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PROPRIEDADES DE PROTEÇÃO GASTROINTESTINAL DA *Rosmarinus officinalis* L. EM ASSOCIAÇÃO A TESTES MICROBIOLÓGICOS E ANTIOXIDANTES *IN VITRO* E *EX VIVO*

TESE DE DOUTORADO

Guilherme Pires Amaral

Santa Maria, RS, Brasil

2016

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por

Guilherme Pires Amaral

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do grau de Doutor em Bioquímica Toxicológica.

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RESUMO

PROPRIEDADES DE PROTEÇÃO GASTROINTESTINAL DA *Rosmarinus officinalis* L. EM ASSOCIAÇÃO A TESTES MICROBIOLÓGICOS E ANTIOXIDANTES *IN VITRO* E *EX VIVO*

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A produção de espécies reativas acima dos níveis fisiológicos representa um alto risco para o surgimento de muitas patologias. Neste contexto, o estresse oxidativo e a inflamação podem estar presentes na etiologia de patologias gastrointestinais, como a úlcera péptica, associadas a ingestão de etanol, e por vezes, apresentam implicações hepáticas e neurais. A úlcera péptica é composta por danos gastrointestinais os quais afetam muitas pessoas em todo o mundo e seu desenvolvimento é resultado do desequilíbrio entre os fatores de proteção e agressão gástricos. A *Rosmarinus officinalis* L. é popularmente conhecida pelas suas propriedades medicinais em várias patologias, principalmente devido a presença de compostos fenólicos em sua composição. Assim, nesta tese o objetivo foi realizar análises *in vitro* e *ex vivo* sobre as propriedades antioxidantes do extrato etanólico de *Rosmarinus officinalis* L. (eeRo) e suas frações (DCM, EA, ButOH), além de investigar as suas atividades antibacterianas e a possível aplicabilidade do eeRo na proteção gastrointestinal. O eeRo foi obtido a partir de folhas secas (40ºC) e submetidas a uma extração alcóolica em aparelho de soxhlet. O eeRo foi separado por diclorometano, acetato de etila a butanol em um funil de separação para obter as frações DCM, EA e ButOH, respectivamente. A quantificação dos constituintes do eeRo e suas frações foi realizada por HPLC–DAD. O eeRo e suas frações foram testados como "scaveging" de radical $DPPH^{\bullet-}$ e sobre capacidade antioxidante total sem a utilização de tecido e em ensaios de peroxidação lipídica induzida por nitroprussiato de sódio e fluorescência de diclorofluorsceína em fígado, cérebro e estômago de ratos adultos machos. O eeRo e suas frações foram testados no ensaio de concentração inibitória mínima em bactérias. Além disso, o eeRo e suas frações foram analizados em modelos de lesão gástrica e intestinal. Após a aplicação desses modelos e o prévio tratamento com eeRo foram realizados os ensaios de fluorescência de dilorofluorceína diacetato, peroxidação lipídica, taxa de GSH/GSSG, atividade da superóxido dismutase, catalase e mieloperoxidase em estômago e intestino de ratos. Além disso, foram mensurados os níveis de nitrito e nitrato, índice de ulceração além da realização de testes histopatológicas apenas em estômago. A atividade da Na^+/K^+-ATPase foi mensurada apenas em intestino. Os eeRo, DCM, EA tiveram, *in vitro*, efeito antioxidante total e "scavenging" de radical $DPPH^{\bullet-}$. O eeRo e a DCM apresentaram efeito antioxidante contra espécies reativas de oxigênio em fígado, estômago e cérebro. O eeRO e a DCM protegeram amostras de fígado e cérebro contra a peroxidação lipídica induzida por nitroprussiato de sódio. O eeRo e as DCM, EA and ButOH tiveram efeitos inibitórios sobre bactérias gram (+) e gram (-). Contudo, o eeRo e a DCM tiveram as menores concentrações inibitórias. O eeRo, após um tratamento *in vivo*, protegeu o estômago e o intestino contra a peroxidação lipídica e aumentou a atividade da catalase em ambos estômago e intestino. O eeRo evitou a alteração nos níveis basais da Na^+/K^+-ATPase e aumentou a atividade da superóxido dismutase apenas no intestino. Além disso, o eeRo reduziu a atividade da mieloperoxidase em estômago e intestino. Os resultados desta tese sugerem que o eeRo, de modo geral, representa uma importante opção contra o estresse oxidativo *in vitro*, *ex vivo* e na prevenção de lesões gastrointestinais com mecanismos associados a ação anti-inflamatória ou vasodilatora.

Palavras-chave: Antioxidante, antibacteriano, proteção gastrointestinal, úlcera, *Rosmarinus officinalis* L.

ABSTRACT

GASTROINTESTINAL PROTECTION PROPERTIES OF *Rosmarinus officinalis* L. IN ASSOCIATION WITH *IN VITRO* AND *EX VIVO* ANTIOXIDANT AND MICROBIOLOGICAL TESTS

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The elevate production of reactive species over physiological levels and associated to pathogenic bacteria could represent a high risk for many diseases. The high levels of oxidative stress and inflammation can be present in the etiology of gastrointestinal pathologies associated with ethanol ingestion, and sometimes with implication in liver or brain. Peptic ulcer includes gastric and intestinal damage that affect many people around the world and its development is a result of the imbalance between aggressive and protective factors. The *Rosmarinus officinalis L.*, more commonly known as rosemary in Europe and as alecrim in Brazil, has exhibited several physiological and medicinal activities against some diseases mainly due its phenolic compounds. So in this thesis, the aim was to perform analyzes *in vitro* and *ex vivo* on the antioxidant properties of the ethanolic extract of *Rosmarinus officinalis* L. (eeRo) and its fractions (DCM, EA, ButOH), and about their antibacterial activities and the possible applicability of eeRo on gastrointestinal protection. The eeRo was obtained from the dried leaves (40ºC) subjected to an alcoholic extraction in the soxhlet apparatus. The eeRo was separately to dichloromethane, ethyl acetate and butanol in a separator funnel to get DCM, EA and ButOH fractions, respectively. The quantification of constituents of eeRo and its fractions were made by HPLC–DAD. The eeRo and its fractions were tested in the $DPPH^{\bullet}$ radical scavenging, total antioxidant capacity assays without tissues and they were tested in sodium nitroprusside-induced lipid peroxidation and H_2DCF-DA in liver, brain and stomach of male adult wistar rats. The eeRo and its fractions were analyzed in bacteria minimum inhibitory concentration assay. Besids, eeRo and its fractions were tested in ethanol-induced gastric and intestinal lesions models. After application of these models and pretreatment with eeRo the dichlorofluorescein fluorescence, lipid peroxidation, ratio of GSH/GSSG, superoxide dismutase activity, catalase activity, myeloperoxidase activity assays were performed in stomach and intestine. Moreover, the measurement of nitrite and nitrate levels, ulcer index and histopathology tests were made only in stomach. The Na^+/K^+ ATPase activity was measured only in intestine. The eeRo, DCM, EA had significant total antioxidant effect and $DPPH^{\bullet}$ radical scavenging activity *in vitro*. The DCM and eeRo got antioxidant effects against basal levels of reactive species in the liver, stomach and brain. The eeRo and DCM protected the liver and brain against lipid peroxidation induced by sodium nitroprussiate. The eeRo, DCM, EA and ButOH had inhibitory effect in the gram (+) and gram (-) bacteria. However, the eeRo and DCM, had the lower minimum inhibitory concentration.The eeRo, after *in vivo* treatment protected stomach and intestine against the lipid peroxidation and they increased the CAT activity. The eeRo prevented the reduction in Na^+/K^+ ATPase and cased the increase of superoxide dismutase activity in intestine. In addition, eeRo reduced the myeloperoxidase activity in stomach and intestine. The results of this tese suggest that the eeRo, in general way, represented the better option as agent against oxidative *stress in vitro*, *ex vivo* e mainly in the prevention of gastrointestinal lesions in association to anti-inflamatory or vasodilatory mechanisms.

Key-words: antioxidante, anti-bacterial, gastrointestinal protection, ulcer, *Rosmarinus officinalis* L.

LISTA DE ABREVIATURAS

eeRo- Extrato etanólico de *Rosmarinus officinalis* L.;

DCM- Fração do eeRo obtida com diclorometano;

EA- Fração do eeRo obtida com acetato de etila;

ButOH- Fração do eeRo obtida com butanol;

ER-Espécies reativas;

ERO-Espécies reativas de oxigênio;

etOH- Etanol;

CAT- Catalase;

SOD- Superóxido dismutase;

Na^+/K^+-ATPase- adenosine trifosfatase;

H^+, K^+-ATPase- H^+/ K^+-adenosina trifosfatase ou bomba de prótons;

GSSG- glutationa oxidada;

GSH- glutationa reduzida;

MPO- mieloperoxidase;

Gpx- Glutationa peroxidase;

GPr- glutationa redutase

SUMÁRIO

1. INTRODUÇÃO

As plantas medicinais têm sido usadas pela população em geral por milhares de anos com a finalidade de explorar as suas propriedades farmacológicas sobre diversas patologias. Entre essas plantas, encontra-se a *Rosmarinus officinalis* L. conhecida no Brasil como alecrim e que tem demonstrado grande potencial farmacológico a partir de propriedades conhecidas como a antioxidante ou ainda desconhecidas que poderiam ser associadas as suas características curativas (SILVA et al., 2015). Os antioxidantes representam a principal forma de proteção contra o estresse oxidativo e estão frequentemente presentes tanto nas plantas como nos animais e seres humanos. Entre os principais mecanismos dos antioxidantes, estão as suas capacidades de neutralizar completamente as espécies reativas ou transforma-las em formas menos reativas (PISOSCHI; POP, 2015). O estresse oxidativo, por sua vez, decorre do desequilíbrio redox caracterizado pelo aumento de espécies reativas e diminuição das defesas antioxidantes. Esse desequilíbrio tem como consequência a oxidação de estruturas celulares como as membranas liproteicas e material genético que podem causar diversas patologias como disfunções hepáticas e nervosas, úlceras pépticas e câncer (PISOSCHI; POP, 2015). Além disso, o processo inflamatório é essencial para a proteção dos seres humanos e animais uma vez que ele possui a finalidade de cessar a causa inicial de uma agressão ao organismo como por exemplo a presença de um patôgeno. Esse processo pode ser inespecífico ou específico respectivamente com eventos vasculares e produção de anticorpos. Em todo o processo inflamatório também ocorre a migração de leucócitos como neutrófilos que ao fagocitar microorganismos produzem espécies reativas a partir de reações catalisadas pela enzima mieloperoxidase (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009; DAI; HARADA; TAKAMATSU, 2016). Dessa forma, existe um grande número de patologias associadas ao estresse oxidativo e a inflamação como as úlceras pépticas as quais são caracterizadas por lesões no estômago e intestino delgado. Essas patologias podem ser geradas por diversas causas como tabagismo, estresse psicológico e álcool etílico principalmente devido ao consumo de bebidas alcóolicas. As lesões encontradas nas úlceras pépticas podem possuir diversos graus de severidade capazes, muitas vezes, de perfurar completamente o órgão afetado levando o paciente a morte; ou inda estarem associadas a patogênese do câncer de estômago ou intestino (BHATTACHARYYA et al., 2014c). Além dessas patologias, é importante destacar a existência de bactérias com grande potencial patogênico que estão frequentemente presentes em infecções nosocomias e muitas vezes associadas ao estresse oxidativo e a inflamação e que poderiam contribuir para o agravamento do estresse oxidativo e processo inflamatório (FU; YUAN; GAO, 2015a).

1.1. Alecrim

Em todo o mundo, diferentes espécies de plantas são usadas na prevenção e tratamento de diversas patologias devido principalmente ao seu conteúdo fenólico (FERLEMI et al., 2015). E entre elas, a *Rosmarinus officinalis* L. conhecida no Brasil como alecrim (fig. 1.) é comumente utilizada como planta ornamental e principalmente como especiaria na culinária mediterrânea devido ao seu sabor e aroma; ou ainda é consumida em infusões na forma de chá (RASKOVIC et al., 2015). O alecrim foi aprovado pela união europeia e considerado seguro para ser utilizado como conservante na indústria alimentícia devido as suas propriedades antioxidantes (ZHAO et al., 2015). Essa planta também é usada comercialmente na composição de cosméticos e como suplemento nutricional (ULBRICHT et al., 2010).

Fig. 1. Alecrim (https://commons.wikimedia.org)

Além dessas aplicações, o alecrim tem sido utilizado pela medicina popular e por pesquisadores a muitos anos com a finalidade de explorar suas propriedades farmacológicas e curativas (FERLEMI et al., 2015; MARA et al., 2015; ROCHA et al., 2015; ULBRICHT et al., 2010). Entre as principais características farmacológicas já descobertas, estão suas propriedades anti-inflamatórias (ROCHA et al., 2015), antioxidantes, antimicrobianas (MARA et al., 2015), de analgesia muscular, articular e na cólica renal (RASKOVIC et al., 2015); antiespasmódica, gastrointestinal (RASKOVIC et al., 2015; ULBRICHT et al., 2010), e como auxiliar em dispepsias leves (FERLEMI et al., 2015; RASKOVIC et al., 2015; ULBRICHT et al., 2010), distúrbios respiratórios (RASKOVIC et al., 2015) e da circulação periférica (ULBRICHT et al., 2010). Essa planta é ainda estudada como potencial agente redutor de peso (ULBRICHT et al., 2010), uma vez que possui características ainda desconhecidas responsáveis por diminuir o ganho de peso e proteger contra a esteatose hepática (ZHAO et al., 2015).

Além disso, extratos e óleos essenciais dessa planta já apresentaram características eficientes na terapêutica do déficit cognitivo (FERLEMI et al., 2015; KENNEDY; SCHOLEY, 2006; ULBRICHT et al., 2010), depressão (FARAHANI et al., 2015; MACHADO et al., 2012), fadiga mental e física, e distúrbios de humor como agitação e histeria (MACHADO et al., 2012).

O alecrim possivelmente possui efeitos anti-inflamatórios agudos devido a sua ação direta sobre os mecanismos de infiltração, locomoção e adesão neutrofílica (SILVA et al., 2015). Ainda existem algumas pesquisas que demonstram a atividade scavenger de seus extratos sobre os ânions superóxido e peróxido nitrito. Alguns estudos têm demonstrado a capacidade dos extratos e óleos essenciais do alecrim no tratamento antitumoral em camundongos, ratos e humanos, *in vitro* e *in vivo*. Alguns extratos com valores padronizados de ácido carnósico foram capazes de induzir apoptose em células cancerígenas de cólon humano, através do aumento do fator de transcrição Nrf2 (PETIWALA; JOHNSON, 2015).

O alecrim possui como principais constituintes óleos essenciais, flavonoides, polifenóis e terpenóides (PETIWALA; JOHNSON, 2015). Entre os flavonoides destacam-se a diosmetina, genkwanina, luteolina, hispidulina e apigenina; acidos cafeico, chlorogenico e rosmarínico; e os terpenos como carnosol, ácido carnósico, rosmanol, epirosmanol, isorosmanol, rosmarino difenol, rosmariquinona, rosmadiol e 7-metil-epirosmanol (CARDOSO et al., 2014). Contudo, mais de 90% da atividade antioxidante do alecrim é atribuída a presença do ácido carnósico e carnosol (PETIWALA; JOHNSON, 2015). Alguns estudos apontam os diterpenos ácido carnósico, carnosol e rosmanol (PETIWALA; JOHNSON, 2015; ULBRICHT et al., 2010) como potenciais agentes de prevenção da carcinogênese.

1.2. Antioxidantes

Para contrabalançar o estresse oxidativo causado pelas ER o sistema antioxidante endógeno utiliza antioxidantes enzimáticos e não enzimáticos com funções “scavenger” ou “quencher”. Os antioxidantes são considerados “scavenger” quando possuem a capacidade de transformar um radical livre em produtos menos reativos (KŁADNA et al., 2016). No entanto, eles são considerados “quencher” quando são capazes de neutralizar completamente os radicais livres (RADOMSKA-LEŚNIEWSKA et al., 2015). Os antioxidantes enzimáticos desintoxicam um organismo vivo ao catalisar reações que transformam espécies reativas de alta reatividade em subprodutos menos

reativos, como exemplo a enzima CAT é capaz de reduzir peróxido de hidrogênio em água e oxigênio livre (KASALA et al., 2016). Esses antioxidantes podem ainda regenerar antioxidantes enzimáticos como a redução da GSSG em GSH pela ação da glutationa redutase (KASALA et al., 2016).

Os antioxidantes não enzimáticos podem ser de origem endógena ou exógena. Entre os de origem endógena, a enzima superóxido dismutase (SOD) e a glutariona reduzida (GSH) estão entre os mais importantes. A SOD pode converter $O2^{\bullet-}$ em H_2O_2 que é substrato para a catalase (CAT). Essa enzima é encontrada nas isoformas CuZnSOD (SOD1) e MnSOD (SOD2), localizadas na mitocôndria respectivamente no espaço intermembranas e na matrix. (Rahman, 2007). O GSH representa um dos principais antioxidantes responsáveis por neutralizar espécies reativas ou regenerar outros antioxidantes como o ácido ascórbico (FERREIRA; ABREU, 2007).

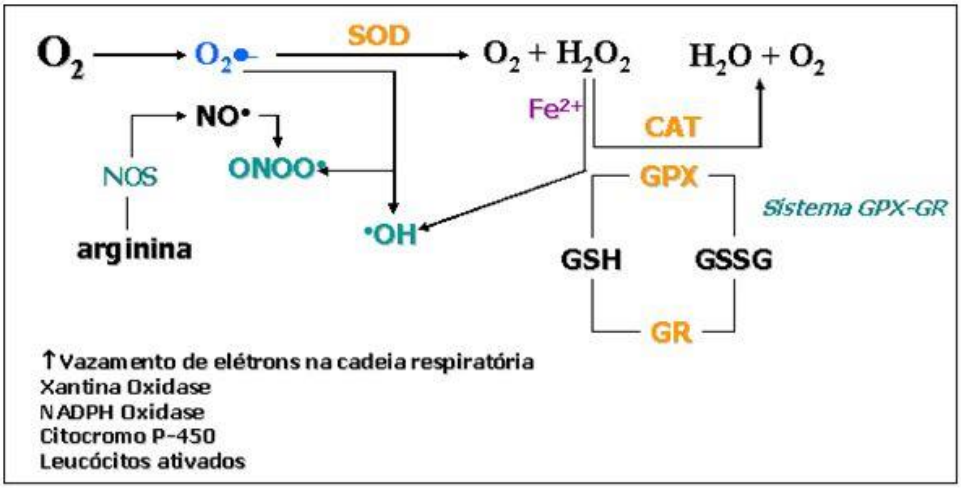
Entre os antioxidantes de origem exógena, existem grande quantidade de substâncias com ação antioxidantes, contudo os de origem natural como os flavonoides são encontrados frequentemente em alimentos e especiarias de origem vegetal. Os flavonoides como quercetina, kaempferol, e miricetina integram a dieta dos seres humanos e animais. Os flavonoides podem desempenhar um importante papel auxiliar em relação ao sistema antioxidante endógeno, permitindo um melhor controle contra ação das espécies reativas (FERREIRA; ABREU, 2007; YAO et al., 2004).

1.3. Estresse oxidativo e suas implicações

Existem diversas espécies reativas, como as derivadas do oxigênio, nitrogênio, cloro, ferro e enxofre. Contudo, entre elas, as ERO estão envolvidas com maior frequência em alterações patológicas; entre as quais, destacam-se os radicais livres como o radical hidroxila ($OH^{\bullet-}$) e superóxido ($O2^{\bullet-}$), os quais são caracterizados por serem moléculas com um ou mais elétrons desemparelhados e por possuírem alta reatividade (HALLIWELL, 2011; RADOMSKA-LEŚNIEWSKA et al., 2015). As mitocôndrias possuem papel fundamental na geração de estresse oxidativo pois são a principal fonte de ROS (90%) celular. Elas são capazes de gerar radicas superóxidos a partir da reação entre oxigênio molecular e elétrons livres. Os superóxidos, embora possuam baixa reatividade, podem ser convertidos em outras espécies reativas como peróxido de hidrogênio que por sua vez pode facilmente gerar radicais livres e causar dano oxidativo (KANDOLA; BOWMAN; BIRCH-MACHIN, 2015).

O adequado equilíbrio entre a proteção antioxidante e as espécies reativas também conhecido como equilíbrio “redox” (Fg. 2.) é essencial para vida de todos os seres aeróbicos (HALLIWELL, 2011), entre os quais encontram-se seres dos reinos Plantae e Animalia. Contudo, quando existe um desequilíbrio “redox” os níveis elevados de espécies reativas são capazes de causar dano oxidativo as estruturas celulares como lipídios, proteínas, DNA nuclear e mitocondrial. Este dano é conhecido como estresse oxidativo que pode gerar citotoxicidade e inflamação, atuando tanto como causa ou consequência desde o envelhecimento a uma patologia previamente estabelecida como úlceras pépticas, gástricas e duodenais, câncer, hepatite e distúrbios neurológicos (VAYA, 2013).

