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RAPHAELLE SOUSA BORGES

Estudo da atividade anti-inflamatória de nanoemulsões a base do óleo essencial de *Rosmarinus officinalis* L.

Macapá - AP
2018

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Tese apresentada ao Programa de Pós-Graduação em Inovação Farmacêutica da Universidade Federal do Amapá como requisito para a obtenção do Título de Doutor em Inovação Farmacêutica.

Orientador: Prof. Dr. José Carlos Tavares Carvalho
Co-orientador: Prof. Dr. Emerson Silva Lima

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Dedico este trabalho aos meus pais,
irmão e esposo.

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LISTA DE ANEXOS

A	Aneurism
ABAP	Azobis (amidinopropane) dihydrochloride
$ABTS^{+}$	Radical preformed monocation of 2,29-azinobis - (3-ethylbenzothiazoline -6 sulfonic)
ACh	Cloreto de acetilcolina
AC	Adenililcilase
AMPc	Adenosine monophosphateb
ATP	Adenosina trifosfato
BD	Bile ducts
CC	Chloride cells
CD	Cell degeneration
CED	Cell disruption
DEL	Detachment of the epithelial lining of the apex of the intestinal villous
DL	Detachment of the lamina própria
CaCM	Complexo Ca2+-Calmodulina
CCh	Cloreto de carbacol
Cd	Caldesmona
CDTC	Cytoplasmic degeneration of tubular cells
CG-MS	Coupled gas chromatography-mass spectrometry
COX-1	Ciclooxigenase-1
COX-2	Ciclooxigenase-2
CVS	Central venous sinusoids
DEC	Displacement of epithelial cells
DMEM	Dulbecco´s modified eagle’s medium
DMSO	Dimethyl Sulfoxide
DPPH	2,2diphenyl-1-picryl-hydrazyl-hydrate
DPL	Detachment of primary lamella
DSL	Detachment of secondary lamella
DCFH-DA	Dichlorofluorescein diacetate
DG	Decreased glycogen
DAG	Diacilglicerol

DML	Degeneration of muscular layer
DGC	Dilation of Glomerular capillaries
DRNF	Decreased relative frequency of nucleus occurrence
EC	Enterocyte cell
ER	Epithelial rupture
EE	Encapsulation efficiency
EC	Epithelial cells
EORO	Essential oil of *Rosmarinus officinalis*
FSL	Fusion of secondary lamellae
FV	Partial or complete fusion of villous
G	Glycogen
G	Glomerulus
GC	Globet cell
GD	Glomerular degeneration
GDP	Difosfato de guanosina
GOLD	Genetic Optimisation for Ligand Docking
H	Hyperemia
H	Hepatocytes
HCI	Histopathological Change Index
HEC	Hyperplasia of epithelial cells
HEC	Hypertrophy of epithelial cells of the lamina própria
HLP	Hemorrhage in the lamina própria
HGC	Hyperplasia of globet cells
HT	Hematopoietic tissue
HTC	Hypertrophy of tubular cells
ICAM	Intercellular Adhesion Molecule
ICV	Increased cell volume
IG	Immunoglobulin
ICV	Increased cell volume
IL	Interleukin
ILI	Leukocyte infiltration
L	Lamina própria
INV	Increased nuclear volume
IP_3	Inositol trifosfato
ISBCA	Increased space of Bowman’s capsule

ITL	Increase in tubular lumen
IFN	Interferons
IL-1β	Interleucina 1β
iNOS	Inducible Nitric Oxide Synthase
ISO	International Organization for Standardization
K_{ATP}	Canais de potássio regulados por ATP
JAK	Janus Kinase
LCC	Loss of cell contour
LNC	Loss of nucleus contour
LCC	Loss of cell contour
LPS	Lipopolysaccharides
LTB_4	Leucotrieno B_4
LT	Leukotriene
MAPKs	Mitogen Activated Protein Kinases
MCP	Monocyte Chemoattractant Protein
MIP	Macrophage Inflammatory Protein
MKP-1	Mitogen-Activated Protein Kinase Phosphatase-1
MLC	Cadeia leve da miosina
MLCK	Miosina quinase
MMP	Matrix Metalloproteinase
N	Necrosis
ND	Nuclear degeneration
NEORO	Nanoemulsion of essential oil of *Rosmarinus officinalis* L.
NOERO	Nanoemulsão obtida com o óleo essencial de *Rosmarinus officinalis* L.
NECHA	Nanoemulsão obtida com o OECHA
NECOM	Nanoemulsão obtida com o OECOM
NECULT	Nanoemulsão obtida com o OECULT
NECONTROL	Nanoemulsão controle contendo Tween 20 e água
NF-κB	Nuclear Factor kappa
NLRP-3	Nod Like Receptor P-3
NOS	Nitric Oxide Synthase
NV	Vacuolization nuclear
OECHA	Óleo essencial obtido das folhas de *R. officinalis* comercializadas para a obtenção de chá
OE	Óleo essencial

