letters.

Table 2
Analyses of ethanolic extract of Rosmarinus officinalis L. effects (eeRo) and its fractions: DCM (from dichloromethane), EA (from ethyl acetate), ButOH (from buthanol) on bacteria minimum inhibitory concentration (MIC). Data are reported as means of three determinations.

Compounds	S. aureus μg/ml	S. epidermidis μg/ml	P. aeruginosa μg/ml	B. cereus μg/ml
eeRo	128	16	128	32
DCM	64	16	128	32
EA	256	32	512	256
ButOH	–	512	512	–

eeRo, DCM, EA and ButOH, it was possible to identify carnosic and rosmarinic acids as the main constituents of the eeRo and DCM fractions. In the EA fraction, rosmarinic acid and quercetin were the major constituents, while carnosic, rosmarinic, and chlorogenic acids were identified as the main compounds in the ButOH fraction (Table 1). The eeRo, DCM, EA, and ButOH fractions had antioxidative or radical-scavenging effects significantly higher than or similar to antioxidant standards (Figs. 1–7). In addition, these compounds significantly inhibited Gram-positive and Gram-negative bacterial colony growth (Table 2). Supraphysiological ROS levels are a main cause of human disease.[22] The eeRo, EA, and DCM at 100 μg/ml and the ButOH at 300 μg/ml were able to exert antioxidative effects *in vitro* (Fig. 3) that were similar to those of ascorbic acid at 30 μg/ml, which is recognized as a standard antioxidant (Fig. 3E). Thus, we hypothesize that these *R. officinalis* L. extracts and fractions could be used in a future as antioxidant medicines.

In the present work, the $DPPH^{\bullet-}$ assay was performed to evaluate the scavenging activity of different compounds.[22] We observed significant radical scavenging by the eeRo, DCM, EA, and ButOH (Fig. 2). It is reasonable to infer that they could neutralize free radicals in biological systems as well (Figs. 3–7) because radicals in an assay and in biological systems are not fundamentally different; they all have one or more unpaired electrons in their structure.[3] However, in this assay, the ButOH only had significant effects at the two higher concentrations, indicating that the eeRo, DCM, and EA are better at scavenging $DPPH^{\bullet-}$ radicals than the ButOH. Moreover, the eeRo, DCM, and EA at their highest concentrations had greater $DPPH^{\bullet-}$ radical scavenging than ascorbic acid (Fig. 2). These findings indicate that the eeRo, DCM, and EA have excellent radical-scavenging activity, similar to the standard tested (Fig. 2). The oxidative stress can be generated by ROS in different organs, and is involved in the etiology of several chronic diseases including hepatic disease, diabetes, cancer, and neurodegenerative disorders.[2] In this context, the DCF is highly reactive with ROS and has been appropriately considered a marker of oxidative stress in animal tissue assays.[19] Thus, the antioxidant effects of an extract of *R. officinalis* L. and its fractions were tested in presence of DCHF (Figs. 3–5) and were effective against physiological levels of ROS present in the liver (Fig. 3), stomach (Fig. 4), and brain (Fig. 5). Thus, they can be considered effective antioxidative compounds.

In the liver, the eeRo, DCM, and EA decreased basal ROS levels similarly to gallic acid (Fig. 3). Therefore, the DCM and EA can be considered to have antioxidant effects, since their effects are present at a concentration smaller than gallic acid (Fig. 3E), a recognized antioxidant standard. It is also reasonable to infer that the DCM and EA fractions have a greater ability than GA to neutralize ROS. The DCM fraction had significant antioxidant effects (Fig. 3) in the liver probably it dues this fraction had the highest rosmarinic acid concentration, as well as the second-highest carnosic acid levels. The EA had the second-highest levels of rosmarinic acid among all the fractions, and the second-greatest variability of identified constituents (Table 1) that could act synergistically to produce antioxidant effects (Fig. 4). Thus, the DCM and EA fractions could potentially be good options in the auxiliary prevention or treatment of pathologies related to oxidative stress in the liver.[23]