Fig. 2. Espécies reativas de oxigênio e antioxidantes (http://www.efdeportes.com)



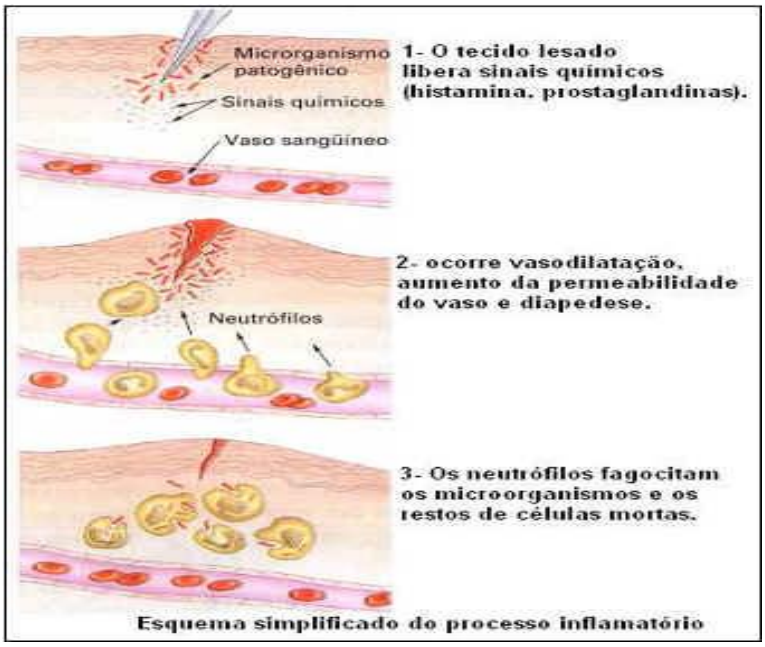
1.4. Inflamação

Qualquer trauma é capaz de levar a uma resposta inflamatória com graus variados de severidade. O processo inflamatório (Fig. 3.) representa um sistema de defesa dos seres humanos e animais, cuja finalidade está em cessar a causa inicial da lesão como exemplo a presença de patógenos (BISWAS, 2016; COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009). A área inflamada, em nível macroscópico apresenta quatro características principais: rubor, edema, calor, dor, diminuição ou perda de função. A reação inflamatória consiste de: uma resposta imunológica inespecífica e outra específica (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009).

A reação inicial é inespecífica e ocorre nos tecidos lesionados com alterações vasculares ou celulares. Entre as alterações vasculares ocorre aumento do fluxo sanguíneo e da permeabilidade celular, causando respectivamente calor e edema (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009). Nos eventos celulares ocorre a sinalização de agentes quimiotáticos

como citocinas e leucotrienos que atraem os leucócitos circulantes até local da lesão (BISWAS, 2016; COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009).

Fig. 3. Processo inflamatório (https://patofisio.wordpress.com).



Na resposta imunológica específica, além da produção de anticorpos pelos linfócitos, ocorre a migração dessas células até o local da lesão onde a exposição as lesões teciduais induzem a liberação de mediadores químicos como o ácido araquidônico e citocinas. No caso do ácido araquidônico, a ativação da enzima fosfolipase A_2 faz com que os fosfolipídios de membrana o liberem para ser metabolizado por ciclo-oxigenases como a COX-2, ou ainda por lipo-oxigenases. Através das ciclo-oxigenases ocorre a síntese de prostaglandinas como a PGE_2 com efeito vasodilatador, contribuindo para a formação de edema, dor e febre. As lipo-oxigenases sintetizam leucotrienos que possuem atividade quimiotática para leucócitos causando produção de superóxidos e dano tecidual (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009).

A regulação do processo inflamatório depende principalmente do equilíbrio entre citocinas pró- e anti-inflamatórias. Estas últimas estão presentes em condições fisiológicas, enquanto que as pró-inflamatórias atuam principalmente em lesões acentuadas (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009). Durante as reações inflamatórias, as citocinas são liberadas para regular a ação celular. As citocinas pró-inflamatórias favorecem a aderência leucocitária ao endotélio, aumentam a síntese de prostaciclina que desencadeia uma cascata de citocinas secundárias. Entre citocinas secundárias estão o TNF-α (Fator de Necrose Tumoral α) e a

IL-1 (Interleucina-1) que são liberadas principalmente por macrófagos ativados. Além disso, existem citocinas secundárias como as quimiocinas que atraem e ativam as células inflamatórias móveis (COUTINHO, MARCELA A. S.; MUZITANO, MICHELE F.; COSTA, 2009).

Existem evidências que apontam para ação de citocinas pró-inflamatórias sobre a causa do processo inflamatório agudo. Durante o processo inflamatório, o aumento da expressão do fator nuclear Kappa B controla a geração de citocinas pró-inflamatórias como o fator de necrose tumoral (TNF-α) e assim ativa os neutrófilos no processo inflamatório (KOLACZKOWSKA; KUBES, 2013). Os neutrófilos são os principais agentes da inflamação aguda e podem também contribuir para condições inflamatórias crônicas. Essas células são leucócitos polimorfonucleares e representam os principais agentes da inflamação aguda e geralmente são os primeiros leucócitos a participarem da resposta inflamatória (KOLACZKOWSKA; KUBES, 2013).

A invasão dos tecidos gastrointestinais por neutrófilos é identificada pela atividade aumentada da mieloperoxidase (MPO) que contribui para a lesão da mucosa gástrica (ARAB et al., 2015). A mieloperoxidase foi descoberta por Kjell Agner em 1941 a partir de neutrófilos presentes em uma amostra de empiema tuberculosa, contudo ela só recebeu esse nome em 1943 após um estudo comparativo com a enzima lacto-peroxidase (NAUSEEF, 2014). A MPO pode exercer ação antimicrobiana através do sistema MPO-H_2O_2-Cl descrito a seguir: A NADPH oxidase gera ânion superóxido (O^{2-}) e peróxido de hidrogênio; e juntamente com o Cl^- citoplasmático e em reações catalisadas pela mieloperoxidase formam ácido hipocloroso e alguns derivados capazes de exercer uma potente ação antimicrobiana no interior de fagossomos neutrofílicos (NAUSEEF, 2014).

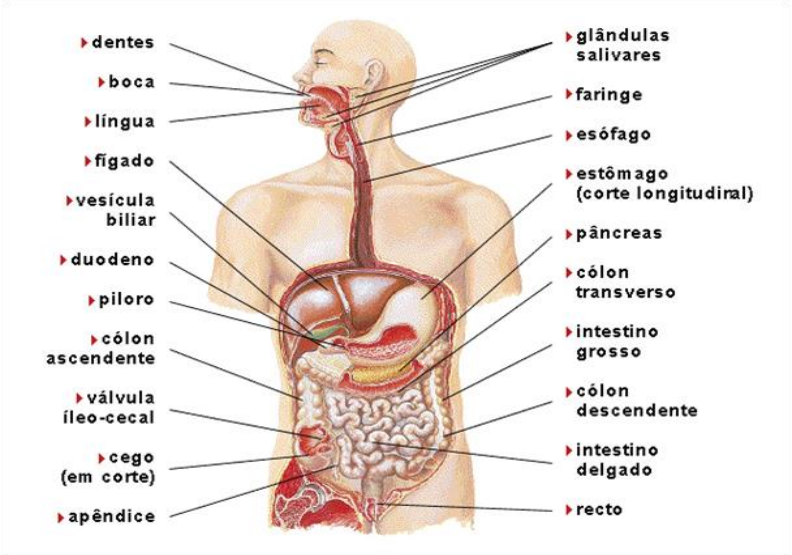
Em animais knock-out para MPO ocorre altos níveis de expressão da enzima óxido nítrico sintase induzível e consequentemente níveis maiores de óxido nítrico (TAKEUCHI et al., 2012). Além disso, a mieloperoxidase corresponde a um marcador anti-inflamatório inespecífico independente de sua ação antimicrobiana (KOLACZKOWSKA; KUBES, 2013; NAUSEEF, 2014). Quando ocorre uma lesão na mucosa gástrica, é desencadeada a invasão de células polimorfonucleares (PMN), indicado pela atividade da MPO que assim gerará ácido hipocloroso, levando a inflamação aguda e dano tecidual (NAUSEEF, 2014).

1.5. Sistema digestivo

A digestão é constituída pela atividade desempenhada pelo aparelho digestivo para transformar os alimentos em substâncias mais simples como nutrientes e minerais que possam ser absorvidas pelo organismo. O processo de digestão é realizado em diferentes etapas. Na etapa mecânica o tamanho dos alimentos é reduzido e nas etapas químicas geralmente moléculas complexas são transformadas em moléculas mais simples para que possam ser absorvidas (COSTANZO, 2014; Guyton & Hall, 2006).

O aparelho digestivo é constituído pela boca, faringe, esôfago, estômago, intestino delgado, intestino grosso, reto e ânus (Fig. 1). O intestino delgado é dividido em duodeno, jejuno e ílio. O intestino grosso, é dividido em seco, apêndice vermiforme, colo ascendente transverso, colo descendente sigmoide, reto e ânus (TAKEUCHI et al., 2012) (Fig. 4.).

Fig. 4. Sistema digestivo e glândulas anexas (Sistema digestório :Revista Biogênese, 2010).



Além disso, o sistema digestivo é auxiliado pelas glândulas anexas (Fig. 1.) como as glândulas salivares, fígado, vesícula biliar e pâncreas. As glândulas salivares produzem a saliva que contém a amilase para decompor o amido e são divididas em labiais, palatinas linguais; molares, parótida; submandibular e sublinguais. O fígado que possui entre outras importantes funções, produzir a bile. A vesícula biliar que armazena e secretada bile no duodeno principalmente para emulsão de gorduras. O pâncreas que possui função exócrina e endócrina, respectivamente produzindo suco pancreático e hormônios como somatostatina, através das ilhotas de Langerhans (TAKEUCHI et al., 2012).

O estômago constitui a parte expandida do trato digestório entre o esôfago e o intestino delgado e externamente recoberto por peritônio. As principais funções do estômago são armazenar grandes quantidades de alimento até que o alimento possa ser processado a partir dele, passando pelo duodeno, até o trato intestinal inferior. Além de misturar o alimento com secreções gástricas até formar uma mistura semifluida chamada quimo; e o esvaziamento lento do quimo do estômago para o intestino delgado, a uma velocidade adequada para a digestão e a absorção (COSTANZO, 2014).O estômago, na posição de decúbito dorsal, geralmente está localizado nos quadrantes superiores direito e esquerdo. Este órgão é dividido anatomicamente da seguinte forma: Cárdia, fundo gástrico, incisura cárdia, corpo gástrico, piloro, curvatura menor, incisura angular, curvatura maior (TAKEUCHI et al., 2012).

O interior do estômago é composto por uma mucosa contínua de castanho-avermelhado ou rósea na parte pilórica com a função de proteger o interior do estômago contra o suco gástrico (ácidos gástricos; pepsina) (TAKEUCHI et al., 2012). Vários tipos de glândulas proporcionam os diferentes tipos de secreções do trato gastrointestinal (TGI). As células de cálice estão presentes na superfície do epitélio do TGI e liberam muco sobre a superfície epitelial, o qual atua como protetor da mucosa contra o suco gástrico ou atrito do bolo alimentar. Entre essas células localizam-se as glândulas oxínticas, principalmente presentes no estômago e no duodeno, que são responsáveis pela secreção ácida e de pepsinogêneo (COSTANZO, 2014; TAKEUCHI et al., 2012).

Ao longo do intestino delgado existem poços profundos chamados de criptas de Lieberkühn e que estão repletos de células secretoras especializadas (COSTANZO, 2014; TAKEUCHI et al., 2012). Mecanismos básicos de estimulação das secreções gástricas ou intestinais pelas glândulas do TGI são controladas pelo SNE. E essas secreções ocorrem quando o contato do alimento com o epitélio causa estimulação tátil, irritação química ou distensão intestinal. Os reflexos nervosos resultantes estimulam ambas as células mucosas na superfície do epitélio intestinal e as glândulas profundas na parede intestinal para aumentar a sua secreção (COSTANZO, 2014). O TGI é regulado, em parte, pelo sistema nervoso autônomo, que tem o componente extrínseco (inervação simpática e parassimpática) e o componente intrínseco (SNE). O SNE está inteiramente contido nos plexos submucoso e mioentérico e se comunica, extensivamente com os SNP e SNS. Contudo, o sistema nervoso intrínseco ou SNE pode coordenar todas as funções do TGI mesmo na ausência de inervação extrínseca (COSTANZO, 2014).

1.5.1. Secreções gastrointestinais

O muco responsável pela proteção da parede gástrica contra enzimas digestivas e ácido hidroclorídrico é secretado principalmente pelas células mucosas das glândulas pilóricas. Essas glândulas também são responsáveis pela secreção do hormônio gastrina formado pelas células G. A gastrina é liberada na corrente sanguínea para ser transportada até as células ECL (Células enterocromafins) que causam a liberação de histamina diretamente nas glândulas oxínticas para estimular a secreção de ácido hidroclorídrico (COSTANZO, 2014).

Diferentes tipos de pepsinogênios com a mesma função são secretados pelas células mucosas e pépticas das glândulas oxínticas e ao entrarem em contato com o ácido hidroclorídrico do estômago formam pepsina. A pepsina é uma enzima proteolítica, com atividade ótima em pH entre 1,8-3,5 que é responsável pela digestão de proteínas presentes no alimento. A regulação da secreção de epsinogênio ocorre pela presença de ácido hidroclorídrico no lúmen do estômago ou a ação da acetilcolina que estimulam a secreção de ácido hidroclorídrico pelas célula pépticas das glândulas oxínticas (COSTANZO, 2014).

1.5.1.1. Hormônios envolvidos nas secreções gastrointestinais

Os hormônios gastrointestinais são liberados na circulação porta e ao atingirem as células-alvo se ligam a receptores específicos para desempenharem seu papel (COSTANZO, 2014). A gastrina é segregada pelas células "G" do antro do estômago em resposta a estímulos associados a ingestão de alimento, e suas principais funções são estimular a secreção de ácido gástrico e o crescimento da mucosa gástrica. A secretina é secretada pelas células "S" da mucosa duodenal e têm como função controlar a secreção pancreática de bicarbonato para neutralizar o suco gástrico no duodeno. Colecistocinina (CCK): é secretada pelas células I do duodeno e jejuno e é responsável por estimular a secreção de $HCO3^-$ no duodeno e jejuno. Somatostatina localizada nas células “D” do antro, controla a secreção ácida estimulada pela gastrina e, em menor proporção pela histamina (COSTANZO, 2014).

1.5.1.2. Processo de formação do ácido hidroclorídrico

O processo de formação do ácido hidroclorídrico ocorre dentro das células parietais, a água é dissociada em H^+ e OH^-. E a partir dessa dissociação, ocorre a concentração de íons H^+ e Cl^- no interior dos canalículos das glandular para a formação de ácido hidroclorídrico. A enzima H^+-K^+-ATPase, catalisa respectivamente a entrada e saída dos íons H^+ e K^+ nos canalículos das células parietais. Esse processo é facilitado pela baixa concentração de Na^+ intracelular criada pela Na^+/K^+-ATPase extracelular que cria um gradiente favorável a entrada de H^+ no interior dos canalículos. Após a dissociação da água, e a secreção de H^+, ocorre o acúmulo de OH^- que reage com CO_2, através de uma reação catalisada pela enzima anidrase carbônica, formando HCO_3^-. $OHCO_3^-$ é então trocado por íons cloreto (Cl^-) do fluido extracelular, que após entrarem na célula, são secretados pelos canais de cloro até os canalículos das células parietais para formarem o ácido hidroclorídrico. Este ácido será secretado posteriormente no lúmen celular, onde juntamente com a água e pequenas concentrações de cloreto de potássio e sódico formarão o suco gástrico (COSTANZO, 2014).

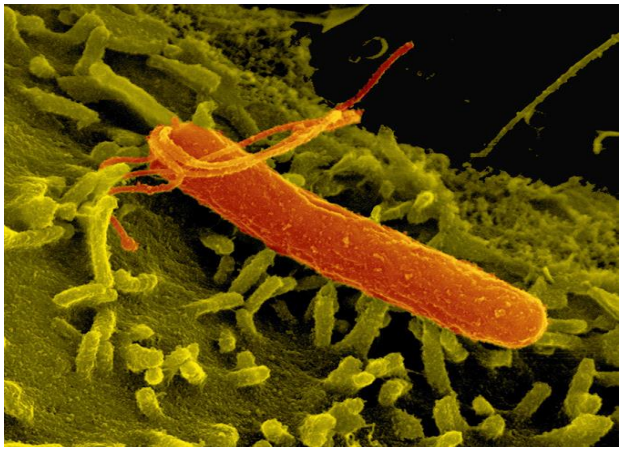
1.6. Úlceras pépticas

A úlcera péptica, que inclui principalmente lesões no estômago e duodeno, tem estado presente na morbidade e mortalidade da população mundial a décadas (COSTANZO, 2014; UYANIKOGLU et al., 2012). E essa patologia em grande parte dos casos está relacionada com o surgimento do câncer gástrico o qual está entre as três maiores causas de morte por câncer em 2011 segundo dados da organização mudial de saúde (OMS, 2011). As principais causas das úlceras pépticas são o desequilíbrio entre os fatores de proteção e agressão da barreira da mucosa gastroduodenal (COSTANZO, 2014). Entre os fatores de agressão destacam-se os níveis de secreção gástrica muito acima do fisiológico, idade, sexo, ou ainda o consumo excessivo de álcool etílico (UYANIKOGLU et al., 2012). Outros agentes agressores são representados pelo consumo frequente de anti-inflamatórios não esteroidais, tabagismo, ou ainda infecções por *Helicobacter pylori*. Esses agentes agressores são capazes de romper a barreira mucosa de proteção gastrointestinal causando lesões hemorrágicas (UYANIKOGLU et al., 2012).

A infecção por *H. pylori* (Fig. 5.) pode causar inflamação crônica que está presente em grande parte dos pacientes com úlceras péptica, gástrica, duodenal, podendo levar a patologias como adenocarcinomas e linfomas (UYANIKOGLU et al., 2012) e merece particular atenção devido a sua grande adaptação ao ambiente gástrico e habilidade de causar ulcerações. Entre os mecanismos usados pela *H. pylori* são conhecidos a sua capacidade de penetrar na barreira mucosa e

principalmente de liberar amônia a qual liquefaz a barreira de proteção e estimula a secreção de ácido hidroclorídrico (CYLWIK et al., 2005). Após a dissolução da barreira da mucosa, os sucos gástricos digestivos ácidos atingem o epitélio desprotegido e causam a destruição tecidual (COSTANZO, 2014;Guyton & Hall - 2006).

Fig. 5. *Helicobacter Pylori* (https://www.mpg.de/9264796/helicobacter-pylori-fingerprint-gastric-cancer).



O consumo de álcool etílico pela população mundial tornou-se muito frequente devido ao grande consumo de bebidas alcóolicas. Contudo, a excessiva ingestão de etanol pela população pode causar anormalidades gastrointestinais como úlceras pépticas, gástricas e duodenais, bacteremia (BECK et al., 2004; SØREIDE; THORSEN; SØREIDE, 2014), diminuição do fluxo sanguíneo local, geração de espécies reativas, diminuição da proliferação celular e resposta inflamatória elevada (JIANG, 2015; TARNAWSKI; AHLUWALIA; JONES, 2014).

1.6.1. Farmacoterapêutica da úlcera

O desenvolvimento de potentes supressores ácidos assim como a descoberta da *H. pylori* são dois importantes passos que causaram a redução da prevalência da úlcera péptica (WU et al., 2010). Entre os principais inibidores ácidos já utilizados no tratamento das úlceras pépticas encontram-se principalmente fármacos que atuam no controle da secreção ácida. E entre eles os principais são os antiácidos, citoprotetores, bloqueadores de receptor H_2 e inibidores da bomba de prótons.

Os antiácidos que possuem a capacidade de neutralizar parcialmente o conteúdo ácido do suco gástrico elevando o pH local, como o bicarbonato de sódio e os hidróxidos de alumínio (Sanofi -Bula HIDRÓXIDO DE ALUMINIO) ou magnésio (Gsk-Bula LEITE DE MAGNESIA DE PHILLIPS). Os inibidores da secreção ácida como os bloqueadores de receptor histamínico H_2, como a cimetidina (Prati Donaduzzi - Bula CIMETIDINA) realizam uma competição reversível pelos sítios dos receptores H_2 nas células parietais. Os inibidores da enzima H^+/K^+-adenosina trifosfatase (H^+/K^+-ATPase) ou bomba de prótons como o omeprazol (Medley - Bula OMEPRAZOL), e o lanzoprazol (Medley - Bula LANSOPRAZOL) são capazes de inibir de forma específica e irreversível a bomba de prótons, impedindo assim a formação de ácido hidroclorídrico pelas células parietais. Os citoprotetores geralmente possuem mais de um mecanismo de proteção da mucosa gastrointestinal. Entre esses fármacos, alguns dos mais utilizados são: Soluções coloidais de Bismuto como peptulan que possuem basicamente 4 mecanismos distintos, como a formação de uma película insolúvel que se deposita sobre o tecido da úlcera e assim protegendo os tecidos lesionados contra os sucos gástricos, inibe a atividade da pepsina, e é bactericida contra *H. pylori*. Contudo é contraindicado em casos de doenças renais, mulheres em estado de amamentação, além de alterar o tempo de esvaziamento gástrico e absorção de alguns medicamentos (Farmasa - Bula PEPTULAN).

Entre os principais tratamentos adotadas para a erradicação da *H. pylori* estão a terapêutica tripla (TT) convencional que inclui inibidores da bomba de prótons (IBP) mais claritromicina e amoxicilina ou metronidazol, de 7 a 14 dias. No entanto, devido ao aumento da resistência a esse tratamento, em alguns casos é necessário fazer uso da terapêutica sequencial (TS). Na TS, os fármacos são empregados em um regime sequencial diferenciado incluindo o uso de IBP associado a amoxicilina nos primeiros cinco dias, seguido de IBP e claritromicina mais metronidazol ou tinidazol ou amoxicilina nos cinco dias subsequentes (MACEDO et al., 2015).

1.7. Bactérias

As bactérias fazem parte do reino Archaea e Bactéria e entre elas encontram-se as comensais que geralmente habitam o meio ambiente ou outros seres vivos sem causar nenhuma patologia. Entre essas bactérias encontram-se as da flora intestinal humana que pertencem as da família Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria (BLOEMENDAAL et al., 2016). Contudo, existe um grande número de bactérias patogênicas que comumente causam sérios problemas a saúde de seres humanos e animais (MATTOCK et al., 1982; PEWZNER-JUNG et al., 2014; SCHAEFFER et al.,

2015). Entre as bactérias com potencial para causar doença destacam-se os *Staphylococcus aureos*, *Staphylococcus epidermidis*, a *Pseudomonas aeruginosa* e o *Bacillus cereus* que foram analisados na presença de eeRo e suas frações.