OECOM	Óleo essencial obtido das folhas de *R. officinalis* comercializado pela empresa Florien
OECULT	Óleo essencial obtido das folhas de *R. officinalis* cultivadas em campo de cultivo no Brasil
OERO	Óleo essencial de *Rosmarinus officinalis* L.
OR	Ostwald ripening
PDB	Protein Data Bank
PdI	Índice de polidispersão
PG	Prostaglandin
PGE_2	Prostraglandina E_2
PGI_2	Prostraciclina
PL	Primary lamellae
PLC	Fosfolipase C
PKA	Proteína quinase A
PKC	Proteína quinase C
PIP_2	Fosfatidilinositol bifosfato
RHOK	Rho-quinase
ROOC	Canal de cálcio operado por receptor
RyR	Receptor de rianodina
ROS	Reactive Oxygen Species
ROS	Espécies reativas de oxigênio
RT	Renal tubules
STAT	Signal Transducer And Activator Of Transcription
SL	Secondary lamellae
SIM	Sloughing of the intestinal mucosa
SV	Sinusoids vessels
TD	Tubular degeneration
TDO	Tubular disorganization
TLR	Toll Like Receptor
TNBS	Trinitrobenzene Sulfonic Acid
TNF-α	Fator de Necrose Tumoral
TPA	12-Otetradecanoyl-phorbol-13-acetate
TREM	Triggering Receptor Expressed on Myeloid Cells
TST	Tail Suspension Test
V	Vacuolization of enterocytes

VA	Villous atrophy
VCAM	Vascular Cell Adhesion Molecule
VD	Vessels dilatation
VD	Villous degeneration
VOOC	Canal de cálcio operado por voltagem
VR	Vessels rupture

Estudo da atividade anti-inflamatória de nanoemulsões a base do óleo essencial de *Rosmarinus officinalis* L.

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RESUMO

Introdução: O alecrim, *Rosmarinus officinalis* L., é um arbusto cultivado em diversos países e popularmente utilizado no tratamento de inflamações. O óleo essencial (OERO) tem sido empregado em estudos farmacológicos, que demonstram a sua atividade antiinflamatória e a atribuem a presença de compostos terpênicos. No entanto, a lipofilicidade do óleo dificulta a sua passagem através das membranas biológicas. Neste sentido, a obtenção de uma nanoemulsão poderia ampliar a permeabilidade através das membranas e potencializar o efeito anti-inflamatório. **Objetivo:** Avaliar a atividade anti-inflamatória de nanoemulsões contendo o OERO em ensaios in vivo e in vitro. Metodologia: Nanoemulsões com OEROs de procedência diversificada foram empregadas em ensaios in vitro, como atividades antioxidante e anti-inflamatória em células, produção de óxido nítrico, viabilidade celular e avaliação de efeito relaxante em músculo liso. As atividades antiálgica e anti-inflamatória foram avaliadas através do teste de contorção por ácido acético em ratos e inibição da formação de edema induzido por carragenina em pata de rato e na região abdominal de zebrafish. Também foi realizado o ensaio de toxicidade em zebrafish. **Resultados e discussões:** As amostras de óleo essencial apresentaram componentes químicos majoritários de acordo com a literatura, 1,8-cineol, α-pineno e cânfora. As nanoemulsões apresentaram tamanho médio de gotícula reduzido e baixo índice de polidispersão, além de indicativos de estabilidade física. Elas apresentaram atividade anti-inflamatória em células, e potente inibição de edema inflamatório em ratos e zebrafish, além de baixa toxicidade para zebrafish. **Conclusão:** O presente estudo possibilitou a obtenção de nanoemulsões capazes de potencializar a atividade antiinflamatória do OERO. Essa formulação aumentou a biodisponilidade do óleo nos modelos animais empregados, tonando os compostos terpênicos proeminentes mais disponíveis nos locais alvo. Este estudo abre perspectiva para o uso sustentável da espécie vegetal através de técnica de baixo aporte de energia, passível de ser reproduzida no âmbito industrial.