Similarly, in the stomach, the DCM and eeRo produced antioxidant effects against ROS at same concentrations as GA. These data prove that the DCM and eeRo fractions had excellent antioxidant activities in the stomach, similarly to the standard used. The DCM and eeRo fractions had the greatest antioxidant effects in the stomach (Fig. 4) as compared to the other fractions, probably due to their compositions previously described (Table 1). The composition of the eeRo included the highest variability of identified compounds, and elevated levels of carnosic acid (Table 1). These findings could be applied in the prevention or treatment of pathologies such as gastric and peptic ulcers, which are associated with oxidative stress.[24] In the brain, the eeRo and DCM had similar antioxidant capacity as gallic acid in reducing ROS levels (Fig. 5). These results are probably due the concentration and variability of their phenolic compounds, as previously described (Table 1). Moreover, the DCM can be reported to have antioxidant effects, since these effect were observed at a lower concentration than the standard. It is reasonable to infer that the DCM has greater antioxidant efficacy than the standard. These data could possibility be used in the prevention or treatment of neurodegenerative diseases that are associated with oxidative stress. The brain is very sensitive to antioxidant protection by the DCM, eeRo, and EA (Fig. 5). The ROS levels in liver were also significantly decreased by the DCM, eeRo, and EA (Fig. 3), but in higher concentrations than in the brain (Fig. 5). The stomach was sensitive to the effects of the DCM and EA (Fig. 4) at the same concentration as in the liver. The DCM and eeRo had the same antioxidant effects in the liver (Fig. 3) and stomach (Fig. 4) mainly due to their high levels of carnosic acid. The Carnosic acid levels (Table 1) are the key in explaining the absence of antioxidant activity in stomach by the EA (Fig. 4). However, in the DCM, the high levels of carnosic acid was complemented by notably greater levels of rosmarinic acid (Table 1), which explains the fact that the DCM had greater antioxidant effects than the others in the liver (Fig. 3), stomach (Fig. 4), and brain (Fig. 5). Additionally, it is reasonable to conclude that, in the liver (Fig. 3), rosmarinic acid (Table 1) is more effective than other compounds (Figs. 3–5), because the EA, with elevated levels of rosmarinic acid (Table 1), had significant antioxidant effects at vastly smaller concentrations than the eeRo (Fig. 3).

The lipid peroxidation is a cause or consequence of oxidative stress and is associated with some dangerous pathological conditions in the liver.[2] Thus, it is common to use SNP, a reagent that is able to generate ROS and stimulate dangerous lipid reactions, as a lipid peroxidation agent.[25] Accordingly, the *R. officinalis* L. extract and fractions were tested in presence of sodium nitroprusside.

In the liver, the eeRo and DCM were similar to GA (Fig. 6), in that at all tested concentrations, they significantly protected against lipid peroxidation. In addition, for the same organ, the EA also produced a significant protective effect at smaller concentrations. In contrast, the ButOH had an antioxidant effect only at the highest concentrations tested (Fig. 6). Thus, the eeRo and DCM can be suggested to have the greatest antioxidant effects (compared to the EA and ButOH), mainly due their phenolic composition, as previously described (Table 1). Protecting the brain against lipid peroxidation and consequently oxidative stress is very important.[4] This organ is very sensible to damage caused by ROS (Fig. 7).[4] The eeRo, DCM, and EA in brain produced excellent effects, similar to the antioxidant standard used against the lipid peroxidation. Because they produced effects at lower concentrations, it can be suggested that they are more effective than the ButOH against lipid peroxidation (Fig. 7). The antioxidative effects of all extract and fractions of *R. officinalis* L. against lipid peroxidation were more pronounced

in the brain (Fig. 7) than the liver (Fig. 6).

These antioxidant effects are similarly observed in basal levels of ROS for same organs (Figs. 5 and 3, respectively). These extracts produced significant effects at lower concentrations against induced lipid peroxidation (Figs. 3 and 5) than against basal ROS levels. This data to indicate that under elevated oxidative stress conditions with high levels of ROS and lipid peroxidation, the eeRo, DCM, EA, and ButOH could offer sufficient antioxidative protection at much lower concentrations (Figs. 6–7) than the concentrations used in basal tests (Figs. 3 and 5). This explanation lets us to hypothesize that the extract and fractions of *R. officinalis* L. could potentially be used at lower concentrations to prevent or treat oxidative imbalance without affecting physiological ROS thresholds and their signaling pathways.

In the brain (Fig. 7), the DCM, eeRo, and EA had similar protective effects, probably due to their composition (Table 1) as previously described. In the liver (Fig. 6), similar to the brain (Fig. 7), the DCM and eeRo had antioxidant effects at the lower concentration tested. However, in the liver, the EA had significantly lower anti-lipid peroxidation activity than the DCM and eeRo (Fig. 6); this is probably attributable to lower levels of carnosic acid as compared to the DCM and eeRo. The ButOH was unable to exert any significant antioxidant effect on basal levels of ROS. However, when this fraction was tested against lipid peroxidation in the brain and liver, it produced concentration-dependent protective effects, possibly due to the synergistic action of equivalent concentrations of the chlorogenic, rosmarinic, and carnosic acids present (Table 1).