A *Staphylococcus aureos* é membro da família Micrococcaceae, formando cocos gram-positivos agrupados. Essas bactérias possuem colônias de coloração dourada. O *Staphylococus aureos* liga-se a células endoteliais através de receptores de adesão e são fagocitados por essas células contribuindo para a progressão de doenças endovasculares. Essas características são capazes de levar ao surgimento de patologias como endocardite, osteomielite, pneumonia, meningite ou ainda sepse (ASLAM et al., 2013; LOWY, 1998). O *Staphylococcus epidermidis* é uma bactéria gram positiva pertencente a família Staphylococcaceae. Essa bactéria é um patógeno oportunista que possui a capacidade de formar biofilmes em instrumentos hospitalares como cateteres. Essa característica, protege as bactérias contra as defesas imunológicas do hospedeiro assim como as torna mais resistentes a ação de antibióticos (VUONG; OTTO, 2002). Assim, o *Staphylococcus epidermides* tem se tornado uma das mais importantes causas das infecções nosocomiais como infecções articulares prostéticas (SCHAEFFER et al., 2015; VUONG; OTTO, 2002).

A *Pseudomonas aeruginosa* é um bacilo gram negativo da família *Pseudomonadaceae* frequentemente encontrado em infecções nosocomiais. Esta bactéria geralmente está presente na etiologia de patologias respiratórias como a pneumonia e se instala principalmente em pacientes tratados com imunossupressores em casos de queimaduras graves, câncer e fibrose cística (MATSUO et al., 2015; PANGHAL et al., 2012; PEWZNER-JUNG et al., 2014; YANG et al., 2011). O *Bacillus cereus* que é um bacilo gram positivo da família Bacillaceae que possui como habitat natural o solo. Essa bactéria é transmitida principalmente por vegetais contaminados e é responsável por um considerável número de infecções gastrointestinais, causando forte diarréia (EHLING-SCHULZ; FRENZEL; GOHAR, 2015; KOTIRANTA; LOUNATMAA; HAAPASALO, 2000).

Assim, devido a importância do estresse oxidativo, assim como do processo inflamatório e antibacteriano sobre a saúde de animais e humanos, com implicações gastrointestinais como úlceras pépticas que atingem grande número de indivíduos da população mundial, torna-se de grande relevância a busca de novos medicamentos ou nutracêuticos que possam combater efetivamente as patologias relacionadas a essas alterações. Dessa forma, a adequada investigação do alecrim poderia ter boas perspectivas curativas a partir do que já se conhece sobre suas aplicações populares e em pesquisas precedentes.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Analisar as propriedades do eeRo e suas frações (DCM, EA e ButOH), com diferentes características constitucionais, buscando um possível suplemento alimentar, nutracêutico ou fitoterápico que possa envolver mecanismos convergentes para o tratamento de patologias gastrointestinais.

2.2. OBJETIVOS ESPECÍFICOS

- Investigar a hipótese do eeRo e suas frações possuirem efeitos antioxidantes semelhantes ou superiores aos encontrados em padrões adequados;
- Analisar as propriedades antioxidantes do eeRo e suas frações em cérebro, estômago e fígado;
- Esclarecer os possíveis efeitos do eeRo e suas frações sobre bactérias com potencial patogênico gram (+), e gram (-).
- Exclarecer os mecanismos antioxidante, vasodilatador e anti-inflamatório do eeRo envolvidos na prevenção de ulcerações gástricas causadas pelo etanol;
- Investigar os possíveis efeitos antioxidantes e anti-inflamatórios do eeRo na prevenção de lesões intestinais causadas por etanol.

3. ARTIGO E MANUSCRITOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo e manuscritos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprios manuscritos e artigo. Os manuscritos e artigo estão dispostos na forma em que foram publicados ou submetidos para as revistas.

3.1. MANUSCRITO 1: ANTIBACTERIAL AND ANTIOXIDANT EFFECTS OF *Rosmarinus officinalis* L. EXTRACT AND ITS FRACTIONS

Manuscrito científico submetido na revista Ethnopharmacology, 2016

ANTIBACTERIAL AND ANTIOXIDANT EFFECTS OF *Rosmarinus officinalis* L. EXTRACT AND ITS FRACTIONS

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

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Abstract

The elevate production of reactive species over physiological levels and associated to pathogenic bacteria could represent a high risk for many diseases. The *Rosmarinus officinalis* L. is world widely used due its pharmacological proprieties against some diseases mainly due its phenolic compounds. So, in this study our aim is to test the hypothesis that *R. officinalis* L. extract (eeRo) and its fractions (DCM, EA, ButOH) could have better or similar antioxidant action to standarts *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, DCM, EA, *in vitro*, had significant total antioxidant and $DPPH^{\bullet-}$ radical scavenging activities. The DCM and eeRo got significant effects against basal levels of reactive species in the 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 (+) and gram (-) bacteria. In conclusion, we inferred that DCM and eeRo, in general form, have the best antioxidant and antibacterial effects that should be used against many related diseases in different organs.

Key words: Oxidative stress; HPLC; microorganisms; medicine; gallic acid; ascorbic acid.

1. Introduction

Pathogenic bacteria can release toxins capable of causing lesions in different organs (WILLIAMS; DEHNBOSTEL; BLACKWELL, 2010) and the oxidative stress is produced as a consequence of these bacterial lesions (FU; YUAN; GAO, 2015b). Thus, oxidative stress caused by bacterial infections represents a potential health risk to various organs such as the liver (MIHAILA et al., 2012) stomach (BHATTACHARYYA et al., 2014a) and brain (DAHLBERG; IVANOVIC, 2015). To synthesize such a medicine in the laboratory would be neither cost- nor time-effective, whereas medicines derived from the constituents of plants (AMARAL et al., 2013a) benefit from having undergone nature's toughest test, natural selection (BOERO, 2015). Under normal conditions, levels of reactive oxygen spcies (ROS) are controlled by endogenous antioxidante systems (VÁZQUEZ-MEDINA et al., 2012). However, elevated production of free radicals over physiological levels, either directly or indirectly by lipid peroxidation (KWIECIEN et al., 2014), is a serious component of many diseases, including gastric ulcers (BHATTACHARYYA et al., 2014a) and carcinogenesis (MA et al., 2013).

The *R. officinalis* L. belongs to the Labiatae family. It is popularly known in Europe as rosemary (CHUN et al., 2014) and in Brazil as Alecrim (PRINS, C.L.; LEMOS, C.S.L.; FREITAS, 2006). In many parts of the world, this plant is used for flavoring food, drinks, and cosmetics (NGO; WILLIAMS; HEAD, 2011). Some studies have demonstrated pharmacological effects of *R. officinalis* L. against inflammatory processes (MARA et al., 2015), hepatotoxicity (NGO; WILLIAMS; HEAD, 2011), atherosclerosis, ischaemic heart disease, respiratory disorders, gastric ulcers, some kinds of cancers (AMARAL et al., 2013a). The known pharmacological effects of this plant are attributable to its phenolic components, such as rosmarinic acid, carnosic acid, and carnosol (MARA et al., 2015). These phenolic compounds have naturally strong antioxidant effects; among them, carnosic acid and carnosol account for 90% of antioxidant activity of *R. officinalis* L. (MULINACCI et al., 2011).

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 (RODRÍGUEZ-MORATÓ et al., 2015; SHAH et al., 2013). 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 (EL HOUFI

et al., 2015; LE MOING et al., 2015). The s*taphylococcus epidermidis* is generally found on human skin and mucosal surfaces (ZIEBUHR et al., 1997) they are considered opportunistic microorganisms responsible for cardiovascular pathologies (SAHAL; BILKAY, 2014) and nosocomial infections (ZIEBUHR et al., 1997). The *P. aeruginosa* is a pathogen that is hospital-acquired. It remains inside mucus and grows under anaerobic conditions (RANGEL et al., 2015) and causes bronchiolitis and pneumonia (WILLIAMS; DEHNBOSTEL; BLACKWELL, 2010). The *B. cereus* is adapted to growth in the intestinal tract of mammals and can cause emesis, diarrhea, gastroenteritis, and foodborne disease (AL-ABRI et al., 2011). 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 *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 *Rosmarinus officinalis* L. (eeRo).

The eeRo was obtained from the dried leaves (40ºC) 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, 60–70ºC) in the Soxhlet apparatus with some modifications in relation to original technique (ASHOK et al., 2010). 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 (KAMDEM et al., 2012).

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 x 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 (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). 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 at 518 nm 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 (PRIETO; PINEDA; AGUILAR, 1999). 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 95ºC 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 hours light: 12 hours 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 Care and Use of Experimental Animal Resources. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Maria, Brazil (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 4000g at 4ºC for 10 min to yield a low-speed supernatant fraction (S_1) for each tissue that was used for SNP-induced lipid peroxidation and H_2DCF-DA assays.

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 37ºC 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 (OHKAWA; OHISHI; YAGI, 1979).

2.8.2. H_2DCF-DA assay

2'-7'-Dichlorofluorescein (DCF) levels was used to evaluate the cellular formation of reactive oxygen species (ROS) (MYHRE et al., 2003). 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 (BRADFORD, 1976).

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).

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 twenty four hours at 37ºC. A total of 50 μL of the standardized microorganism suspension 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. The plates were incubated for twenty four hours at 37ºC. 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% (PALOMINO et al., 2002).

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).

3.2. Total antioxidant capacity (TAC) assay

The ascorbic acid at 30 μg/ml was used as the control (data not shown). 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 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 (data not shown).

3.4.The H_2DCF-DA assay

In liver, the eeRo at 300 μg/ml decreased basal levels of ROS (Fig. 3) similarly to gallic acid (data not shown). 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). Similar to the antioxidant standard (data not shown), 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. 5) and gallic acid (data not shown) 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 (data not shown).

3.5. SNP-induced lipid peroxidation assay

In liver, the gallic acid (data not shown) 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 (data not shown). 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 (data not shown) and the eeRo, DCM, and EA significantly protected brain against the effects of lipid peroxidation induced by SNP (Fig. 7). Furthermore, gallic acid (data not shown) 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 (Tab. 2). The order of approximate decrease in efficiency for bacteria were as follow as:

a) The S. aureus : DCM (better efficiency) DCM > eeRo > EA > ButOH (anyone effect);

b) The S. epidermidis: DCM (better efficiency) eeRo = DCM > EA > ButOH (worst efficiency);

c) The P. aeruginosa: DCM (better efficiency) DCM = eeRo > EA = ButOH (worst efficiency);

d) The B. cereus: DCM (better efficiency) DCM = eeRo > EA > ButOH (anyone effect).

4. Discussion

Various plants have been used for years in traditional medicine due to their advantageous effects (ALLAHYARI; DELAZAR; NAJAFI, 2014). After thorough analysis of the 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 (Figs. 1-7) effects significantly higher than or similar to antioxidant standards (data not shown). In addition, these compounds significantly inhibited Gram (+) and Gram (-) bacterial colony

growth (Table 2). Supraphysiological ROS levels are a main cause of human disease (Stefanello et al. 2013). 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 (data not shown). 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 (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). 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 (BHATTACHARYYA et al., 2014b). 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 (Table 1), similar to the standard tested (data not shown). 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 (PISOSCHI; POP, 2015). In this context, the DCF is highly reactive with ROS and has been appropriately considered a marker of oxidative stress in animal tissue assays (MYHRE et al., 2003). 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) (LAVIE, 2015). Thus, they can be considered effective antioxidative compounds.

In the liver, the eeRo, DCM, and EA decreased basal ROS levels (Fig. 3) similarly to gallic acid (data not shown). Therefore, the DCM and EA can be considered to have antioxidant effects, since their effects are present at a concentration smaller than gallic acid (data not shown), a recognized antioxidant standard (ROSENBLAT et al., 2015). 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 (CARVALHO et al., 2013; PUNTEL et al., 2013).

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 (Tables 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 (AMARAL et al., 2013a; INEU et al., 2013; SØREIDE; THORSEN; SØREIDE, 2014). 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 (TATARANNO; PERRONE; BUONOCORE, 2015). 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 (STEFANELLO et al., 2015). 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 (AMARAL et al., 2012). Accordingly, the *R. officinalis* L. extract and fractions were tested in presence of sodium nitroprusside.

In the liver, the eeRo and DCM (Fig. 6) were similar to GA (data not shown), 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. This organ is very sensible to damage caused by ROS (Fig. 7) (DA SILVA et al., 2012). 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 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 (RAY; HUANG; TSUJI, 2012).

In the brain (Fig. 7), the DCM, eeRo, and EA had similar protective effects, probably due to their composition (Tables 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 (FU; YUAN; GAO, 2015b). 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 (LOWY, 1998) and endocarditis; *S. epidermidis*, present in cases of endocarditis (CHU et al., 2004); *B. cereus*, a common bacteria in gastroenteritis pathogenesis (AL-ABRI et al., 2011); and *P. aeruginosa*, a dangerous pathogenic microorganism in endocarditis (BHATTACHARYYA et al., 2014a; HASSAN; AL-RIYAMI, 2012) and respiratory infections (WILSON; DOWLING, 1998) (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 (+) and Gram (-) 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).

5. Conclusions

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 (+) and Gram (-) 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 and associated bacterial infections.

Conflict of Interest

None declared

Acknowledgments

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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

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.

	S.aureus	*S.epidermidis*	*P.aeruginosa*	*B.cereus*
Coumponds	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**
eeRo	128	16	128	32
DCM	64	16	128	32
EA	256	32	512	256
ButOH	-	512	512	-

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.

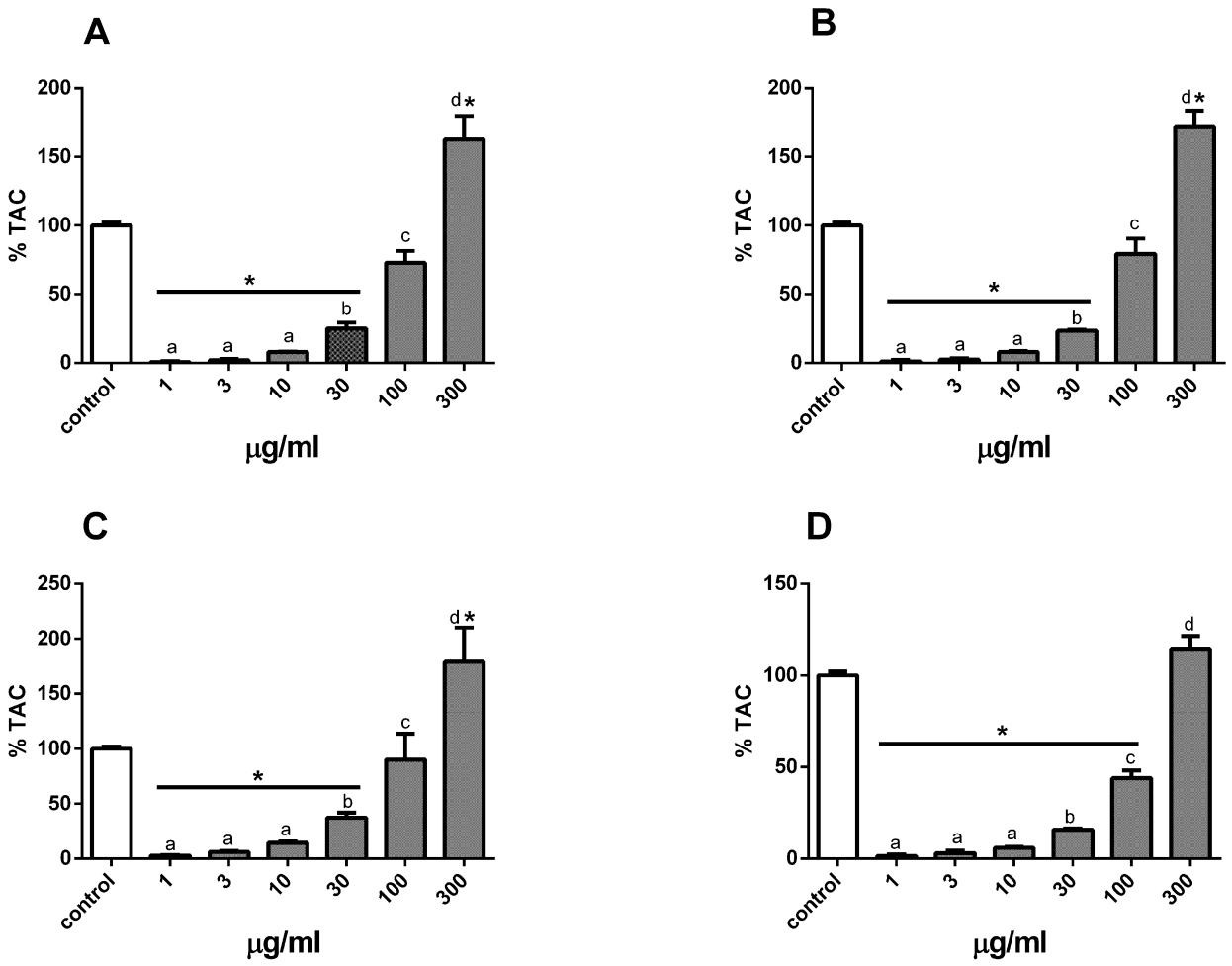

Figure 1. Assay of total antioxidant capacity assay (TAC) of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D). The ascorbic acid was used as control at 30 μg/ml. Data are reported as means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

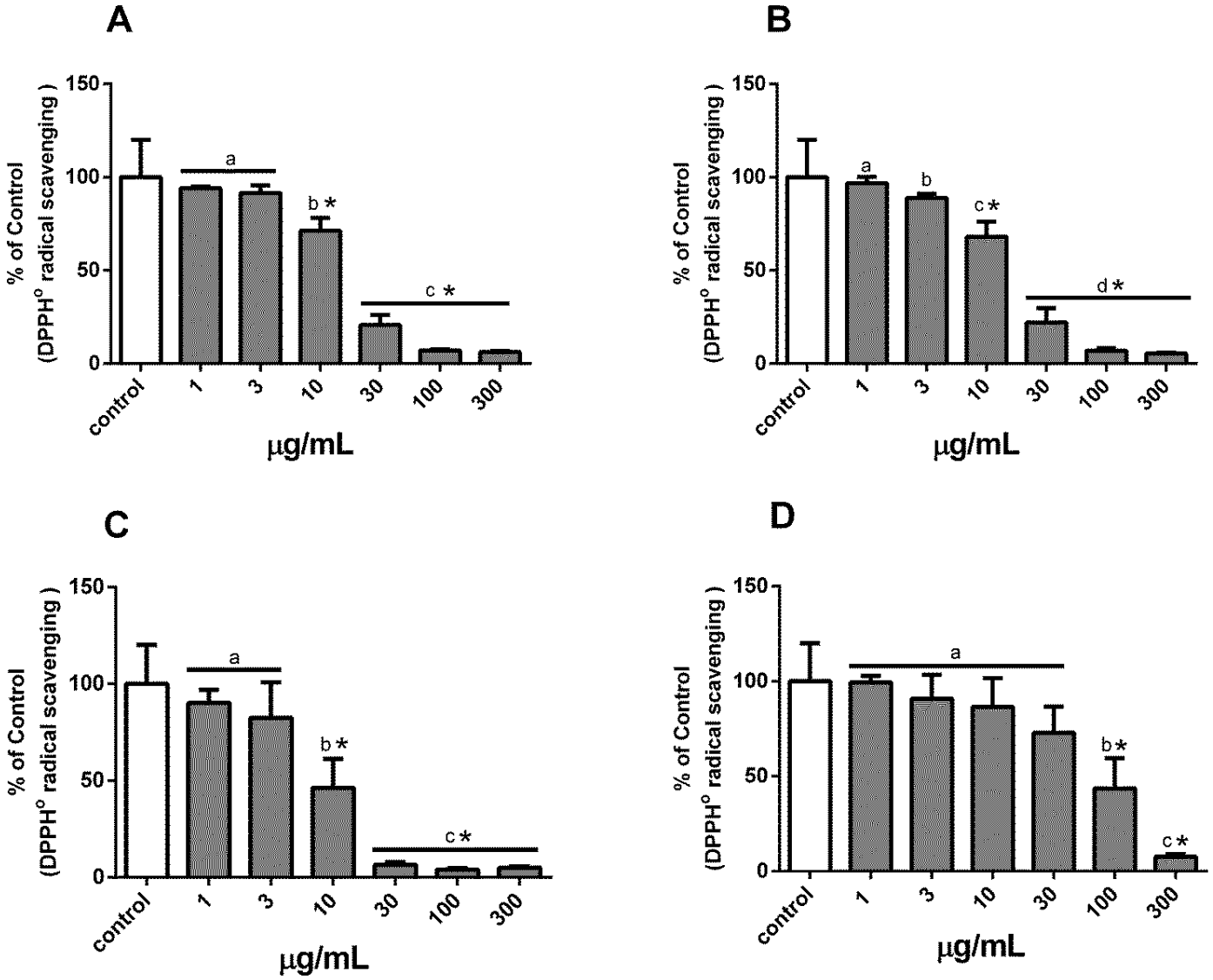

Fig. 2. Analyze of $DPPH^{\bullet}$ radical scavenging capacity of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D). Data are reported as means ± SEM of three to six determinations. One-way 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.