Palavras-Chave: *Rosmarinus officinalis*; nanoemulsão; anti-inflamatória.

Agradecimentos: CAPES, CNPq e PREI-UNAM.

Study of the anti-inflammatory activity of nanoemulsions containing the essential oil of *Rosmarinus officinalis* L.

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ABSTRACT

Introduction: Rosemary, *Rosmarinus officinalis* L. is a shrub type plant cultivated in various countries, popularly used in the treatment of inflammations. The essential oil of R. officinalis (OERO) has been evaluated in pharmacological studies, which report antiinflammatory potential, mainly attributed to its terpenoid compounds. However, its lipophilic nature stunts the passage through biological membranes. This can be bypassed using nanoemulsions, which can improve its permeability and consequently its anti-inflammatory potential. **Objective:** Evaluate the anti-inflammatory activity of nanoemulsions containing EORO performing in vivo and in vitro assays. Methodology: Nanoemulsions containing OERO samples from different sources were tested in vitro for antioxidant and antiinflammatory activity in cells, nitric oxide production, cellular viability and smooth muscle relaxant effect on isolated trachea of guinea pigs. In addition, antialgic and antiinflammatory activity were evaluated through acetic acid-induced writhing test using rats, and carrageenan-induced inflammatory edema both in rats paw and zebrafish peritoneal region. **Results and discussion:** The tested samples of EORO presented the major compounds according the published literature, 1,8-cineol, α-pinene and camphor. The nanoemulsions showed small average size, low polydispersity index, and physical stability. **Conclusion:** This study allowed obtaining nanoemulsions capable of improve the antiinflammatory activity of EORO. This formulation increased the bioavailability of the oil in the employed animal models, increasing the availability of the major terpenic compounds in the target tissue. This study open perspective toward sustainable use of this vegetal specie through a low energy load technique, passible of use in the industrial level.

Keywords: *Rosmarinus officinalis*; nanoemulsion; anti-inflammatory.

Acknowledgements: CAPES, CNPq and PREI-UNAM.

1 INTRODUÇÃO

A espécie vegetal *Rosmarinus officinalis* L., popularmente conhecida como alecrim, é um arbusto de até 2 m de altura com folhas verdes de aroma intenso e pertencente à família Lamiaceae. É nativo da região do Mediterrâneo e seu desenvolvimento ocorre preferencialmente em solo seco a moderadamente úmido.

Tem sido popularmente utilizada para fins medicinais. É reconhecida por diversas atividades biológicas. As partes aéreas são usadas principalmente na forma de decocção e infusão ou óleo essencial por administração oral para dores de cabeça e da região abdominal e também para tratar diferentes enfermidades de origem inflamatória.

O óleo essencial de *Rosmarinus officinalis* L. (OERO) é um líquido incolor de sabor canforado e aroma intenso. Tem relevância comercial devido à sua aplicação nas indústrias farmacêutica e alimentar. É composto, principalmente, por monoterpenos, alguns diterpenos e sesquiterpenos. O teor desses compostos pode variar de acordo com fatores geográficos e partes da planta utilizadas. Diversos estudos demonstram que os componentes terpênicos, 1,8-cineol, cânfora e α-pineno, são os principais responsáveis pelas atividades biológicas relatadas.

Apesar de seu uso popular para o tratamento de inflamações diversas, a intrínseca baixa solubilidade dos constituintes desse óleo e a imiscibilidade em água dificultam a sua passagem através das membranas biológicas. Diante deste fato, o encapsulamento do OERO torna-se uma alternativa para ampliar a sua biodisponilidade e promover o uso sustentável da espécie vegetal.