There are various species of bacteria that are able to cause disease in animals and humans.[1] In the present study, the effect of the eeRo and its fractions on bacterial colonies was investigated. The bacteria evaluated included: *S. aureus*, responsible for intestinal infections and endocarditis; *S. epidermidis*, present in cases of endocarditis [26]; *B. cereus*, a common bacteria in gastroenteritis pathogenesis [14]; and *P. aeruginosa*, a dangerous pathogenic microorganism in endocarditis[3] and respiratory infections[27] (Table 2). Our data indicate that the DCM could be used in association with other compounds for the prevention or treatment of pathologies caused by *S. aureus*.

The DCM and eeRo were demonstrated to have the best antibacterial effects compared to the others against *S. epidermidis, P. aeruginosa*, and *B. cereus*. The DCM and EA in small concentrations were able to inhibit some kinds of Gram-positive and Gram-negative bacterial colonies. The DCM had an antibacterial effect on *S. aureus* colonies at a 50% smaller concentration than the eeRo (Table 2); rosmarinic acid is the only constituent of DCM present at concentrations higher than in eeRo (21.5 times). We can speculate that the antibacterial effects against *S. aureus* were due to rosmarinic acid. However, the eeRo had excellent antibacterial effects at a 50% smaller concentration than in the EA for the same bacteria. The single substance found at higher levels in the eeRo than in the EA is carnosic acid (9.36 times) (Table 1); thus, we could conclude that a significant portion of the anti-bacterial effect against *S. aureus* is produced by carnosic acid. The ButOH had antibacterial effects only on the *S. epidermidis and P. aeruginosa* colonies (Table 2), probably due to the synergic action of the equivalent concentrations of rosmarinic, carnosic, and chlorogenic acids (Table 1). Among all the tested compounds, the DCM and eeRo had the lowest MIC (Table 2), suggesting that their composition of phenolic compounds (Table 1) could potentially be used as a treatment for animals and humans against the bacteria tested in the present study (Table 2).

In conclusion, the extract and of *R. officinalis* L. had excellent antioxidant action *in vitro* and *ex vivo*, in the liver, stomach, and brain of rats. Moreover, the extract and fractions were able to inhibit pathogenic Gram-positive and Gram-negative bacterial colony growth. We demonstrate that the DCM and eeRo have the better phenolic compound composition, with effective antioxidant and antibacterial activity against numerous diseases whose pathogenesis is associated with oxidative stress and bacterial infection. In this context, the DCM and eeRo could be a significant option for an important multi-mechanistic approach to oxidative stress associated bacterial infections.

Conflict of interest

None.

Acknowledgments

This work was supported by the PRONEM #11/2029-1 research grant by DCIT/SCTIE-MS/CNPQ/FAPERGS. G.P.A., F.A.A.S., R.F., R.L.P, M.M.A.C and A.S.L.M. receive a fellowship from CNPq. S.T.S. and C.M. fellowship by CAPES. We would like to thanks botanical garden of UFSM by cultivation and supply of *R. officinalis* L. The **English language** of our manuscript was edited by native English-speaking experts from **Editage**.