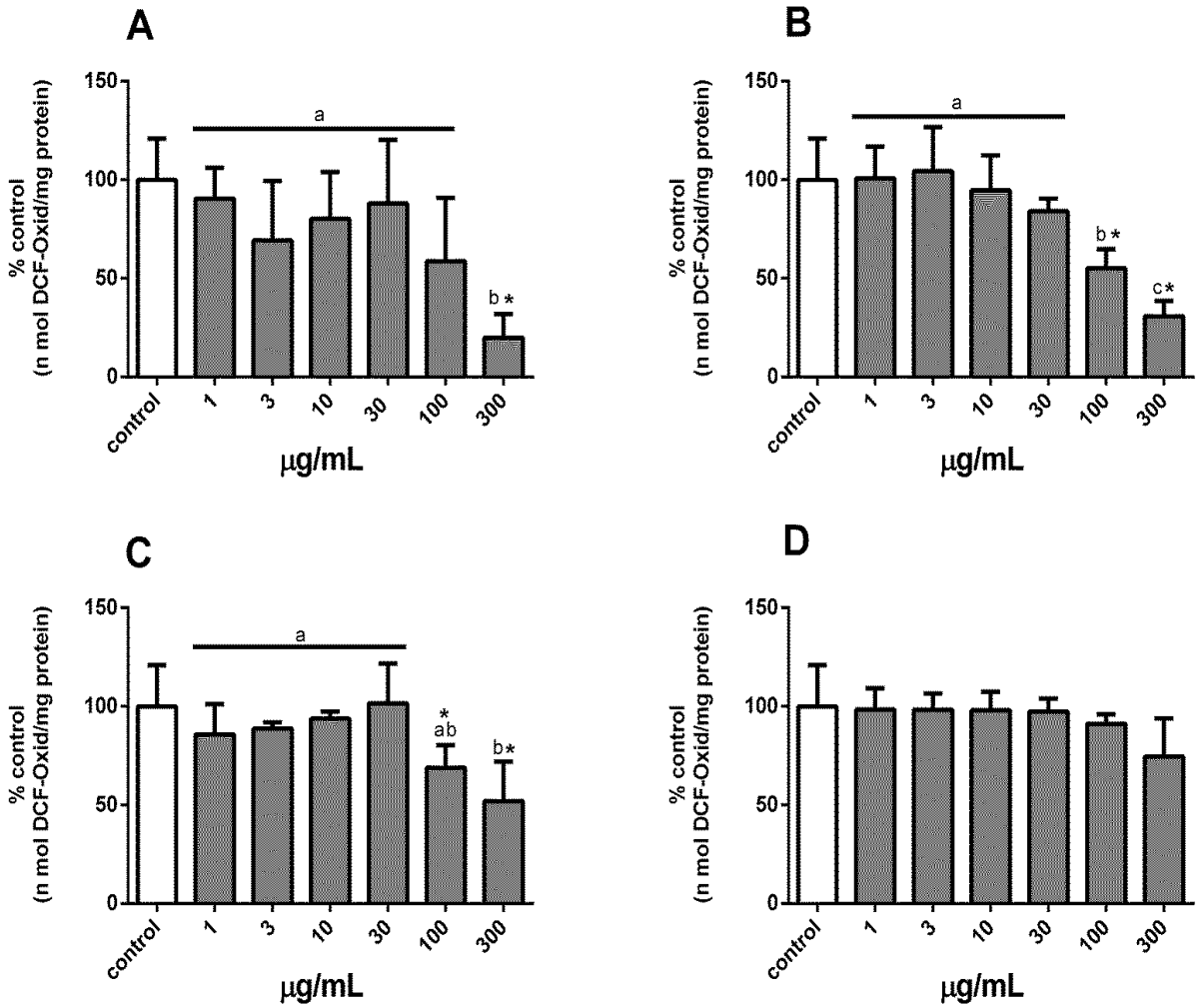

Fig. 3. Test of protective effect of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) on basal formation of reactive oxygen species (ROS) in the **liver**. Data are reported as means ± 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.

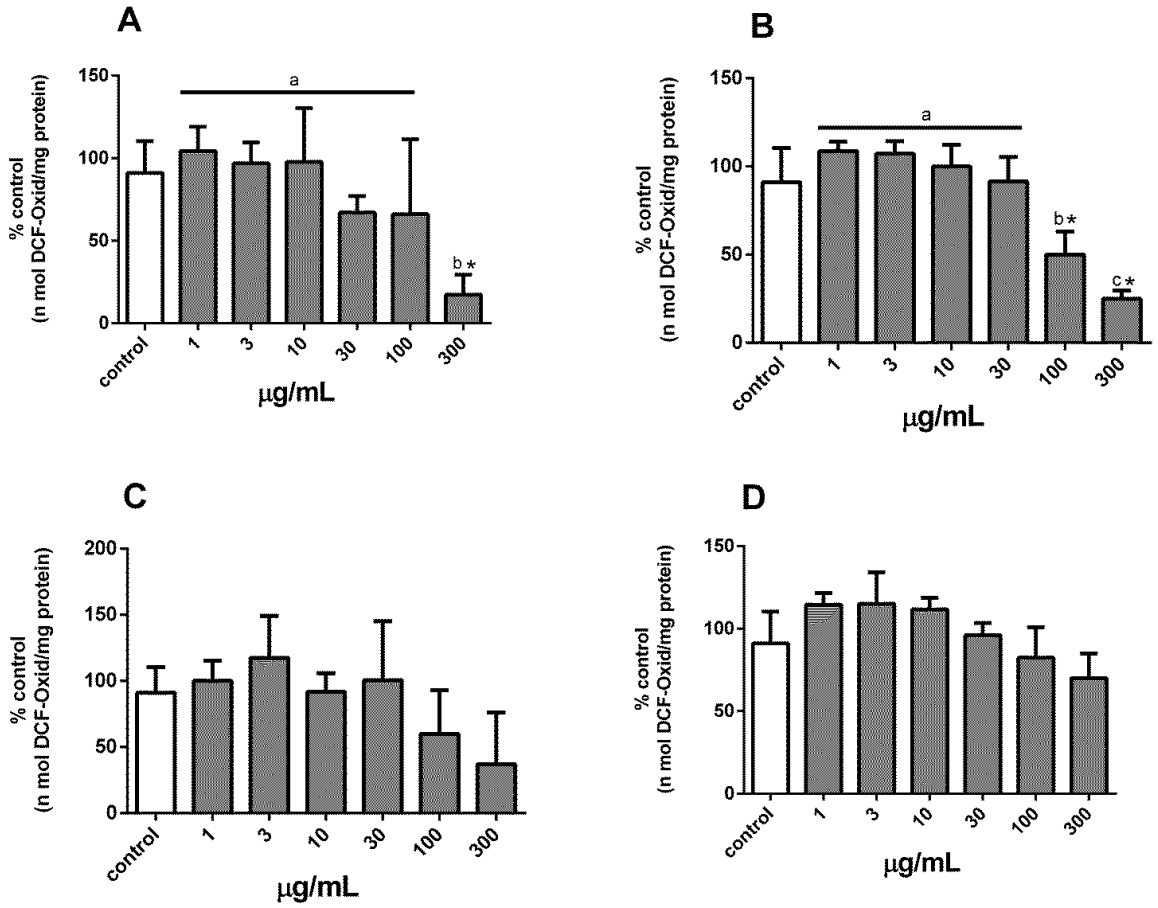

Fig. 4. Test of protective effect of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) on basal formation of reactive oxygen species (ROS) in the **stomach**. Data are reported as means ± 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.

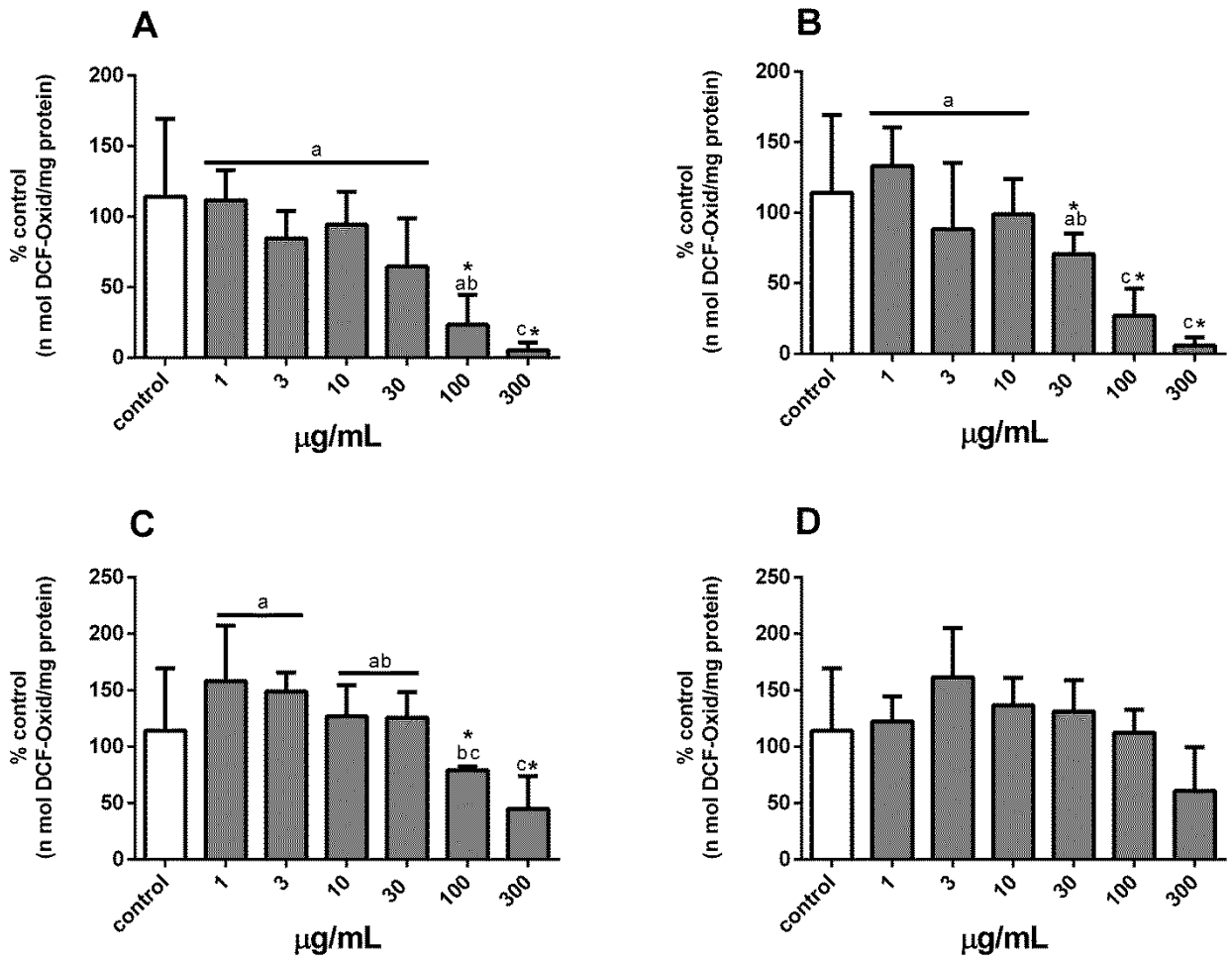

Fig. 5. Test of protective effect of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) on basal formation of reactive oxygen species (ROS) in the **brain**. Data are reported as means ± 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.

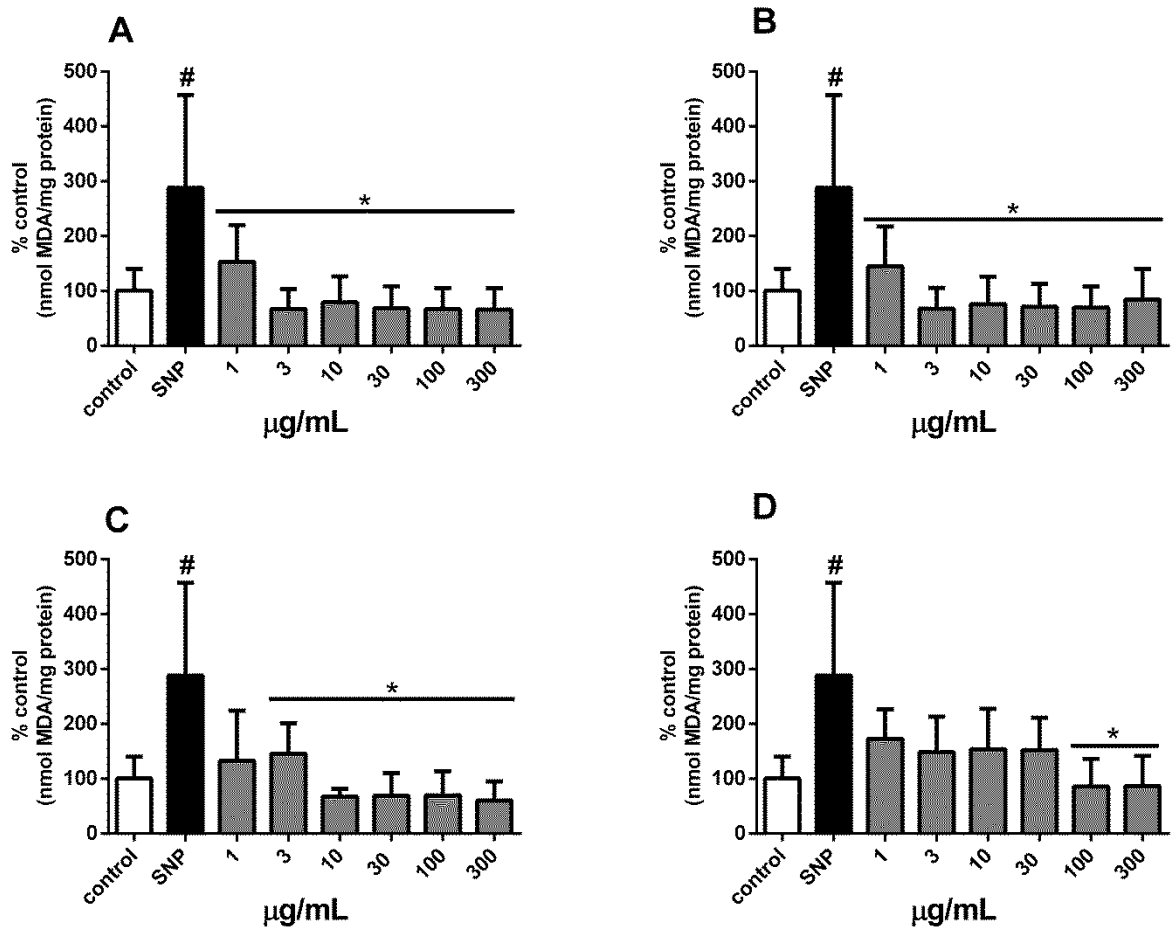

Fig. 6. Analysis of the protective action of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) on lipid peroxidation induced by sodium nitroprusside (SNP) at 10 µM in the **liver**. Data 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.

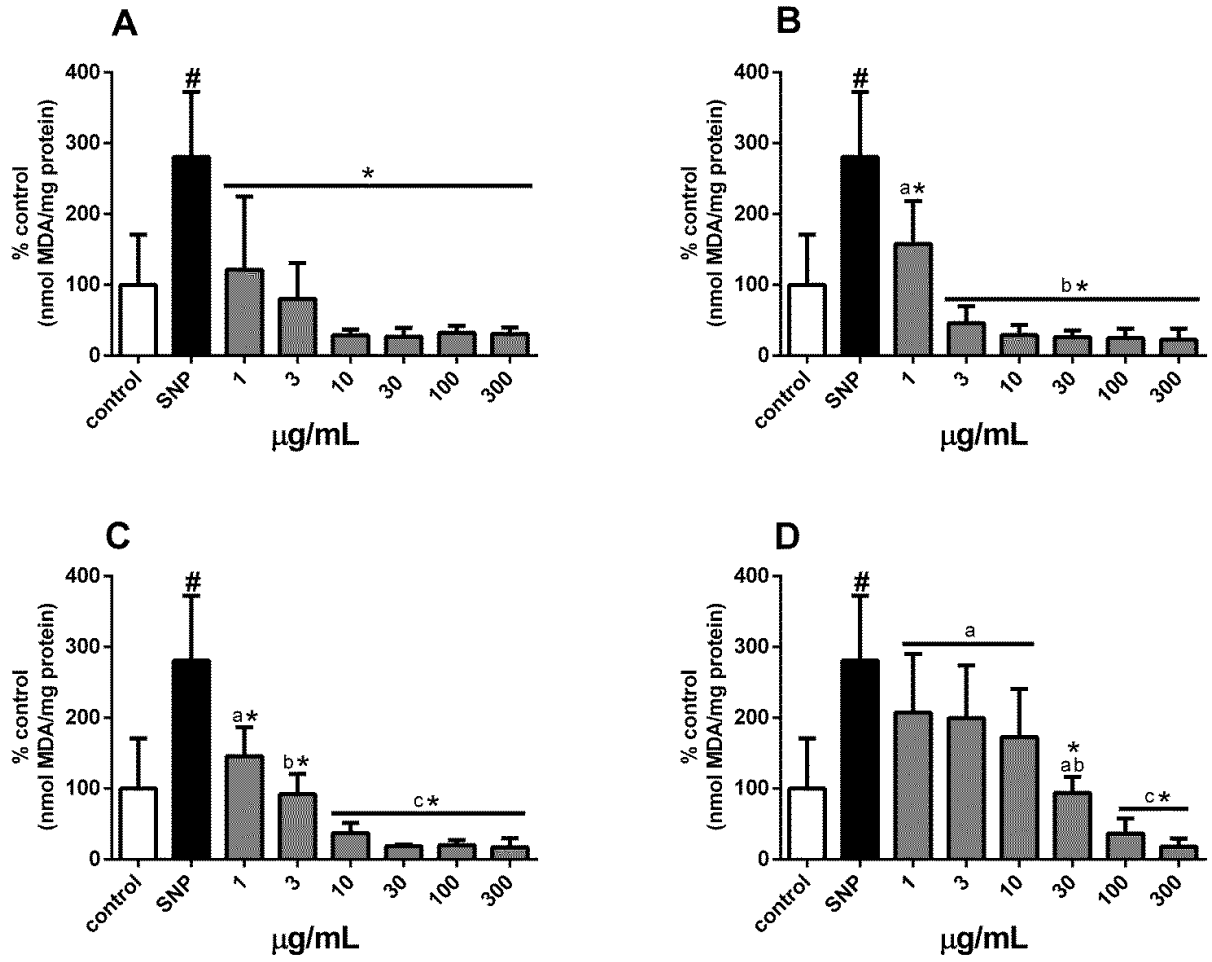

Fig. 7. Analysis of the protective action of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) (A) and its fractions: DCM (from dichloromethane) (B), EA (from ethyl acetate) (C), ButOH (from buthanol) (D) on lipid peroxidation induced by sodium nitroprusside (SNP) at 10 µM in the **brain**. Data 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.

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3.2. ARTIGO 1: PROTECTIVE ACTION OF ETHANOLIC EXTRACT OF *Rosmarinus officinalis* L. IN GASTRIC ULCER PREVENTION INDUCED BY ETHANOL IN RATS

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Protective action of ethanolic extract of *Rosmarinus officinalis* L. in gastric ulcer prevention induced by ethanol in rats

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ABSTRACT

The pathology of a gastric ulcer is complex and multifactorial. Gastric ulcers affect many people around the world and its development is a result of the imbalance between aggressive and protective factors in the gastric mucosa. In this study, we evaluated the ethanolic extract of *Rosmarinus officinalis* L. (eeRo); this plant, more commonly known as rosemary, has attracted the interest of the scientific community due to its numerous pharmacological properties and their potential therapeutic applications. Here, we tested the preventive effects of eeRo against gastric ulcer induced by 70% ethanol in male Wistar rats. In addition, we aimed to clarify the mechanism involved in the preventive action of the eeRo in gastric ulcers. Based on the analysis of markers of oxidative damage and enzymatic antioxidant defense systems, the measurement of nitrite and nitrate levels and the assessment of the inflammatory response, the eeRo exhibited significant antioxidant, vasodilator and antiinflammatory properties.

1. Introduction

Gastric ulcers are common pathologies (Ineu et al., 2008; Shaker et al., 2010) that affect a significant number of people around the world. Some authors have referred to gastric ulcers as the new "plague of the 21st century" (O'Malley, 2003). The development of gastric ulcers is a complex and multifactorial process, occurring from an imbalance between aggressive and protective factors present in the gastric mucosa (Choi et al., 2009; Shaker et al., 2010). Some etiologies of gastric ulcers include increased acid secretion and pepsin activity, reduced mucus and bicarbonate secretion, imbalanced bile salt secretion, the presence of *Helicobacter pylori*, increased gastric contractions and decreased blood flow (Galuska et al., 2002; Hoogerwerf and Pasricha, 2001a).

Furthermore, the increased incidence of gastric ulcers (Choi et al., 2009; Correa and Houghton, 2007) is associated with aggressive factors against the gastric mucosa such as ethanol exposure, stress, smoking, nutritional deficiencies and frequent ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) (Belaiche et al., 2002; Shaker et al., 2010). The key defense factors of the gastric mucosa include the secretion of bicarbonate and prostaglandin, increased levels of antioxidants (Hoogerwerf and Pasricha, 2001b), and maintaining adequate levels of nitric oxide (NO). NO dilates blood vessels, increases blood flow and stimulates gastric angiogenesis in the healing process of ulcers (Yang et al., 2000). NO also stimulates cell proliferation of the gastric mucosa and granulation tissue formation at the base of an ulcer (Yang et al., 2000).

There are many different experimental models of gastric ulcer induction, including ethanol and acetic acid (Shaker et al., 2010). Using such animal models, researchers simulate conditions to which humans may be exposed and, as a result, develop gastric ulcers.

Ethanol is known as a cause of gastric damage by altering protective factors, including decreasing mucus production and blood circulation within the mucosa (Choi et al., 2009; Ineu et al., 2008). In addition, the gastric damage caused by ethanol may be due to the generation of reactive species (RS), decreased cell

Abbreviations: eeRo, ethanolic extract of *Rosmarinus officinalis* L.; Omep, omeprazole; etOH, ethanol.