A nanotecnologia tem caráter multidisciplinar e tem sido empregada para a obtenção de novos produtos em diversos segmentos industriais, na forma de nanocápsulas, nanodispersões ou nanoemulsões, que promovem o aumento da área superficial dos materiais em escala nano. As nanoemulsões consistem em sistemas onde uma substância com características lipofílicas se encontra encapsulada em micelas cercadas por moléculas anfifílicas. Isso a torna um veículo importante para a passagem de substâncias de reduzida solubilidade, como os óleos essenciais, através das membranas biológicas.

Desta maneira, a obtenção de nanoemulsões usando o óleo essencial de *Rosmarinus officinalis*, espécie cultivada em diversas regiões do mundo, representa uma alternativa viável para aumentar a biodisponibilidade do OERO, mantendo seu potencial

anti-inflamatório e ampliando sua eficácia terapêutica.

O presente estudo encontra-se apresentado na forma de artigos de acordo com as normas do Programa de Pós-Graduação em Inovação Farmacêutica. Os capítulos que o compõem, são:

Capítulo 1: Phytochemical and pharmacological studies of *Rosmarinus officinalis* L. essential oil related to anti-inflammatory activity;

Capítulo 2: Anti-inflammatory and antialgic actions of a nanoemulsion of *Rosmarinus officinalis* L. essential oil and molecular docking study of its major chemical constituents;

Capítulo 3: Anti-inflammatory activity of nanoemulsions of essential oil from *Rosmarinus officinalis* L.: *in vitro* and in zebrafish studies;

Capítulo 4: Toxicidade de nanoemulsões contendo o óleo essencial de *Rosmarinus officinalis* L. em zebrafish (*Danio rerio*);

Capítulo 5: Efeito relaxante de nanoemulsões contendo o óleo essencial de *Rosmarinus officinalis* L. sobre o músculo liso de cobaias.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Realizar o estudo da atividade anti-inflamatória de nanoemulsões contendo o óleo essencial de *Rosmarinus officinalis* L. (NOERO), em ensaios *in vivo* e *in vitro*.

2.2 OBJETIVOS ESPECÍFICOS

Determinar a atividade anti-inflamatória da NOERO *in vivo*, através dos ensaios de avaliação de edema induzido por carragenina em pata de rato e na região abdominal de zebrafish;

Avaliar a atividade antiálgica da NOERO através do teste de contorção induzida por ácido acético;

Analisar a atividade gastroprotetora da NOERO com o ensaio de produção de H_2S em estômago de ratos;

Estimar a potência anti-inflamatória da NOERO *in vitro*, através de métodos padrão, como ensaio de viabilidade celular, atividade anti-inflamatória, atividade antioxidante e determinação da produção de óxido nítrico em células;

Avaliar a toxicidade da NOERO aplicada em zebrafish via oral para observação do comportamento dos peixes e alterações histológicas de brânquias, fígado, rins e intestino;

Avaliar o efeito relaxante da NOERO em traquéia isolada de cobaia.

3 CAPÍTULO 1

PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF *ROSMARINUS OFFICINALIS L.* ESSENTIAL OIL RELATED TO ANTI-INFLAMMATORY ACTIVITY

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Abstract

Ethnopharmacological relevance

The plant species *Rosmarinus officinalis* L. is widely used all over the world for many purposes ranging from use in cooking, spiritual healing and as a popular medicine for inflammatory processes in general. Several authors have demonstrated in several studies that the essential oil of *Rosmarinus officinalis* L. (EORO) presents biological activities, as its bioactive compounds. The major components, such as 1,8-cineol, camphor, and α-pinene, have been associated with the antiinflammatory activity.

Aim of the study

This review aimed to describe the chemical composition of EORO and the main studies related to anti-inflammatory activity and its possible mechanisms of action.

Materials and methods

We researched Medline, Embase, BVS Regional Portal, Science Direct, CAPES Journals, Scopus, using the keywords Rosmarinus officinalis L., anti-inflammatory and essential oil, complementary information was obtained from related textbooks.

Results

150 chemical compounds were identified in EORO samples. Studies report that the anti-inflammatory effect of EORO may possibly occurs through inhibition of arachidonic acid metabolites formation and possibly inhibition of NF-κB transcription, which is synergistic with the antioxidant and relaxing activities of smooth muscle.

Conclusions

Although it has been widely evaluated in recent years, EORO will most likely continue to be subject of research, since more trials are needed to elucidate the various biological activities reported and consolidate it as an anti-inflammatory agent.