References

1. Fu H, Yuan J, Gao H. Microbial oxidative stress response: novel insights from environmental facultative anaerobic bacteria. *Arch Biochem Biophys*. 2015;584:28–35. https://doi.org/10.1016/j.abb.2015.08.012.
2. Stefanello ST, Flores da Rosa EJ, Dobrachinski F, et al. Effect of diselenide administration in thioacetamide-induced acute neurological and hepatic failure in mice. *Toxicol Res (Camb)*. 2015;4(3):707–717. https://doi.org/10.1039/c4tx00166d.
3. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev*. 2014;94(2):329–354. https://doi.org/10.1152/physrev.00040.2012.
4. da Silva MH, da Rosa EJ, de Carvalho NR, et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. *Neurotox Res*. 2012;21(3):334–344. https://doi.org/10.1007/s12640-011-9288-1.
5. Boero F. From Darwin's Origin of Species toward a theory of natural history. *F1000 Prime Rep*. 2015;7:49. https://doi.org/10.12703/P7-49.
6. Vázquez-Medina JP, Zenteno-Savín T, Elsner R, Ortiz RM. Coping with physiological oxidative stress: a review of antioxidant strategies in seals. *J Comp Physiol B*. 2012;182(6):741–750. https://doi.org/10.1007/s00360-012-0652-0.
7. Ngo SNT, Williams DB. Head RJ. Rosemary and cancer prevention: preclinical perspectives. *Crit Rev Food Sci Nutr*. 2011;51(10):946–954. https://doi.org/10.1080/10408398.2010.490883.
8. Mohamed WAM, Abd-Elhakim YM, Farouk SM. Protective effects of ethanolic extract of rosemary against lead-induced hepato-renal damage in rabbits. *Exp Toxicol Pathol*. 2016;68(8):451–461. https://doi.org/10.1016/j.etp.2016.07.003.
9. Mara A, Oliveira D, Machado ID, et al. Aqueous extract of Rosmarinus officinalis L. Inhibits neutrophil influx and cytokine secretion. *Phyther Res*. August 2014;2015(133):125–133.
10. Kamdem JP, Stefanello ST, Boligon AA, et al. In vitro antioxidant activity of stem bark of Trichilia catigua Adr. *Juss Acta Pharm*. 2012;62(3):371–382, 10.2478/v10007-012-0026-x/j/acph.2012.62.issue-3/v10007-012-0026-x/v10007-012-0026-x.xml [pii].
11. Macahilig C, Stephens JM, Chambers R, Li JZ. *Early-switch/Early-discharge Opportunities for Hospitalized Patients with Methicillin-resistant Staphylococcus aureus Complicated Skin and Soft Tissue Infections: Proof of Concept in the United Arab Emirates*. 2015:173–179.
12. Sahal G, Bilkay IS. Multi drug resistance in strong biofilm forming clinical isolates of Staphylococcus epidermidis. *Braz J Microbiol*. 2014;544:539–544.
13. Williams BJ, Dehnbostel J, Blackwell TS. Pseudomonas aeruginosa: host defence in lung diseases. *Respirology*. 2010;15(7):1037–1056. https://doi.org/10.1111/j.1440-1843.2010.01819.x.
14. Al-Abri SS, Al-Jardani AK, Al-Hosni MS, Kurup PJ, Al-Busaidi S, Beeching NJ. A hospital acquired outbreak of Bacillus cereus gastroenteritis, Oman. *J Infect Public Health*. 2011;4(4):180–186. https://doi.org/10.1016/j.jiph.2011.05.003.
15. Ashok P, Koti BC, Thippeswamy AHM, Tikare VP, Dabadi P, Viswanathaswamy AHM. Evaluation of antiinflammatory activity of centratherum anthelminticum (L) kuntze seed. *Indian J Pharm Sci*. 2010;72(6):697–703. https://doi.org/10.4103/0250-474X.84577.
16. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol*. 1995;28(1):25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.
17. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific

application to the determination of vitamin E. *Anal Biochem*. 1999;269(2):337–341. S0003-2697(99)94019-8 [pii]10.1006/abio.1999.4019.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95(2):351–358. http://www.ncbi.nlm.nih.gov/pubmed/36810. Accessed June 15, 2014.
19. Myhre O, Andersen JM, Aarnes H, Fonnum F. Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem Pharmacol*. 2003;65(10):1575–1582. http://www.ncbi.nlm.nih.gov/pubmed/12754093. Accessed June 16, 2014.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254. http://www.ncbi.nlm.nih.gov/pubmed/942051. Accessed May 26, 2014.
21. Palomino J, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. *Antimicrobail Agents Chemother*. 2002;46(8):2720–2722. https://doi.org/10.1128/AAC.46.8.2720.
22. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol*. 1995;28(1):25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.
23. Carvalho NR, da Rosa EF, da Silva MH, et al. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. *PLoS One*. 2013;8(12), e81961. https://doi.org/10.1371/journal.pone.0081961PONE-D-13-12843.
24. Ineu RP, Oliveira CS, Oliveira VA, Moraes-Silva L, da Luz SCA, Pereira ME. Antioxidant effect of zinc chloride against ethanol-induced gastrointestinal lesions in rats. *Food Chem Toxicol*. 2013;58:522–529. https://doi.org/10.1016/j.fct.2013.05.022.
25. Amaral GP, Puntel GO, Dalla Corte CL, et al. The antioxidant properties of different phthalocyanines. *Toxicol Vitro*. 2012;26(1):125–132. https://doi.org/10.1016/j.tiv.2011.10.006.
26. Chu VH, Cabell CH, Abrutyn E, et al. Native valve endocarditis due to coagulase-negative staphylococci: report of 99 episodes from the international collaboration on endocarditis merged database. *Clin Infect Dis*. 2004;39(10):1527–1530. https://doi.org/10.1086/424878.
27. Wilson R, Dowling R. Pseudomonas aeruginosa and other related species. *Thorax*. 1998;53(3):213–219. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1745165/.