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proliferation, and an exacerbated inflammatory response (Choi et al., 2009; Ineu et al., 2008). The production of RS and a concomitant reduction of antioxidant capacity are responsible for cell damage and death due to their extreme reactivity. The RS attack essential cell constituents such as proteins, lipids and nucleic acids, and causing the formation of toxic compounds (Kaharaman et al., 2003). Another key factor in the pathogenesis of gastric ulcers is the presence of gastric acid secretion. The high acidity of gastric acid can worsen existing damage or potentiate the action of an aggressive agent on the gastric mucosa (Savegnago et al., 2006).

Therefore, controlling the formation of RS and secretion of gastric acid are essential for the treatment of these pathologies. The plant *Rosmarinus officinalis* L. (*Labiatae*), popularly known in Europe as rosemary and in Brazil as alecrim, is native of Europe, but it is widely distributed throughout the Brazilian territory (Machado et al., 2013). This plant is known for its many uses in food, but it is gaining interest for its pharmacological properties (Mulinaccia et al., 2009; Nabekuraa et al., 2010). Two groups of compounds are primarily responsible for the biological activity of this plant, the volatile fraction and phenolic constituents as rosmarinic acid (Pereira et al., 2005) and fractions of flavonoids and diterpenes, which are structural derivatives of carnosic acid (Pérez-Fons et al., 2006).

There is evidence that the aqueous and alcohol of extracts of the leaves of *R. officinalis* L. have a large number of pharmacological (Nabekuraa et al., 2010; Pereira et al., 2005) properties, including hepatoprotective (Sotelo-Félix et al., 2002), antibacterial (Del Campo et al., 2000), antithrombotic (Yamamoto et al., 2005), antiulcerogenic (Dias et al., 2000), diuretic (Haloui et al., 2000), antidiabetic (Bakirel et al., 2008), antioxidant (Bakirel et al., 2008), antinoceptive (González-Trujano et al., 2007), antiinflammatory (Altinier et al., 2007), and antidepressant (Heinrich et al., 2006) activities. The extracts are also effective in treating gastrointestinal disorders (Dias et al., 2000). The properties of extracts of *R. officinalis* L. are closely related to their phenolic compounds, especially the more abundant constituents, carnosic and rosmarinic acids, which are also known for various biological properties (Mulinaccia et al., 2011; Pereira et al., 2005).

Thus, we believe that the study of the possible beneficial effects of the ethanolic extract of rosemary in the pathogenesis of gastric ulcers is of great importance. Particularly, the macroscopic, microscopic and biochemical changes involved in gastric ulcers induced by ethanol deserve attention. Our goal is to clarify the protective mechanisms of the ethanolic fraction of *R. officinalis* L. (eeRo) against gastric ulcers induced by ethanol.

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 and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC–DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1. Other reagents were supplied by Sigma–Aldrich Chemical.

2.2. Animals

Male Wistar rats weighing 270–320 g from our own breeding colony were kept in cages of five animals each, with food and water ad libitum, in a controlled temperature room (22 ± 2 °C) with a 12 h light/dark cycle and with lights on at 07.00 h. The animals received a chow diet as basal food from Puro Trato Company. The rats were maintained and used in accordance with the guidelines of Care and Use of Experimental Animal Resources of Federal University of Santa Maria (Brazil) (044/2012).

2.3. 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 (Ashok et al., 2010).

2.4. Quantification the ethanolic extract constituents of R. Officinalis L. by HPLC–DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (*A*) and methanol (*B*), and the composition gradient was: 5% of *B* until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70%, and 100% *B* at 10, 20, 30, 40, 50, and 60 min, respectively. The flow rate was 0.8 ml/min, injection volume 40 μl and the wavelength were 285 nm for carnosic acid, 325 nm for caffeic, chlorogenic and rosmarinic acids, and 365 nm for quercetin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg/ml for kaempferol, quercetin and rutin; and 0.006–0.250 mg/ml for rosmarinic, carnosic, chlorogenic and caffeic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curve for chlorogenic acid: $Y = 13257x + 1096.8$ ($r = 0.9998$); caffeic acid: $Y = 16862x + 1108.3$ ($r = 0.9987$); rosmarinic acid: $Y = 15534x + 1284.1$ ($r = 0.9993$); carnosic acid: $Y = 16278x + 1365.4$ ($r = 0.9989$); rutin: $Y = 20976x - 1521.7$ ($r = 0.9996$); quercetin: $Y = 25734x - 1492.3$ ($r = 0.9996$) and kaempferol: $Y = 17923x - 1853.9$ ($r = 0.9978$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.5. The experimental protocol and ethanol-induced gastric lesions method

The animals were randomly divided into six groups and they were submitted to three administrations of drugs every 24 h. It was used 2% tween 80 as drug vehicle (vehicle *A*), except for omeprazole that was solubilized with 0.5% carboximetilhylcellulose (vehicle *B*). The gastric lesions were induced with 70% ethanol (2 mL/kg), oral way (p.o.), in animals submitted to 36 h fasted, according to the method described by Robert (1979). Animals were submitted to 36 h of fasting starting 12 h after first drug doses. For this purpose, rats were kept in a cage equipped with a gate apparatus that hindered the animals feeding. The fasting period was necessary for the right ethanol-induced gastric lesions and the analysis of possible protective effects of tested drugs (see Fig. 1).

The treatment groups and experimental protocol are detailed below:

(1) Control group. This group received only a daily dose of vehicle *A* (15 mL/kg, p.o.), along the treatment time.
(2) eeRo control group. It received only eeRo at 1000 mg/kg (15 mL/kg), only one dose every 24 h, during every experimental period.
(3) Omeprazole group (omep + etOH). The animals of this group got only one omeprazole dose (30 mg/kg, 2 mL/kg, p.o.), 1 h after ethanol administration (70%, 2 mL/kg, p.o.) given to animals only once.
(4) 500 + etOH. The animals of this group were submitted to eeRo treatment at 500 mg/kg, every 24 h, they received only one ethanol dose (70%, 2 mL/kg, p.o.), 1 h after eeRo administration.
(5) 1000 + etOH. The animals of this group were submitted to eeRo treatment at 1000 mg/kg, respectively, every 24 h, they received only one ethanol dose (70%, 2 mL/kg, p.o.), 1 h after eeRo administration.
(6) Ethanol group (etOH). It received vehicle *A*, every 12 h, along the experimental period. Furthermore, this group got only one ethanol administration (70%, 2 mL/kg, p.o.), 1 h after last vehicle dose.

In this protocol, all the animals were sacrificed by decapitation, 1 h after ethanol administration. After this, the stomachs were immediately removed to determine the gastric lesion Index and biochemical parameters. All drugs were administrated by oral way. Data are reported as three or six animals per group.

2.6. The gastric lesion index

Animals were sacrificed 1 h after ethanol administration, or 1 h after treatment with vehicle for the control group. The stomachs were quickly removed, opened along the greater curvature and fixed to determine the gastric lesion index. The ulcerative lesion index of each animal was calculated according to Gamberini et al. (1991) and using scored as follows: loss of mucosal folding, mucosal discoloration, edema or

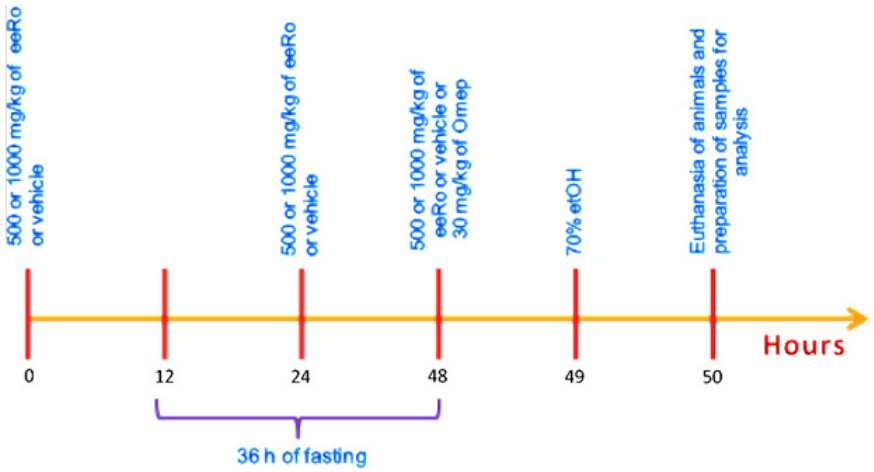

Fig. 1. Diagram showing the design and time-course of experimental procedures (eeRo, ethanolic extract of *Rosmarinus officinalis* L. at 500 or 1000 mg/kg; vehicle, distilled water (98%) + tween 80 (2%); Omep, omeprazole at 30 mg/kg; etOH, 70% ethanol). All drugs were administrated by oral way. Data are reported as three or six animals per group.

hemorrhage (score 1 each); ulcers/cm^2 less than 1 mm (score – number of ulcer × 2); ulcers more than 1 mm/cm^2 (score – number × 3); perforated ulcers (score – number × 4).

2.7. Histopathology

Stomachs were fixed in 10% formalin. For microscopic analysis, the tissues are immersed in paraffin and stained with hematoxylin and eosin (Bancroft and Stevens, 1977).

2.8. Biochemical analysis

After sacrifice of the animals, the stomachs were quickly removed, homogenized in saline 150 mM (1:4 dilution, w/v) and maintained on ice. After homogenization, was made centrifugation of the stomach samples to 2000*g* at 4 °C for 10 min to get a supernatant (S1) from low speed which was used for the determination of oxidative damage markers . Aliquots of S1 stomach were frozen (−20 °C) for later analysis of Enzymatic antioxidant defense system and also to determination of proteins related to these tests.

2.9. Markers of oxidative damage

2.9.1. Dichlorofluorescein fluorescence assay (DCF)

The dichlorofluoresceine fluorescence assay was used to measure cellular peroxide production and other reactive species (Myhre et al., 2003). Aliquots of stomach (S1) was added to a medium containing Tris–HCl buffer (0.01 mM, pH 7.4) and dichlorofluoresceine diacetate (7 μM). After the addition of dichlorofluoresceine diacetate, the medium was incubated in the dark for 1 h until the fluorescence measurement (excitation at 488 nm and emission at 525 nm, with both slit widths at 1.5 nm). Oxidized dichlorofluoresceine was determined using a standard curve of oxidized dichlorofluoresceine and results were expressed as μmol of oxidized DCF/mg protein (Pérez-Severiano et al., 2004).

2.9.2. Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as described by Ohkawa et al. (1979) in stomach samples (S_1). We added 200-ml aliquots of S_1 obtained for the color reaction. The concentration of thiobarbituric acid reactive substances was measured at 532 nm using a standard curve of malondialdehyde, and the results were expressed as nmol MDA/mg protein.

2.9.3. GSH/GSSG Levels assay

The levels of GSH and GSSG (Hissin and Hilf, 1976) were measured by the fluorimetric method as previously described by Hissin and Hilf (1976). According this method we added 250 mg protein/ml (S_1) to 2 ml final phosphate–EDTA buffer (100 mM, pH 8.0) and HPO3 (25%) and this mixture is centrifuged at 100,000*g* for 30 min, at 4 °C and the supernatant was separated in two aliquots for measurement of GSH and GSSG. Results are expressed as ratio of GSH/GSSG.

2.10. Enzymatic antioxidant defense system

2.10.1. Superoxide dismutase activity (SOD) assay

Superoxide dismutase activity was assayed spectrophotometrically as previously described by Misra and Fridovich (1972). This method is based on the capacity of SOD to inhibit the autoxidation of epinephrine to adrenochrome. The color reaction was measured at 480 nm (Spectrophotometer U-2001 Hitachi – Japan). One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 36 °C.

2.10.2. Catalase activity assay (CAT)

The stomach was homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400 g for 15 min. The supernatant was assayed spectrophotometrically (Spectrophotometer U-2001 Hitachi – Japan) by the method of Aebi (1984), which involves monitoring the degradation of H_2O_2 in the presence of cell homogenate at 240 nm.

2.11. Measurement of nitrite and nitrate levels (Nox)

The nitrite and nitrate levels (degradation products of NO) (Nox) are measured in the stomach samples and were prepared according to Guerra et al. (2006). The sulting pellet is resuspended in NaOH (3 M) for protein determination (Guerra et al., 2006; Miranda et al., 2001).

The content of Nox in the supernatant is estimated in a medium containing VCl3 (in 5% HCl), 200 mL of N-(1-naphthyl)-ethylene diamine dihydrochloride (0.1%), 2% sulfanilamide (5% HCl). After incubation at 37 °C for 60 min, the nitrite levels were determined spectrophotometrically at 540 nm, based on the reduction of nitrite to nitrate by VCl3 (Guerra et al., 2006; Miranda et al., 2001). The levels of nitrite and nitrate tissue were expressed in nmol Nox/mg protein.

2.12. Myeloperoxidase activity assay

Myeloperoxidase activity was determined in stomach supernatant fraction as previously described Grisham et al. (1986), with some modifications. Briefly, a sample of the stomach preparation (20 ml) was added to a medium containing potassium phosphate buffer (50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%) and N, N, N0, N0-tetramethylbenzidine (1.5 mM). The kinetic analysis of myeloperoxidase was started after the addition of hydrogen peroxide (0.01%), and the color reaction was measured at 655 nm at 37 °C.

2.13. Protein determination

The protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Table 1
Phenolics and flavonoids composition of ethanolic extract of *Rosmarinus officinalis* L. (eeRo) at mg/1 g of eeRo. Results are expressed as mean ± standard deviations (SDs) of three determinations.

Compounds	eeRo (mg/g)
Chlorogenic acid	11.2 ± 0.17
Caffeic acid	7.63 ± 0.09
Rutin	3.07 ± 0.12
Rosmarinic acid	38.5 ± 0.04
Quercetin	5.10 ± 0.07
Kaempferol	2.53 ± 0.45
Carnosic acid	26.4 ± 0.15

2.14. 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 analysis of variance (ANOVA), followed by Newman–Keuls's Test for post hoc comparison for all assays, except for index ulcer which we used the Kruskal–Wallis followed post test Dunns. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Quantification of the ethanolic extract constituints of R. officinalis L.

3.1.1. HPLC analysis

HPLC fingerprinting of eeRo revealed the presence of the chlorogenic acid (t_R = 20.67 min; 12.2 mg/g), caffeic acid (t_R = 26.17 min; 7.63 mg/g), rutin (t_R = 36.09 min; 3.07), rosmarinic acid (t_R = 38.43 min; 38.5 mg/g), quercetin (t_R = 47.03 min; 5.10 mg/g), kaempferol (t_R = 54.78 min; 2.53 mg/kg), and carnosic acid (t_R = 63.97 min; 26.4 mg/g) (Table 1). The HPLC analysis revealed that flavonoids (quercetin, rutin and kaempferol) and phenolics acids (chlorogenic, caffeic, rosmarinic and carnosic acids) are the major components of the extract.

3.2. Macroscopic analysis

3.2.1. Gross appearances of stomach and gastric ulcer index

The animals that received 70% ethanol developed a consistent macroscopic damage which was evidenced by presence of ulceration hemorrhagic (Fig. 2A$_2$), it is attenuated by the prior administration of omeprazole (30 mg/kg) with a few fields of hyperemia

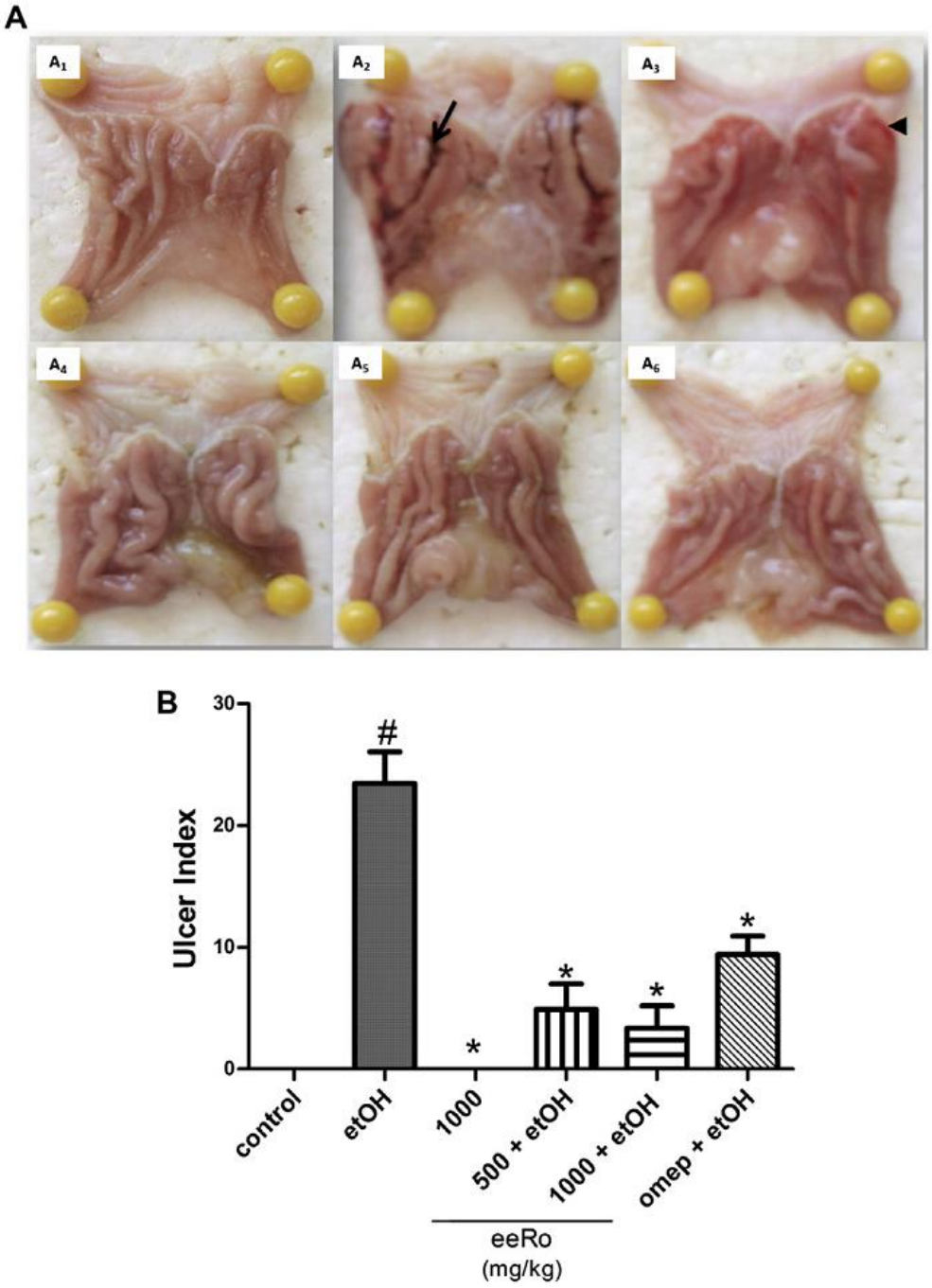

Fig. 2. Effect of ethanolic extract of *Rosmarinus officinalis* L. on Gross appearances of stomach (A) and ulcer index (B). Control (A$_1$), 70% ethanol (A$_2$), omeprazole at 30 mg/kg + 70% ethanol (A$_3$), eeRo at 1000 mg/kg (A$_4$), eeRo at 500 mg/kg + 70% ethanol (A$_5$), eeRo at 1000 mg/kg + 70% ethanol (A$_6$). eeRo (ethanolic extract of *Rosmarinus officinalis* L.). etOH (70% ethanol). Omep (omeprazole at 30 mg/kg). All drugs were administrated by oral way. ↑ Ulcerations with hemorrhagic regions. ◄ damage in the gastric mucosa with hyperemia regions. (A) Representative analysis of three animals per group. (B) Data are reported as means ± standard error of means (SEMs) of six animals per group.

(Fig. 2A_3). In addition, the eeRo did not show any macroscopic toxicity, preserving the morphological integrity of the gastric mucosa (Fig. 2A_4) compared to non-treated control group. Furthermore, the animals treated with eeRo at 500 and 1000 mg/kg was able to prevent the damage induced by ethanol (Fig. 2A_5 and A_6, respectively), with very similar aspect to the control group (Fig. 2A_1).

In addition, the administration of ethanol induced a significant increase of ulcer index in relation to control animals. However, the groups treated with eeRo at 500 and 1000 mg/kg and omeprazole at 30 mg/kg showed a significant decrease of ulcer index compared to animals treated with ethanol. Moreover, the animals that received eeRo did not show any significantly different of the ulcer index compared to control animals ($p < 0.0001$) (Fig. 2B).

3.3. Histopathology

The administration of 70% ethanol induced consistent microscopic damage, when compared to control animals (Fig. 3A – 4×), with the presence of severe swelling in the tissues structure (Fig. 3C – 4×), loss of continuity epithelial (Fig. 3C_1 – 10×) and pronounced infiltration of neutrophils (Fig. 3C_2 – 40×).

Moreover, the animals which received eeRo at 500 and 1000 mg/kg were able to substantially prevent the damage induced by ethanol (Fig. 3D and E, respectively). The group which received eeRo at 500 mg/kg presented a small swelling caused by the aggressive action of ethanol, as a characteristic small inflammation, but without the presence of inflammatory cells (Fig. 3D – 4×).

Beside, the gastric mucosa of animals which received eeRo at 1000 mg/kg was completely protected against the ethanol action, preserving all histological aspects when compared to control animals (Fig. 3E – 4×). Furthermore, the omeprazole group at 30 mg/kg showed a relative protection against of ethanol, with swelling similar to animals which received ethanol-only (Fig. 3F and F_1 – 4× and 10×, respectively) and moderate infiltration of neutrophils inflammatory cells (Fig. 3F_2 – 40×).