Keywords: reviews, essential oil, inflammation, cyclooxygenase, antioxidant

Abbreviations:

EORO: Essential oil of *Rosmarinus officinalis*; NF-κB: Nuclear Factor kappa B; ISO: International Organization for Standardization; TST: Tail Suspension Test; DPPH: 2,2diphenyl-1-picryl-hydrazyl-hydrate; LT: Leukotriene; PG: Prostaglandin; TNF-α: Tumor Necrosis Factor; IL: Interleukin; MMP: Matrix Metalloproteinase; COX: Cyclooxygenase; iNOS: Inducible Nitric Oxide Synthase; ICAM: Intercellular Adhesion Molecule; VCAM: Vascular Cell Adhesion Molecule; MIP: Macrophage Inflammatory Protein; TNBS: Trinitrobenzene sulfonic acid; ROS: Reactive Oxygen Species; NOS: Nitric Oxide Synthase; TREM: Triggering Receptor Expressed on Myeloid Cells; NLRP-3: Nod Like

Receptor P-3; MAPKs: Mitogen Activated Protein Kinases; MKP-1: Mitogen-Activated Protein Kinase Phosphatase-1; LPS: Lipopolysaccharides; TLR: Toll Like Receptor; TPA: 12-OTetradecanoylphorbol-13-acetate; Ig: Immunoglobulin; IFN: Interferons; MCP: Monocyte Chemoattractant Protein; JAK: Janus Kinase; STAT: Signal Transducer And Activator Of Transcription.

1 Introduction

Rosmarinus officinalis L., is a worldwide cultivated plant, known for its diverse nutritional and medicinal properties. It's widely used in cooking to aromatize foods and industrially as a food preservative, due to its antioxidant potential. It's also used in cosmetic products. The plant has long been used in folk medicine for centuries due to its pharmacological activities on many diseases (Borrás-Linares et al., 2014).

Andrade et al. (2018) showed the growing interest in studies with this plant, with a considerable amount of research since the year 2010, with an average of 120 per year. Studies report mainly: antibiotic, antifungal, antiviral, antidiabetic, antimutagenic, antiprotozoal, antioxidant, antidepressive and anti-inflammatory activities (Selmi et al., 2017; Ghasemian et al., 2016; Ali et al., 2015; Shakeri and Boskabady, 2015; Machado et al., 2013; Fahim et al., 1999). These diverse biological activities are attributed to the vast presence of bioactive molecules in the composition of EORO (BorrásLinares et al., 2014). Mainly terpenes, such as 1,8-cineole, borneol, pinene, limonene, camphene, camphor and myrcene (Borges et al., 2017; Selmi et al., 2017; Bajalan et al., 2017; Vilela et al., 2016; Takayama et al., 2016; Chávez-González et al., 2016; Akbari et al., 2015; Machado et al., 2013; Faria et al., 2011; Juhás et al., 2009).

In folk medicine, *R. officinalis* is used in the form of tincture or infusion obtained from the stem and leaves (Marchiori, 2004). Infusion is used in the treatment of gastric disorders, pain, and inflammatory origin diseases. The EORO, after ingestion, can act both physiologically and psychologically (Marchiori, 2004). It has been used for the treatment of dyspepsia and milder forms of gastrointestinal tract spasmodic disorders; as a complement in the treatment of inflammations and pain of muscular and articular origin; also in circulatory system anomalies (Rašković et al., 2014). Due to its broad application, studies have been carried out to investigate its biological activities, and mechanism of action.

The anti-inflammatory activity attributed to EORO instigated the interest of researchers worldwide and has been applied in several studies in the field of pharmacology, both *in vitro* (de Melo et al., 2011) and *in vivo* assays (Takaki et al., 2008,

Inoue et al., 2005; de Melo et al., 2011), to evaluate its chemical compounds and their mechanisms of action behind it's known pharmacological properties.

Essential oils are complex mixtures obtained mainly by distillation of the plant material by steam. They are widely marketed worldwide for different applications, being purchased mainly by alimentary and fragrance industries, used to obtain cleaning products, cosmetics, candles, incense and herbal derived products (de Groot and Schmidt, 2016). They usually present large amounts of terpenes, mainly monoterpenes and sesquiterpenes, among other plant secondary metabolites, such as aromatic, phenolic and alcoholic compounds (de Melo et al., 2011; Patel, 2015). EORO has a strong aroma and transparent or pale yellow color (Rašković et al., 2014). Different chemotypes of this oil have been reported in the literature named according to the predominant chemical compound.