3.4. Markers of oxidative damage and enzymatic antioxidant defense system

The ethanol group showed significant changes on oxidative markers with an increase on lipid peroxidation ($F_{5.35} = 4.61$, $p < 0.0025$) (Fig. 4A) and oxidized DCF levels ($F_{5.24} = 11.37$, $p < 0.0001$) (Fig. 4B). In addition the ethanol group showed a decrease on GSH/GSSG ratio ($F_{5.35} = 5.082$, $p < 0.0013$) (Fig. 4C) and catalase activity ($F_{5.28} = 4.85$, $p < 0.0026$) (Fig. 4D) in relation to respective control animals.

However, the animals which received eeRo at 500 and 1000 mg/kg were protected against the damage induced by ethanol (Fig. 4). The eeRo, at all doses tested, was able to significantly prevent the increase on lipid peroxidation ($F_{5.35} = 4.61$, $p < 0.0025$) (Fig. 4A) and oxidized DCF levels ($F_{5.24} = 11.37$, $p < 0.0001$) (Fig. 4B), and decrease on GSH/GSSG ratio ($F_{5.35} = 5.082$, $p < 0.0013$) (Fig. 4C) and catalase activity ($F_{5.28} = 4.85$, $p < 0.0026$) (Fig. 4D), maintaining similar levels to the control animals.

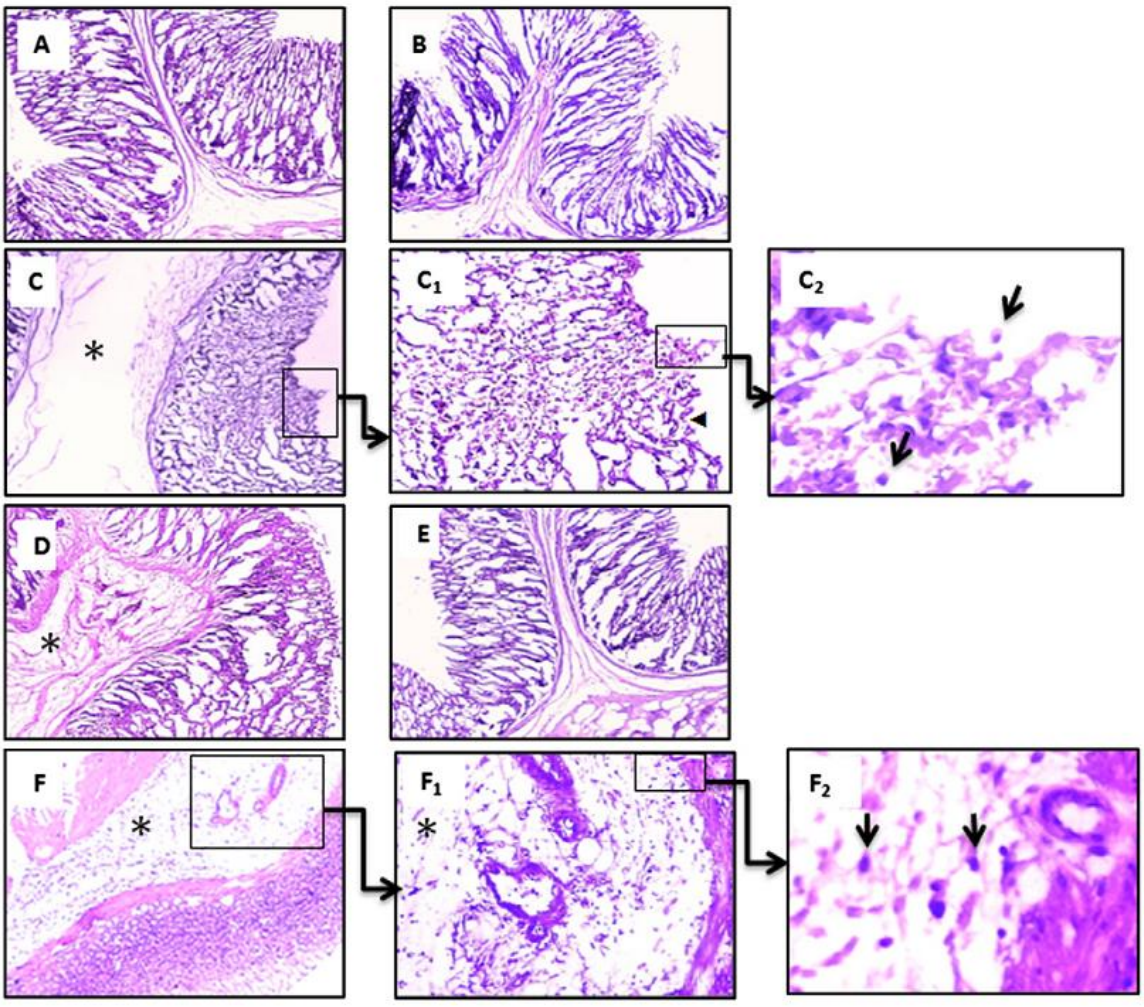

Fig. 3. Histopathology analysis of stomach from control (4×) (A), eeRo at 1000 mg/kg (4×) (B), 70% ethanol (C – 4×, C_1 – 10×, C_2 – 40×), eeRo at 500 mg/kg + 70% ethanol – 4× (D), eeRo at 1000 mg/kg + 70% ethanol – 4× (E), omeprazole at 30 mg/kg (F – 4×, F_1 – 10×, F_2 – 40×). *Swelling. ◄ Loss of continuity epithelial. ↑ Inflammatory cells. eeRo (ethanolic extract of *Rosmarinus officinalis* L.). All drugs were administrated by oral way. Representative analysis of three animals per group.

Furthermore, omeprazole at 30 mg/kg was able to prevent the increase on lipid peroxidation ($F_{5.35} = 4.61$, $p < 0.0025$) and oxidized DCF levels ($F_{5.24} = 11.37$, $p < 0.0001$), and decrease the GSH/GSSG ratio ($F_{5.35} = 5.082$, $p < 0.0013$) and catalase activity in relation to ethanol group ($F_{5.28} = 4.85$, $p < 0.0026$) (Fig. 4A–D, respectively). Moreover, the eeRo did not present any effect per se on the lipid peroxidation, oxidized DCF, GSH/GSSG ratio levels and catalase activity when compared to control animals (Fig. 4A–D, respectively).

In addition, the animals treated with ethanol, eeRo (500 and 1000 mg/kg), and omeprazole at 30 mg/kg did not show changes on SOD activity (antioxidant enzyme) in relation to control animals ($F_{5.35} = 1.907$, $p = 0.118$) (data not shown).

3.5. Nitrite and nitrate levels (Nox) and myeloperoxidase activity assays

The animals that were treated with ethanol showed significant changes of Inflammatory response markers and nitrite and nitrate levels (Nox) with respective decrease of Nox levels ($F_{5.22} = 5.667$, $p < 0.0017$) and increase of myeloperoxidase activity ($F_{5.31} = 9.956$, $p < 0.0001$) compared to respective control animals (Fig. 5A and B, respectively).

However, in animals treated with eeRo at 500 and 1000 mg/kg was able to significantly prevent the decrease of Nox levels ($F_{5.22} = 5.667$, $p < 0.0017$) and increase myeloperoxidase activity ($F_{5.31} = 9.956$, $p < 0.0001$) (Fig. 5A and B, respectively). Moreover, the treatment with omeprazole at 30 mg/kg prevented the decrease of Nox levels ($F_{5.22} = 5.667$, $p < 0.0017$), but did not prevent the increase of myelopeoxidase activity ($F_{5.31} = 9.956$, $p < 0.0001$) when compared to respective control animals (Fig. 5A and B, respectively). In addition, the eeRo had none effect per se on the Nox levels and myeloperoxidase activity in comparison with the control animals (Fig. 5A and B, respectively).

4. Discussion

After thorough analysis of the eeRo, we identified the primary active constituents and observed that carnosic and rosmarinic acids are the most abundant constituents of eeRo (Table 1). The antiulcerogenic properties of eeRo, resulting from different mechanisms, may be attributed to carnosic acid and rosmarinic acid, in addition to its antioxidant effects already reported in the literature (Mulinaccia et al., 2011). Furthermore, we could not rule out some antiulcerogenic activity of other constituents found in eeRo that might act synergistically.

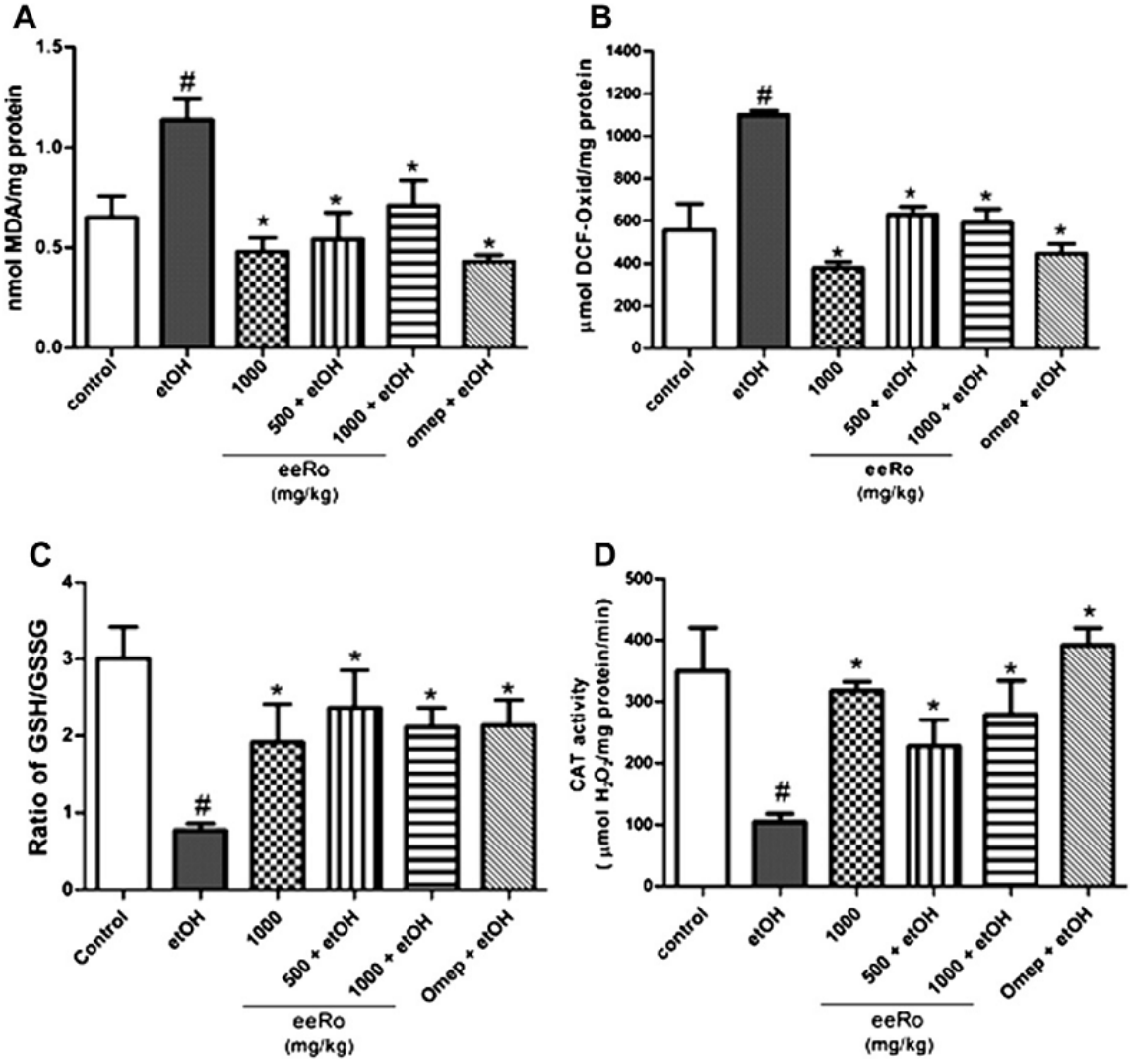

Fig. 4. Effect of ethanolic extract of *Rosmarinus officinalis* L. on lipid peroxidation (A), dichlorofluorescein fluorescence (B), ratio of GSH/GSSG (C) and catalase activity assays (D). eeRo (ethanolic extract of *Rosmarinus officinalis* L. at 500 or 1000 mg/kg). etOH (70% ethanol). Omep (omeprazole at 30 mg/kg). All drugs were administrated by oral way. One-way/ANOVA, followed by Newman–Keuls's Test for post hoc. #$p < 0.05$, significant difference when compared to control. *$p < 0.05$, significant difference when compared to ethanol group. Data are reported as means ± standard error of means (SEMs) of six animals per group.

We believe that the eeRo was able to act as a gastroprotective agent against the damage caused by ethanol through various mechanisms. Among these mechanisms, we believe that eeRo exerted a potent antiinflammatory effect in the gastric mucosa (Fig. 5B), confirmed by microscopic evidence obtained in our analysis, decreasing the infiltration of inflammatory cells (neutrophils) (Fig. 3D and 3E) in relation to samples from stomachs of rats that received ethanol-only (Fig. $3C_2$). This finding was confirmed by the protection of the normal activity of myeloperoxidase (Fig. 5B), an enzyme present in neutrophils (Queiroz et al., 2012). Furthermore, the eeRo at 1000 mg/kg was able to protect the histological structure of the gastric mucosa, preventing swelling (Fig. 3E) and preventing the infiltration of inflammatory cells (neutrophils) at 500 and 1000 mg/kg (Fig. 3D and E, respectively).

Moreover, another significant mechanism displayed by the constituents of the eeRo was the protection of normal physiological levels of NO (Fig. 5A). This protective mechanism of the extract was identified by measuring the Nox (degradation products of NO) (Fig. 5A). The increased levels of NO plays an important role in gastric protection through the dilation of gastric blood vessels, resulting in an increased supply of nutrients that contributes to the multiplication of cells that constitute the granulation tissue (the first tissue to be formed in the regeneration process). This, in turn, contributes to the healing process of the gastric mucosa (Yang et al., 2000).

The antioxidant effects of the eeRo were evidenced by the increased levels of reduced glutathione (GSH) associated with decreased levels of oxidized glutathione (GSSG) (Fig. 4C). This data revealed that the constituents of the eeRo could acted as antioxidant, oxidizing itself to decrease the RS levels, which were elevated due to the mucosal damage caused by ethanol. The antioxidant potential of the eeRo likely prevented the consumption of gastric GSH reserves, which would have been oxidized to GSSG. In addition, the constituents of the eeRo protected normal catalase activity, an important antioxidant enzyme that is responsible for the degradation of hydrogen peroxide (H_2O_2) to oxygen and water (Fig. 4D). However, the ethanol or eeRo were not able to change the physiological levels of SOD activity by mechanism not yet known.

The eeRo also exhibited protection against lipid peroxidation, which was demonstrated by the ability of the constituents of the eeRo to protect cell membranes against attack by RS. The abnormal elevation of RS corresponds to one of the main aggressive mechanisms of ethanol, which can cause gastric cell damage and death (Ineu et al., 2008; Savegnago et al., 2006). The eeRo antioxidant mechanism was enhanced by its capacity to prevent the damage oxidative (Fig. 4A–D). The broad antioxidant properties of eeRo were demonstrated by lipid peroxidation, DCF, GSH/GSSG ratio and CAT activity assays.

In addition, we believe that the aggressive action of ethanol, which caused macroscopic evidence of hemorrhagic damage (Fig. $2A_2$), could create an imbalance in the gastric tissues that results in an increased amount of RS (Fig. 4B) and an enhanced inflammatory process (Fig. 5B).

Thus, we believe that the inflammatory process, which happens as a part of the body's natural defense against tissue damage, is generally associated with an increase in oxidative stress (Reuter et al., 2010). The results of this study represent direct evidence that the eeRo has a marked antiinflammatory effects (Fig. 5B), which can be directly linked to its isolated compounds (Table 1). These findings are in accordance with the literature (Benincá et al., 2011). These properties are achieved by a decrease in pro-inflammatory mediators, including myeloperoxidase (Fig. 5B) (Benincá et al., 2011), IL-1b and TNF-α, and a reduction in leukocyte activation (Benincá et al., 2011).

Moreover, our study demonstrated in accordance with the literature that eeRo has, among other constituents, a high concentration of carnosic acid and rosmarinic acid recognized as natural antioxidants (Erkan et al., 2008; Pereira et al., 2005) which reinforce the importance of eeRo as antioxidant (Bakirel et al., 2008) and anti-ulcerogenic (Altinier et al., 2007).

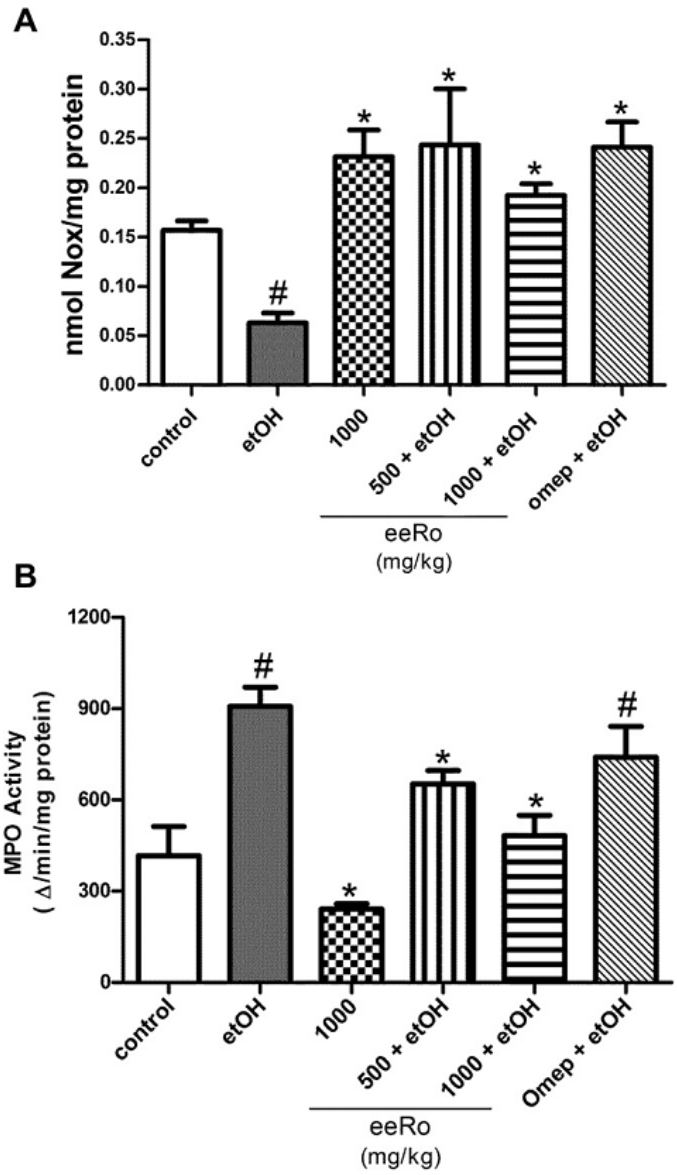

Fig. 5. Effect of ethanolic extract of *Rosmarinus officinalis* L. on Nox levels (A) and myeloperoxidase activity (B). eeRo (ethanolic extract of *Rosmarinus officinalis* L. at 500 or 1000 mg/kg). etOH (70% ethanol). Omep (omeprazole at 30 mg/kg). All drugs were administrated by oral way. One-way/ANOVA, followed by Newman–Keuls's test for post hoc. #$p < 0.05$, significant difference when compared to control. *$p < 0.05$, significant difference when compared to ethanol group. Data are reported as means ± standard error of means (SEMs) of six animals per group.

Lastly, the eeRo induced an anti-ulcerogenic effect with on antioxidant aspects and NOx levels similar to animals treated with omeprazole (proton pump inhibitor) (Figs. 4 and 5A, respectively), a drug widely used by humans throughout the world. Moreover, the eeRo at 500 and 1000 mg/kg induced a better gastroprotective effects than omeprazole at 30 mg/kg, a dose clinically used in humans, because the eeRo showed an antiinflammatory potential action in addition to its other gastroprotective properties (Fig. 5B).

5. Conclusions

In conclusion, we believe that the eeRo tested in this investigation deserve further attention due its importance in the prevention of gastric ulcerations induced by ethanol through three different

mechanisms: antioxidant, antiinflammatory and vasodilator. This work presents the main mechanisms of *R. officinalis* L. extract, analyzing the macroscopic, microscopic and biochemical aspects.

In addition, it is relevant to point out that the *R. officinalis* L. is widely used by the majority people of the world as nutraceutical and drug (Mulinaccia et al., 2009; Nabekuraa et al., 2010). Moreover, it has an expressive development from the european continent (natural habitat) up to american continent, where this plant is perfectly adapted (Machado et al., 2013). In this context, we believe that the ethanolic extract of *R. officinalis* L. should be seen as a promising and accessible gastroprotective drug.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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3.3. MANUSCRITO 2: MULTI-MECHANISTIC ACTION OF *Rosmarinus officinalis* L. EXTRACT AGAINST ETHANOL EFFECTS IN AN ACUTE MODEL OF INTESTINAL DAMAGE

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MULTI-MECHANISTIC ACTION OF *Rosmarinus officinalis* L. EXTRACT AGAINST ETHANOL EFFECTS IN AN ACUTE MODEL OF INTESTINAL DAMAGE

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Multi-mechanistic action of *Rosmarinus officinalis* L. extract against ethanol effects in an acute model of intestinel damage

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Abstract

The high levels of oxidative stress and inflammation can be present in the etiology of degenerative intestinal pathologies associated with ethanol ingestion. The *Rosmarinus officinalis L.*, more commonly known as Rosemary in Europe and as Alecrim in Brazil, has exhibited several physiological and medicinal activities. In this investigation, we intended to clarify for the first time the antioxidant and anti-inflammatory effects of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) against an acute damage induced by ethanol in intestine of rats. The rats were treated three times, at every 24 hours, with eeRo at 500-1000 mg/kg or vehicle, oral way. All groups got an only dose of ethanol (2ml/kg), oral way, after 36 hours of food fasting and 1 hour after the last dose of eeRo or vehicle administration. This analyzes were done by the mensuration of oxidative stress profile in lipid peroxidation in serum and intestine; Na^+/K^+ ATPase, catalase and superoxide dismutase activities assays only in intestine; and inflammatory clouds of eeRo in myeloperoxidase activity assay only in intestine. The eeRo was able to protect against the lipid peroxidation in serum and intestine. It prevented the reduction in Na^+/K^+ ATPase and catalase levels induced by ethanol in intestine. In addition, eeRo increased the superoxide dismutase activity when compared to control and protected the intestine against elevations in myeloperoxidase activity caused by ethanol. Our results suggested that eeRo exerted a significant intestinal protective effect by antioxidant and anti-inflammatory mechanisms. Thus the eeRo represented a promising agent against intestinal lesions induced by ethanol.