The aim of the article is to update our present knowledge of the chemical composition of EORO, highlighting the major chemical compounds, especially those associated with the anti-inflammatory action.

2 Materials and Methods

The literature review was analyzed using the following databases: Medline, Embase, BVS Regional Portal, Science Direct, CAPES Periodicals and Scopus using the keywords *Rosmarinus officinalis* L., anti-inflammatory, and essential oil. For the research, keywords were used combined as follows: *Rosmarinus officinalis* L., plus anti-inflammatory; *Rosmarinus officinalis* L. plus essential oil; *Rosmarinus officinalis* L., anti-inflammatory plus essential oil. Among the found articles, repeated ones were excluded, those which described the phytochemistry of EORO were selected, together with articles that related the major chemical compounds with anti-inflammatory activity, demonstrating the possible mechanisms of action. Complementary information was obtained from related textbooks: Rang et. al (2011), Marchiori (2004), Muñoz-Centeno (2002).

3 *Rosmarinus officinalis* relevance in pharmacological research

The pharmacological importance of *Rosmarinus officinalis* is remarkable. Figure 1 shows the number of results found in each database with the terms used. 944 items in total were found, 51 in Medline, 47 in Embase, 62 in the BVS Regional Portal, 567 in Science Direct, 174 in the CAPES Periodicals and 43 in Scopus. The diversity of studies found in general search evidences the growing interest of researchers to identify the chemical components of EORO and demonstrate their activity as an anti-inflammatory

agent.

3.1 Botanical Description

Rosmarinus officinalis L. − Taxonomic Serial N°.: 32677 (ITIS, 2018) − belongs to the Lamiaceae family, formerly called Labiatae (Begum et al., 2013). It is known in some countries by the common name of alecrim (Portuguese), rosemary (Spanish) (Muñoz-Centeno, 2002). It is a shrub type plant that grows up to 2 m tall with perenial green leaves, native of the Mediterranean region, with an intense aroma and a rough, bitter and slightly spicy taste due to its chemical compounds present in its volatile oil, accumulated in glandular trichomes (Ribeiro-Santos et al., 2015; Kokkini et al., 2003).

The plant has brown, erect, rarely crawling branches with bright dark green, linear, coriaceous leaves with inward-facing edges measuring about 10-36 x 1.2-3.5 mm with white tomento covered posterior portion. The flowers are small, arranged in axillary pauciflorus verticillasters. The corolla, measuring about 10 to 12 mm, is slightly white to pink. Androecium is formed by two distinctly exposed parallel stamens, and its filaments have a small lateral tooth (Ribeiro-Santos et al., 2015); (Kokkini et al., 2003; Muñoz-Centeno, 2002; Tawfik et al., 1998).

It usually grows in dry or moderately humid soil, does not tolerate anaerobic or soaked soil and exhibits average tolerance to salinity. The flowering period often occurs between May to June in Mediterranean climate, and its fructification takes place between spring and summer. It's a temperate climate plant that grows naturally in the Mediterranean region (Ribeiro-Santos et al., 2015).

3.2 History and traditional use of *Rosmarinus officinalis* L.

Since ancient times, *R. officinalis* has been employed in several ways. In ancient Egypt, its leaves and flowers were used to deodorize bodies of dead pharaohs. In China, India and Mesopotamia, it was consumed in their diet, and applied in preparation of medicines and cosmetics (Ribeiro-Santos et al., 2015).

In Hungary, the plant was used in the treatment of rheumatism, utilizing an infusion called "water of the queen of Hungary," whose name is due to the possibility that the distillate was used in the treatment of rheumatic pain of queen Isabel of Hungary in the XIII century. This infusion made the *R. officinalis* popular among the medicinal plants used by the court of Louis XIV. In Europe, it was considered a revitalizing agent, subsequently being used in the production of the first alcohol-based perfume. Around 1330, the essential oil was for the first time obtained by the philosopher Raimundo Lúlio, since then it has

been used in perfumeries (Muñoz-Centeno, 2002).