Key-words: ethanol; intestinal; bowel, oxidative stress; inflammation.

1. Introduction

The breakdown of the normal mucosal defense mechanisms in intestine (BHATTACHARYYA et al., 2014a) by stressors, for example ethanol, (BAGCHI et al., 1999) can induce peptic and duodenal ulcers, dyspepsia, bacteremia or translocation of bacterial products (BECK et al., 2004; SØREIDE; THORSEN; SØREIDE, 2014). Moreover, the peptic ulcer-related gastric cancer ranked fifth among the top 10 causes of cancer (WU et al., 2010).

Several factors including environmental and emotional stress, age, diet, genetics and individual behavior among others have been attributed to either predispose or potentiate the gastrointestinal mucosal to injury through enhanced ROS production mucosal oxidative stress could result from the disruption of redox control and the subsequent alteration in redox signaling (BAGCHI et al., 1999; BHATTACHARYYA et al., 2014b). These can contribute to the development of degenerative pathologies of the intestine, such as inflammation and cancer (CIRCU; AW, 2012).

Ethanol is widely consumed and associated with development of gastrointestinal ulcer and cancers (ELAMIN et al., 2013b). Experimental and clinical studies have demonstrated that ethanol is able to induce the intestinal barrier dysfunction which have been associated with the oxidative stress generation and also the rise of inflammatory process in intestine (ELAMIN et al., 2013b; LAMBERT et al., 2003; PARLESAK et al., 2000).

In this context, natural products have been proposed as an alternative to avoid several pathological process since the plants are a good source of agents with pharmacological effects either by blocking oxidative stress cascade or acting in cellular targets (DEVI; MAZUMDER; DEVI, 2015; SARKHAIL, 2014). Therefore, the *Rosmarinus officinalis* (Lamiaceae) is a perennial herb native to the Mediterranean area and is widely distributed in many parts of the world, including Brazil (BARBOSA et al., 2015). The *Rosmarinus officinalis L.* extracts are used as condiment for flavouring food, and as a source of antioxidant compounds employed in food conservation (BENINCÁ et al., 2011). Moreover, this plant extracts have showed evidences of medicinal properties, as antibacterial (BARBOSA et al., 2015), antioxidant (SOTELO-FÉLIX et al., 2002), antidermatophytic (ENDO et al., 2015) among others. The antioxidant activity of these extracts is related to the presence of phenolic abietane diterpenes, such as carnosic acid and its derivatives, carnosol, rosmadial, rosmanol, rosmanol isomers, and methyl carnosate, and phenolic acids such as rosmarinic acid (BORRÁS LINARES et al., 2011; JORDÁN et al., 2012; PÉREZ-FONS; GARZÓN; MICOL, 2010).

Our goal, in this investigation, is to clarify, for the first time, the antioxidant effects and anti-inflammatory clues about eeRo in intestine against an acute damage induced by ethanol and its

possible applications in the future as dietary supplement or medicine to contribute the health of humans and animals intestines.

2. Materials and methods

2.1. Chemical, apparatus and general procedures

Ethanol was obtained from local suppliers with purity of 99%. Methanol, hydrogen peroxide (H_2O_2), acetic acid, chlorogenic acid and caffeic acid were purchased from Merck (Darmstadt, Germany). The Carnosic acid, quercetin, rutin, kaempferol and other reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan).

2.2. Animals

Male wistar rats weighing 270–320 g from breeding colony of UFSM were kept in cages of five animals each, with food and water *ad libitum*, in a controlled temperature room (22±2ºC) with a 12h light/dark cycle and with lights on at 07.00 h. The animals were maintained and used in accordance with the guidelines of the National council of control of animal experimentation (CONCEA) and approved by Committee on Care and Use of Experimental Animal of the UFSM, Brazil (044/2012).

2.3. Obtaining of the ethanolic extract of *R. officinalis L.* (eeRo)

The leaves of *R. officinalis L.* were dried and subjected to an ethanolic extraction (100% ethanol, 1.5 h, 60-70°C) in the Soxhlet apparatus as previously described (BARBOSA et al., 2015) with some modifications to obtain eeRo. The voucher specimen was deposited in the herbarium of UFSM under the number of SMDB 15.050. The access to genetic patrimony was authorized by CNPq under the number 010757/2014-7.

2.4. Quantification of eeRo constituints by HPLC-DAD.

Reverse phase chromatographic analyses were carried out under gradient conditions in accordance with Aamaral et al., 2013 (AMARAL et al., 2013b). All the samples and mobile phase

were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg/ml for kaempferol, quercetin and rutin; and 0.006 – 0.250 mg/ml for rosmarinic, carnosic, chlorogenic and caffeic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

2.5. The treatments and ethanol-induced intestinal damage

The intestinal damages were induced by 70% ethanol according to the method previously described (ROBERT et al., 1979). The animals were submitted to 36h of fasting before the ethanol administration. For this purpose the rats were kept in a cage equipped with a gate apparatus that hindered the animals feeding. The control rats received vehicle (tween 80 at 2%; 15 mL/kg; p.o.). The eeRo was administered p.o. in different doses (500 and 1000 mg/kg, 15 mL/kg) 1 h before oral administration of 70% ethanol (v/v, 2 mL/kg, p.o.). After 1h the animals were killed by decapitation. The experimental procedure is depicted on Fig. 1.

2.6. Biochemical analysis

The intestine, especially the duodenum and small portions of the jejunum and ileum, were quickly removed, homogenized in 150 mM NaCl (1:4 dilution, w/v) and maintained on ice. After, the samples were centrifuged to 2,000 x g at 4°C for 10 min to get a low speed supernatant (S_1) which was used for determining markers of oxidative damage. Aliquots of S_1 were frozen (-20 °C) for later analysis of enzymatic antioxidant defense system and to protein determination.

2.6.1. Markers of oxidative damage

2.6.1.1. Dichlorofluorescein fluorescence assay (DCF)

This assay was used to measure the reactive species levels (MYHRE et al., 2003). Aliquots of 50μl S_1 were added to a medium containing tris-HCl buffer (10 mM, pH 7.4) and 2′, 7′-*dichlorofluorescein* diacetate (1 mM). After the addition of 2′, 7′-*dichlorofluorescein* diacetate, the medium was incubated in the dark for 1h until the fluorescence measurement (excitation at 488 nm

and emission at 525 nm, with both slit widths at 1.5 nm). Oxidized *dichlorofluorescein* (DCF) was determined using a standard curve and results were expressed as nmol of oxidized DCF/mg protein (PÉREZ-SEVERIANO et al., 2004).

2.6.1.2. Lipid peroxidation assay

The Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as described by Ohkawa and colleagues (OHKAWA; OHISHI; YAGI, 1979) in intestinal samples (S_1). We added 200µl aliquots of S_1 obtained for the color reaction. The concentration of thiobarbituric acid reactive substances was measured at 532 nm using a standard curve of malondialdehyde, and the results were expressed as nmol MDA/mg protein.

2.6.1.3 GSH/GSSG Levels assay

The levels of GSH and GSSG (Hissin and Hilf, 1976) were measured by the fluorimetric method as previously described by Hissin and Hilf (1976). According this method we added 250 mg protein/ml (S1) to 2 ml final phosphate–EDTA buffer (100 mM, pH 8.0) and HPO_3 (25%) and this mixture is centrifuged at 100,000g for 30 min, at 4°C and the supernatant was separated in two aliquots for measurement of GSH and GSSG. Results are expressed as ratio of GSH/GSSG.

2.6.1.4. Enzymatic antioxidant defense system

2.6.1.5. Superoxide dismutase activity (SOD) assay

This enzyme activity was spectrophotometrically measured at 480 nm (MISRA; FRIDOVICH, 1972). This method is based on the capacity of SOD to inhibit the auto-oxidation of epinephrine to its adrenochrome. The aliquots of S_1 (40 µL) were added to tris-HCl (10mM, pH 7.4). One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50% at 36°C.

2.6.1.6. Catalase activity (CAT) assay

The intestine was homogenized in 50 mM tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400xg for 15 min. The supernatant (40µL) was added to TFK buffer (50mM, pH 7,0) and H2O2 (10 mM) assayed spectrophotometrically at 240 nm (AEBI, 1984). The kinetic analysis of catalase was

started after H_2O_2 addition and the rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. One unit of the enzyme was considered as the amount of enzyme which decomposes 1 μmol H_2O_2/min at pH 7.

2.6.2. Anti-inflammatory assay

2.6.2.1. Myeloperoxidase (MPO) activity assay

Myeloperoxidase activity was measured in intestine supernatant fraction (GRISHAM; HERNANDEZ; GRANGER, 1986), with some modifications. The intestine samples (70 μL) were added to a medium containing potassium phosphate buffer (50 mM, pH 6.0) with hexadecyl trimethylammonium bromide (0.5%) and N, N, NO, NO-tetramethylbenzidine (1.5 mM). This test was started after the addition of 0.01% H_2O_2, and the color reaction was measured at 655 nm at 37°C.

2.6.3. Protein determination

The protein content was determined according to Bradford (1976) (BRADFORD, 1976) using bovine serum albumin (BSA) as standard.

2. 7. 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. Five to nine animals were analyzed per group. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Quantification of eeRo constituents

3.1.1. HPLC analysis

The eeRo's constituents used in this work are the same analyzed in a previously published paper (AMARAL et al., 2013b) and revealed the presence of the chlorogenic acid (12.2 mg/g), caffeic acid (7.63 mg/g), rutin (3.07 mg/g), rosmarinic acid (38.5 mg/g), quercetin (5.10 mg/g), kaempferol (2.53 mg/kg) and carnosic acid (26.4 mg/g). The HPLC analysis revealed that phenolics acids (rosmarinic and carnosic) are the major components of the eeRo extract.

3. 2. Markers of oxidative damage and enzymatic antioxidant defense system

The ethanol group showed significant changes on oxidative markers with an increase of lipid peroxidation in serum and intestine (Figs 2A-B, respectively). The eeRo extract at 500 and 1000 mg/kg avoided the lipid peroxidation either serum or intestine.

In addition the ethanol group showed, in intestine, a significant decrease on Na^+, K^+-ATPase activity and CAT in relation to respective control animals (Figs 3A-B, respectively). Furthermore, the animals which received eeRo at 500 and 1000 mg/kg were protected against the alterations induced by ethanol (Figs 2A-B, 3A-B), maintaining similar levels to control. Nevertheless, the SOD activity levels were not altered by ethanol treatment, but the eeRo at 500 and 1000 mg/kg increased these levels in relation to the control group (Fig. 3C).

In addition, the animals treated with ethanol and eeRo (500 and 1000 mg/kg) did not show changes on DCF activity and GSH/GSSG ratio in relation to control animals (data not shown).

3. 3. Myeloperoxidase activity assay

The animals that were treated with ethanol showed significant changes on inflammatory response, in intestine, with a relevant increase on myeloperoxidase activity compared to respective control animals (Fig. 4). However, the eeRo at 500 and 1000 mg/kg was able to significantly prevent the increase on myeloperoxidase activity induced by ethanol, maintaining similar levels to the control group (Fig. 4).

4. Discussion

In agreement with previous findings in the literature (MANZO-AVALOS; SAAVEDRA-MOLINA, 2010; VARELLA MORANDI JUNQUEIRA-FRANCO et al., 2006), rats that received

only ethanol showed a marked rise in oxidative stress (Figs 2 and 3), represented by increasing in lipid peroxidation in both intestine and serum (Figs 2A and 2B, respectively). On the other hand, animals pretreated with eeRo showed lipid peroxidation levels similar to the control group in intestine and serum, except for the biggest dose in intestine which presented higher levels in relation to control (Fig. 3A and 3B, respectively). These data showed that the eeRo was able to protect against the damage induced by ethanol in a non dependent dose way (Fig. 3A).

The oxidative stress generation induced by ethanol (Fig. 2) can be associated with a decrease in the CAT and SOD activity which are antioxidant enzymes (Figs 3B and 3C), involved in the detoxication of ROS (BHATTACHARYYA et al., 2014b; XUE et al., 2014). The SOD is a scavenging enzyme of superoxide anion that has hydrogen peroxide as product and it is able to decrease the toxic effects of ROS (CARILLON et al., 2013; FERREIRA; BARROS; ABREU, 2009) . Besides, CAT was involved in detoxification of H_2O_2 through its reduction to H_2O and O_2 (FERREIRA; BARROS; ABREU, 2009; SCIBIOR; CZECZOT, 2006). Therefore, the decrease in these enzymes activity could be related with the vulnerability of animals against harmful effects of oxidative stress induced by ethanol (Figs 3B and 3C). However, when the animals were pretreated with eeRo at all doses, the CAT and SOD activity levels were protected against the ethanol alterations (Figs 3B and 3C). These enzymes had levels similar to the control group (Figs 3B and 3C, respectively), except for the lowest eeRo dose in CAT activity which was higher than control group activity (Fig. 3B). These protective effects of eeRo in the antioxidant enzymes probably were due its constituents' antioxidant properties (Figs. 3B and 3C).

There are evidences that the ethanol is able to induce various damages, for example alterations on mitochondrial membranes (BROZINSKY et al., 1978; ROSSI; ZUCOLOTO, 1977) which can cause an impairment in the inner mitochondrial membranes and induce formation of megamitochondrias (KLEIN; HARMJANZ, 1975; ROSSI; ZUCOLOTO, 1977). Moreover, this whole of alterations can cause impairment in mitochondrial oxidative phosphorylation and a reduction in the ATP production (TAIT; GREEN, 2012).

Thus, these literature data to point out a possibility of ethanol could act doubly in the reduction of intestinal Na^+/K^+ ATPase activity (Fig. 3A), when decreases the ATP production (SKOU, 1957) and causes elevation of reactive species levels (KUEZELLA et al., 1997) (Figs 2A and B). This is probably due to the ATP dependency of Na^+/K^+-ATPase activity (SKOU, 1957), so when ATP levels are not adequate they can inhibit this enzyme activity. Another possibility of Na^+/K^+-ATPase inhibition mechanism is the oxidation of its thiol groups by the presence of high levels of reactive species (PUNTEL et al., 2013; SHAIK et al., 2012).

Consequently, when the Na^+/K^+-ATPase activity was reduced (Fig. 3A) it was able to cause harmful alterations in the intestine, such as the inhibition of secondary active transport of glucose because there was not formation of adequate electrochemical gradient (THORSEN; DRENGSTIG; RUOFF, 2014). Moreover, the reduction of Na^+/K^{+-}ATPase activity could impair the formation of membrane potential and permit the loss of osmotic balance by entry of Na^+ and Cl^-, leading to swelling and cell rupture (GUGGINO; OBERLEITHNER; GIEBISCH, 1985). On the other hand, animals pretreated with eeRo presented levels of Na^+/K^+-ATPase activity similar to the control group (Fig. 3A), showing that eeRo was able to protect the intestine from damage induced by ethanol in a dose non-dependent way (Fig. 3A). Moreover, in accordance with the evidences presented in literature (KIRPICH et al., 2013), the ethanol was able to increase the MPO activity (Fig. 4), an enzyme that was widely used as an indirect marker of inflammation (LUPP et al., 2007) because its ample presence of neutrophils this process (RELJA et al., 2013). Neutrophils are important inflammatory cells directly involved in the early stages of inflammation (RODRIGUEZ et al., 2004) which was an important part of the damage mechanism in the damage´s acute models induced by ethanol (ELAMIN et al., 2013a). On the other hand, animals that were pretreated with eeRo before receiving ethanol maintained the MPO activity similar to control. These data raise the strong hypothesis that constituents of eeRo could have significant anti-inflammatory properties on the initial phase of this process (Fig. 4).

Thus, in previous studies our research group identified the major components of eeRo and reported that carnosic and rosmarinic acids were more abundant constituents (AMARAL et al., 2013b). The protective effects of eeRo in intestine act mainly through antioxidant (Figs 2 and 3B-3C) and anti-inflammatory (Fig. 4) mechanisms that can be attributed mainly to the presence of carnosic and rosmarinic acids, both with recognized antioxidant properties (JORDÁN et al., 2012; PÉREZ-FONS; GARZÓN; MICOL, 2010) and anti-inflammatory evidence (KAMATOU; VILJOEN; STEENKAMP, 2010). In addition, its constituents could be acting synergistically with a summation of properties which can play a significant protection against intestinal disorders (Figs. 2-4) induced by ethanol.

5. Conclusions

In conclusion, we demonstrated in this investigation a relevant antioxidant and important anti-inflammatory effects about eeRo in intestine against an acute damage induced by ethanol. The eeRo has constituents that could act naturally through different mechanisms which probably converge for

an effective intestinal protection against the main damage`s mechanisms as oxidative stress and acute inflammation.

Besides, the eeRo would have an important benefit use due the natural compatibility among its constituents whose are not easy to find in synthetic compounds or in associations of commercial drugs. Moreover, eeRo could present a high limit of safety because there was not yet in the literature anyone potential toxic effects of the products obtained from *R. officinalis* L. Thus, we believe that eeRo could be a promising antioxidant and anti-inflammatory extract used as medicine or dietary supplement for intestinal protection.

Conflict of Interest

None declared

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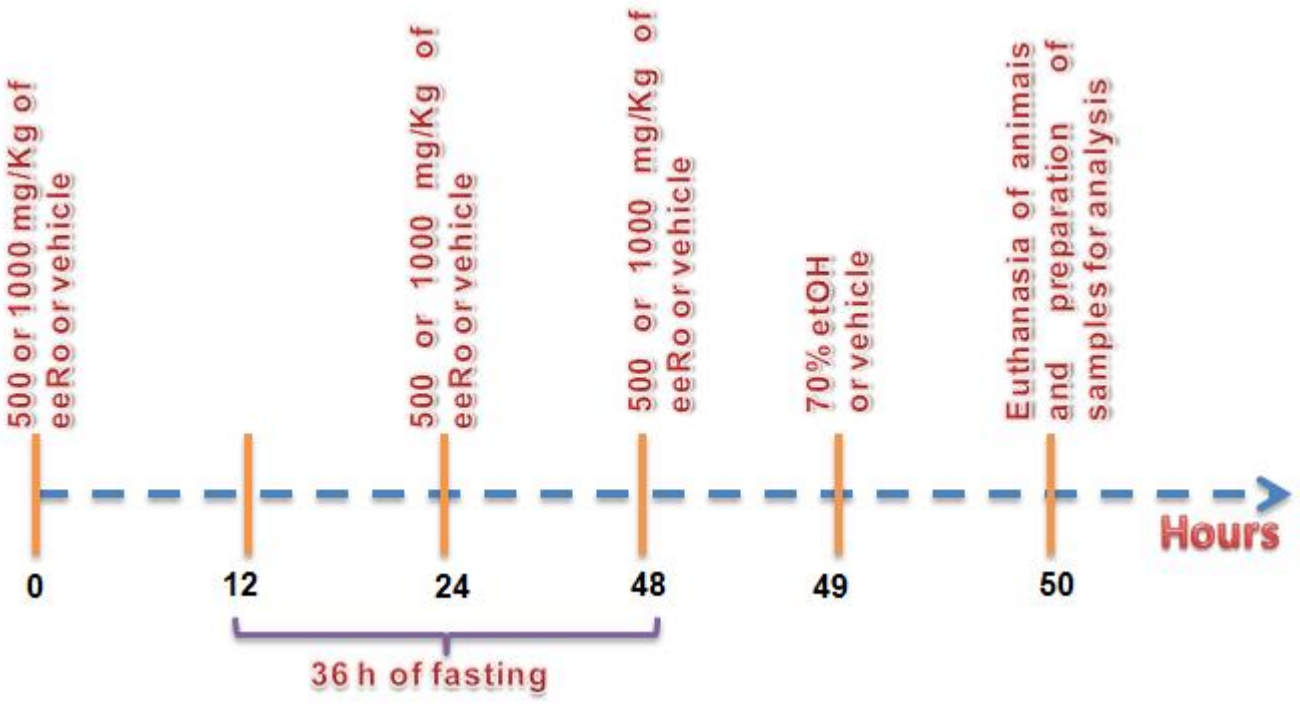

Fig. 1. Diagram showing the design and time-course of experimental procedures (eeRo, ethanolic extract of *R. officinalis* L. at 500 or 1000 mg/kg; vehicle, distilled water (98%) + tween 80 (2%); etOH, 70% ethanol). All drugs were administrated by oral way. Data are reported as five to nine animals per group.

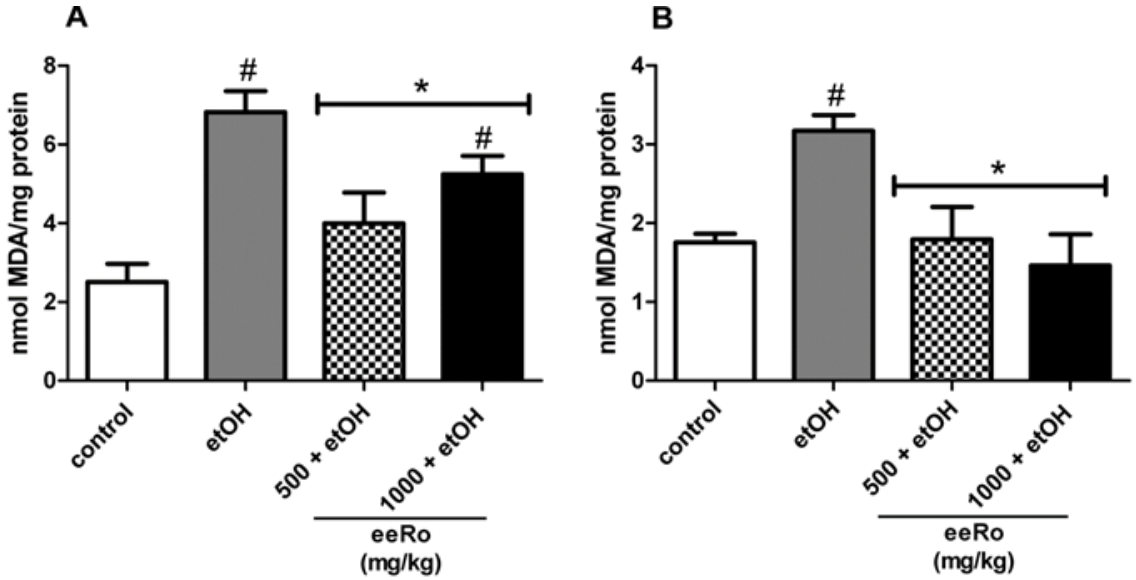

Fig. 2. Effect of *Rosmarinus officinalis* L. crude extract (eeRo) on lipid peroxidation in serum (A) and intestine (B). Data are reported as means ± SEM of five to nine animals per group. One-way ANOVA, followed by Newman–Keuls test for post hoc. etOH (70% ethanol). $^{\#}p < 0.05$, significant difference when compared to non-treated control. $*p < 0.05$, significant difference when compared to ethanol group.

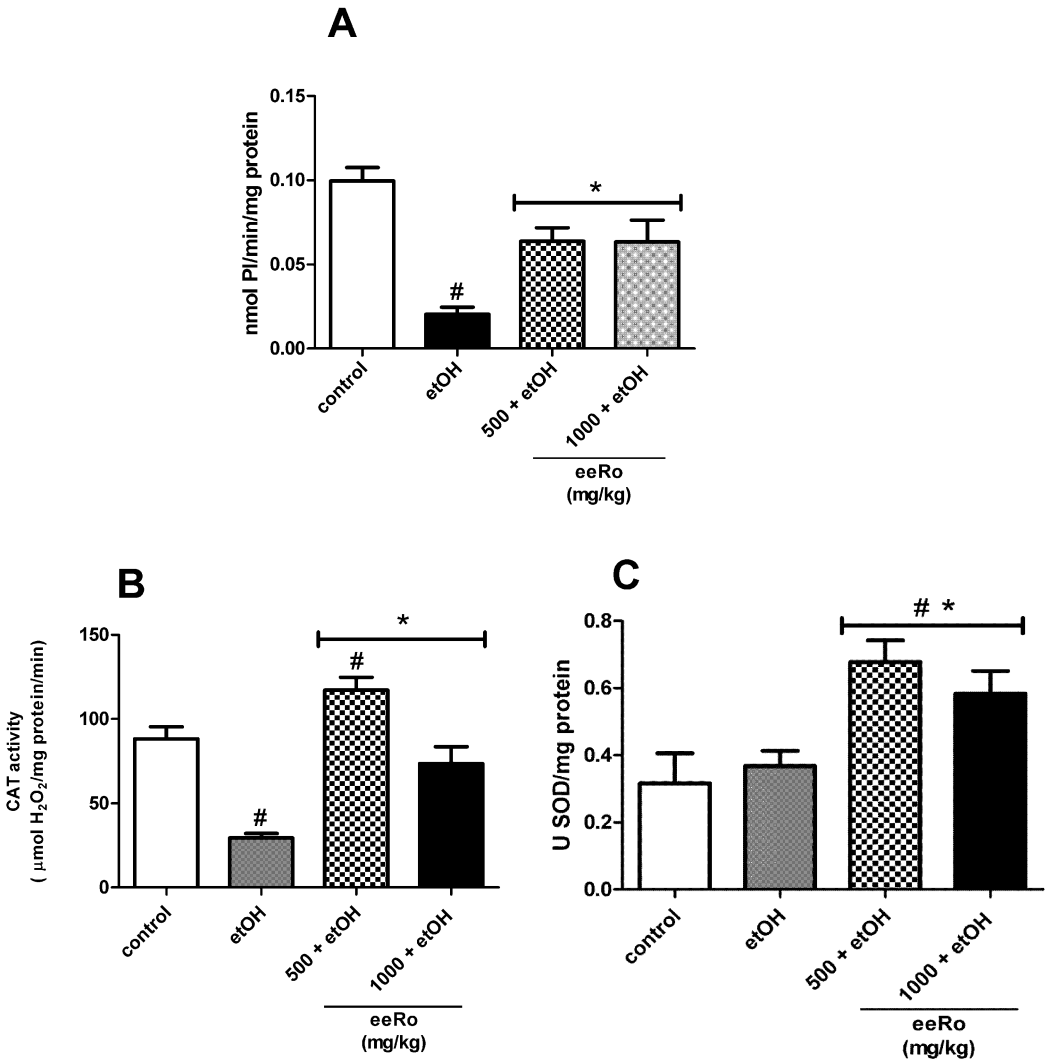

Fig. 3. Effect of *Rosmarinus officinalis* L. crude extract (eeRo) on Na^+/K^+ ATPase (A), CAT (B) and SOD activities (C). Data are reported as means ± SEM of five to nine animals per group. One-way ANOVA, followed by Newman–Keuls test for post hoc. $^{\#}p < 0.05$, significant difference when compared to non-treated control. $^{*}p < 0.05$, significant difference when compared to ethanol group.

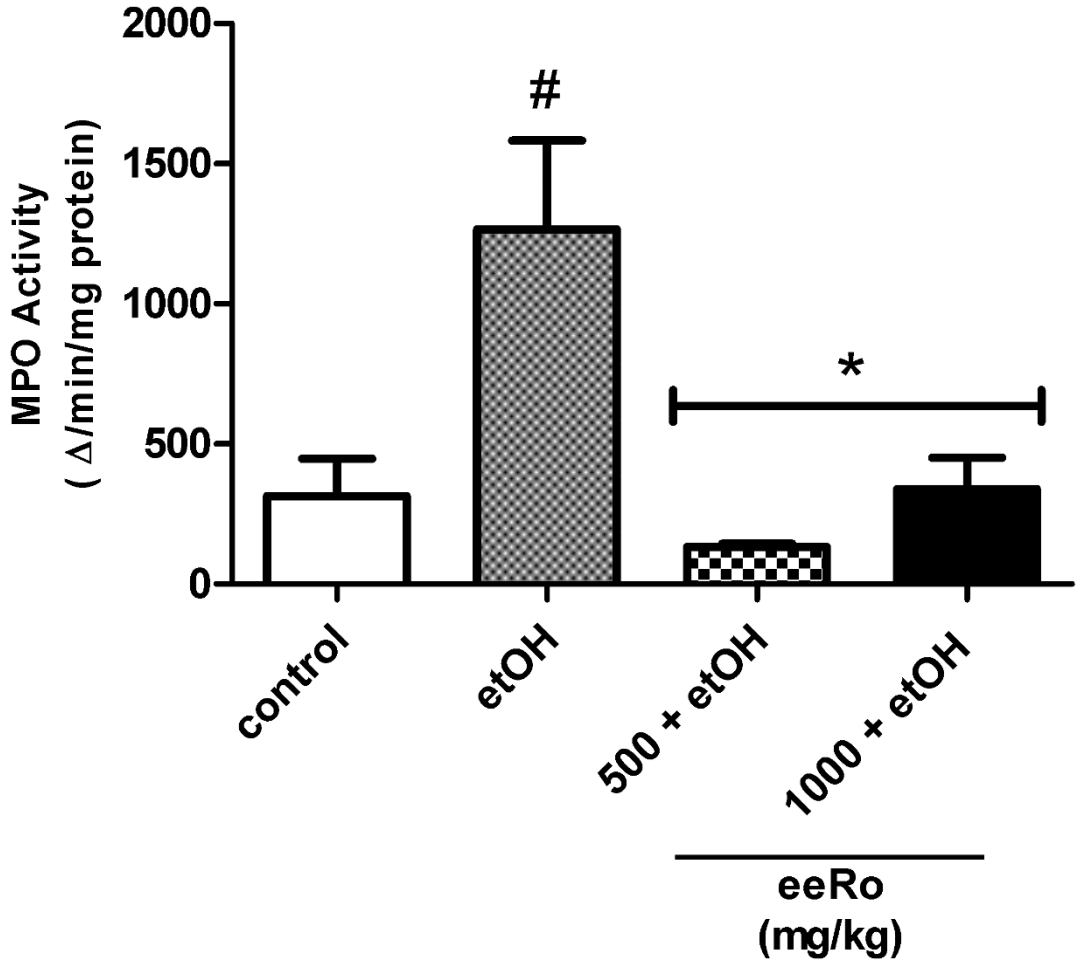

Fig. 4. Effect of *Rosmarinus officinalis* L. crude extract (eeRo) on MPO activity. Data are reported as means ± SEM of five to nine animals per group. One-way ANOVA, followed by Newman–Keuls test for post-hoc. $^{\#}p < 0.05$, significant difference when compared to non-treated control. $^{*}p < 0.05$, significant difference when compared to ethanol group.

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4. DISCUSSÃO

Ao longo de milhares de anos, reações químicas diversas, associadas a mutações genéticas criaram inúmeras espécies de plantas que se distinguiram entre si pelas suas características constitucionais (BOERO, 2015). Essas características proporcionaram uma maior ou menor capacidade de adaptação e sobrevivência conceituada primeiramente por Darwin e melhor definida por pesquisadores modernos sobre a teoria neodarwinista (BOERO, 2015). Os espécimes das espécies que apresentaram maior adaptação ao meio ambiente, sendo por suas características externas ou internas, foram selecionados (BOERO, 2015). Entre as características íntimas das plantas, o seu sistema antioxidante endógeno, principalmente constituído por compostos fenólicos, merece uma atenção especial pelo seu importante papel na dieta de humanos e animais, como possível ação nutraceutica (BHATTACHARYYA et al., 2014a; RODRÍGUEZ-MORATÓ et al., 2015; ROSENBLAT et al., 2015; WANG et al., 2015), e como matéria prima para preparações farmacológicas como extratos (ENDO et al., 2015), decoctos (ALTINIER et al., 2007) em seu estado bruto ou submetidos a seletivos fracionamentos através de solventes de diferentes polaridades (MARQUES et al., 2015). Dessa forma, esses derivados de plantas, ao longo da história da humanidade, tem servido de meio útil para o tratamento popular de diversas patologias (FARAHANI et al., 2015; MACHADO et al., 2012; SEBAI et al., 2014). E com desenvolvimento científico, muitos pesquisadores tem demonstrado um crescente interesse pelo grande potencial curativo das plantas a partir do uso popular e de preparações em estudo (FARAHANI et al., 2015; MACHADO et al., 2012; SEBAI et al., 2014) ou já descobertos e usados como fitoterápicos (CHUA, 2013).

Entre as mais antigas especulações e características farmacológicas atribuídas e constantemente investigadas, encontram-se as propriedades antioxidantes das plantas que nessa tese foram testadas para preparações de *Rosmarinus officinalis* L. em diferentes tipos de investigação. Primeiramente, foram realizados ensaios in vitro sem qualquer tecido de origem animal, para investigar a capacidade *per se* do eeRo e suas frações, sem levar em consideração as alterações metabólicas de sistemas biológicos. Dessa forma, nesses ensaios, entre os compostos testados o eeRo apresentou juntamente com a DCM uma alta ação antioxidante semelhante ou superior ao ácido ascórbico, tanto na sua capacidade antioxidante total, quanto no seu potencial "scavenging" de radical $DPPH^{\bullet-}$. Demonstrando assim, que o eeRo poderia ser usado, em baixas concentrações, com efeitos iguais ou superiores ao ácido ascórbico. Além disso, os resultados obtidos frente ao radical $DPPH^{\bullet-}$ demonstraram um forte indício de que o eeRo poderia também atuar diretamente sobre os radicais

livres em sistemas biológicos devido a características comum a todos os radicais livres, ou seja, um ou mais elétrons desemparelhados em suas estruturas.

Essas propriedades antioxidantes foram confirmadas em ensaios *ex vivo*, envolvendo amostras de fígado, cérebro e estômago de ratos, uma vez que esses órgãos são vitais e de grande importância. No caso do fígado, este órgão está envolvido em inúmeras funções metabólicas como a detoxificação e eliminação de medicamentos (REZANIA et al., 2013); e no caso do cérebro com particular relevância devido a sua fragilidade e muito baixa capacidade de regeneração diante do estresse oxidativo e lesões relacionadas (KHAN et al., 2011). Nos ensaios em questão, além dessas propriedades *in vitro*, quando o eeRo e frações foram testadas juntamente com amostras de fígado, cérebro e estômago, tanto o eeRo quanto o DCM apresentaram efeitos antioxidantes significativos contra um amplo espectro de espécies reativas de oxigênio, em níveis basais, em fígado, estômago e cérebro. Sendo que nas amostras de estômago só o eeRo e a DCM tiveram resultados significativos, demonstrando assim um melhor potencial antioxidante nos tecidos gástricos do que as demais frações.

Além desses testes, foram analisadas as capacidades protetoras do eeRo e de suas frações sobre a peroxidação lipídica, uma vez que as membranas celulares, que possuem como um dos principais constituintes os lipídios e estes são um dos principais alvos das ERs (KWIECIEN et al., 2014). A eeRo e a DCM apresentaram propriedades antioxidantes frente a peroxidação lipídica induzida por nitroprussiato de sódio, demonstrando assim que o eeRo poderia ser usado, com eficiência, em situações de alta dano tecidual, com elevadas concentrações de ER, uma vez que a peroxidação lipídica representa uma das principais causas ou consequências do estresse oxidative (KWIECIEN et al., 2014).

Na terceira etapa dos ensaios antioxidantes, os testes foram direcionados para os tecidos de maior interesse nessa tese, o estômago e o intestino delgado. Assim, quando o eeRo foi testado em um tratamento *in vivo*, ele foi capaz de proteger significativamente tanto o estômago quanto o intestino delgado contra os mecanismos agressores do etanol, que incluem o elevado estresse oxidativo (KOLACZKOWSKA et al., 2015); confirmando assim, *in vivo*, a grande capacidade antioxidante do eeRo com potencial aplicação gastrointestinal. Além disso, o eeRo também demonstrou *in vivo*, mecanismos protetores que não foram possíveis de serem observados *in vitro* ou *ex vivo*, como as suas características anti-inflamatórias, tanto em estômago como intestino, e vasodilatadora em estômago, que poderiam convergir com os mecanismos antioxidantes na proteção gastrointestinal diante de um significativo dano agudo.

Após um tratamente agudo *in vivo* com eeRo, ele foi capaz de proteger o estômago contra ulcerações causadas pela ingestão de etanol. No estômago, o eeRo demonstrou potente gastro-

proteção observada macroscopicamente pela ausência ou diminuição de lesões características do etanol como hemorragia, edema e alterações morfológicas (INEU et al., 2013). Os achados macroscópicos foram sustentados por análises histopatológicas nas quais o eeRo foi capaz de prevenir ou diminuir o edema, perda de continuidade epitelial assim como a infiltração neutrofílica característica do processo inflamatório agudo. Essa prevenção foi confirmada pela mensuração da atividade da enzima mieloperoxidase, presente em neutrófilos, que foi mantida em níveis semelhantes ao controle. Além dos efeitos anti-inflamatórios, o eeRo também teve relevante ação antioxidante pela prevenção contra o aumento dos níveis de ERO e da peroxidação lipídica, mantendo a atividade normal da CAT e dos níveis de GSH possivelmente devido a concentração elevada de ácido carnósico e rosmarínico no eeRo que poderiam prevenir a oxidação da GSH pela neutralização das ERO. Em especial a proteção que eeRo demonstrou contra a peroxidação lipídica pode possivelmente ser atribuída ao aumento das defesas do sistema antioxidante endógeno como a enzima CAT tanto no estômago como no intestino, além de níveis aumentados da razão GSH/GSSG no estômago. Além disso, a proteção gástrica foi confirmada pelos níveis significativamente menores de espécies reativas de oxigênio nos grupos tratados com eeRo.

Após um tratamento semelhante ao realizado para o estômago, o eeRo no intestino desempenhou uma importante ação protetora através dos mecanismos antioxidante e anti-inflamatório. Devido as diferenças absortivas entre o estômago e o intestino, o etanol, no intestino, não foi capaz de causar alterações sobre os níveis de ROS, razão GSH/GSSG e atividade da SOD. Contudo, devido as mesmas particularidades absortivas o eeRo foi capaz de aumentar a atividade da SOD no intestino diferentemente do que ocorreu no estômago possivelmente devido a melhor absorção de parte dos componentes do eeRo no intestino. Contudo, a atividade antioxidante do eeRo sobre as alterações causadas pelo etanol na atividade da CAT e sobre a peroxidação lipídica foram semelhantes as encontradas no estômago, mantendo níveis similares ao controle. Os aspectos anti-inflamatórios observados no eeRo foram analisados em estômago e intestino através da mensuração da atividade da MPO uma vez que essa enzima está presente em infiltrados de neutrófilos liberados principalmente na fase exsudativa do processo inflamatório e evidenciada em inflamações agudas (ARAB et al., 2015; KOLACZKOWSKA; KUBES, 2013). Nesses ensaios, os grupos tratados com eeRo, v.o., apresentaram níveis diminuídos de MPO tanto no estômago como no intestino quando comparados aos respectivos grupos tratados somente com etanol; evidenciando assim a ação anti-inflamatória do eeRo na fase celular do processo inflamatório agudo.

Além disso, testes bacterianos foram realizados devido ao crescente número de infecções em humanos e animais que poderiam afetar diretamente ou indiretamente órgãos como o fígado, cérebro

e o sistema gastrointestinal, tanto em aspectos toxicológicos como na mudança da flora intestinal (EHLING-SCHULZ; FRENZEL; GOHAR, 2015; HE; YANG; LU, 2016). Assim quando o eeRo e as frações foram testados em bactérias gram (+) e gram (-), eles foram capazes de inibir o crescimento dessas colônias a partir de concentrações muito baixas. Isso demonstra um forte indício de uma potencial aplicação antibacteriana do eeRo, juntamente com suas características antioxidantes e anti-inflamatórias, em patologias nas quais a ação simultânea desses três mecanismos seria positivamente somada, levando a prevenção, alívio ou cura.

5. CONCLUSÕES

De acordo com os resultados apresentados nesta tese, é possível inferir que entre o eeRo e suas frações, o próprio eeRo e a DCM apresentaram os melhores resultados in vitro e ex vivo. O eeRo e a DCM destacaram-se entre as demais frações por exercerem uma ação antioxidante mais eficiente *in vitro*, sem a utilização de tecidos animais, ou ainda quando foram testadas *ex vivo*, em amostras de fígado, cérebro e estômago. Além disso o eeRo, após um tratamento *in vivo*, foi capaz de exercer uma significativa proteção antioxidante e anti-inflamatória de fase celular aguda contra lesões gástricas e intestinais. Somado a isso, em amostras de estômago, o eeRo apresentou uma possível ação vasodilatadora que poderia contribuir para cura de lesões gástricas.

Contudo, devido a maior facilidade de obtenção do eeRo associado aos seus efeitos semelhantes a DCM seria mais viável a utilização da eeRo em futuros estudos que possam talvez conduzir a um melhor entendimento e uma real aplicação do eeRo. O eeRo poderia ser usado como suplemento alimentar, nutraceutico ou ainda como fitoterápico na prevenção ou tratamento de patologias gastrointestinais, com implicações hepáticas ou neurológicas com suas patogêneses relacionadas ao estresse oxidativo, inflamação ou infecção bacteriana.

6. PERSPECTIVAS

- Investigar a influência de tratamento com eeRo e DCM sobre os mecanismos específicos antioxidantes, anti-inflmatórios, vasodilatador e de secreções gastrointestinais envolvidos em lesões agudas induzidas por etanol;
- Investigar a influencia do eeRo e da DCM sobre a atividade da enzima H^+/K^+-ATPase;
- Mensurar a atividade e expressão gênica da enzima óxido nítric induzível e constitucional;
- Mensurar a expressão gênica e dosagem sérica de citocinas direta ou indiretamente relacionadas ao processo inflamatório gastrointestinal em estômago e intestino;
- Mensurar a expressão gênica das ciclo-oxigenases como COX-1 e COX-2 e prostaglandinas relacionadas com o precesso inflamatório e secreções gastrointestinais, em estômago e intestino;
- Caso o eeRo e a DCM continuarem demonstrando potenciais gastroprotetores, buscas formulações farmacêuticas apropriadas para uma melhor farmacológica delas em animais e humanos.

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8. CARTAS DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Ação protetora do extrato bruto de *Rosmarinus officinalis* na prevenção das úlceras gástricas induzidas por etanol em ratos machos da raça wistar."

Numero do Parecer: 044/2012

Pesquisador Responsável: Félix Alexandre Antunes Soares

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 13 de agosto de 2012.

Roselei Fachinetto
Coordenadora da Comissão de Ética no Uso de Animais-UFSM

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação dos efeitos de Rosmarinus officinallis L. e suas frações in vitro e sobre as alterações bioquímicas em modelo de úlcera gástrica induzida por etanol em ratos."

Número do Parecer: 068/2014

Pesquisador Responsável: Prof. Dr. Félix Alexandre Antunes Soares

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 07/08/2014.

Santa Maria, 07 de Agosto de 2014.

Sonia Lucia Loro

Prof. Drª. Vania Lucia Loro
Vice-coordenadora da Comissão de Ética no Uso de Animais- UFSM