There are two possible sources for the generic name *Rosmarinus*. One possibility is that the name derives from Latin ros (dew) and marinus (sea), sea dew; the other says its derived from the two Greek words, rhos (shrubs) and mirrinos (aromatic), due to the characteristics of the plant. The name of the specie, officinalis, is used to denote its application as a medicinal plant (Ribeiro-Santos et al., 2015; Begum et al., 2013; Faria et al., 2011; Napoli et al., 2010; Muñoz-Centeno, 2002; Aqel, 1992).

Currently, *R. officinalis* is a worldwide known aromatic plant widely used in folk medicine due its therapeutic properties. Aerial parts (fresh or dried) are mainly used by decoction or infusion with oral administration in the treatment of headaches; illnesses of the abdominal region, as antispasmodic; arthritis, and finally to minimize the symptoms of gout. In the form of poultice, the plant is used for wounds healing (Aqel, 1992). EORO is also used in the treatment of digestive disorders and respiratory diseases, due to its anti-inflammatory, analgesic and antimicrobial effect (Raut and Karuppayil, 2014; Ribeiro-Santos et al., 2015).

4 Essential oils

Essential oil is defined by ISO as the product obtained from raw plant material by steam distillation, including hydrodistillation, or alternative processes with the same principle (de Groot and Schmidt, 2016). They are complex mixtures of volatile compounds, usually with large amounts of terpenes, mainly monoterpenes and sesquiterpenes (de Melo et al., 2011), among other plant secondary metabolites, such as aromatic, phenolic and alcoholic compounds (Patel, 2015).

The most commonly used extraction technique on industrial scale is the steam distillation, which is faster and more suitable for large quantities. In laboratories, the predominant method is hydrodistillation using Clevenger type devices, that despite being slower, volatilizing the oil at lower temperature, it maintains the integrity of thermolabile substances that could be degraded by steam distillation (de Groot and Schmidt, 2016). Other methods include cold press distillation (Patel, 2015), dry distillation (Filly et al., 2014), and hydrodistillation with microwaves, a modified hydrodistillation method that takes up to 65% less extraction time due to the higher temperature gradient caused by microwaves (Karakaya et al., 2014).

Essential oils are widely used as flavorings, food additives, fragrances, in folk medicine and phytotherapy. It is also widely by cosmetic, pharmaceutical and general chemical industries (de Groot and Schmidt, 2016). The chemical composition of essential

oils depends not only of the plant species, but also of its age, variety, part collected, origin, climate, soil, agrochemicals employed, stocking time, preparation, among other factors. This variation is more quantitative than qualitative. Due to these variations, performed essays with essential oils should always provide the botanical characterization of the plant material and phytochemical profile of the extracted oil, enabling the reproducibility and accuracy of data (Freires et al., 2015).

4.1 Phytochemical studies of *Rosmarinus officinalis* essential oil

The essential oil of *Rosmarinus officinalis* L. (EORO) – ISO N° 1342:2012 (ISO, 2018) − has commercial importance due its application in pharmaceutical and food industry (Kfoury et al., 2015). It is a colorless or pale-yellow liquid with intense and spicy aroma. It tastes like camphor due its chemical composition, consisting mainly of monoterpenes, some diterpenes, and sesquiterpenes. EORO represents about 1-2.5% of the of the plant total weight. The phytochemical profile of EORO, as other essential oils, may vary according to geographical area where the plant is collected, climate, parts of the plant used for extraction, and lastly the extraction method (Mouahid et al., 2017; Tawfeeq et al., 2016; Yosr et al., 2013; Graber et al., 2010; Napoli et al., 2010; Tawfik et al., 1998). The characteristic chemical components of this oil are 1,8-cineol, α-pinene, camphor, bornyl acetate, borneol, camphene, α-terpineol, limonene, β-pinene, β-caryophyllene and myrcene (Chávez-González et al., 2016).

EORO originating of Morocco and Tunisia shows high content of 1,8-cineol, while EORO from Spain shows small amounts of this compound (Muñoz-Centeno, 2002). The EORO obtained from leaves cultivated in the region of Tunisia shows increased extraction yield during the flowering phase (1.43%) compared to vegetative phase (1.23%); plants collected in the summer shows almost double of the yield compared to those collected in the winter (Tawfik et al., 1998).

Likewise, there is a significant variation in the quantity and type of compounds found in the essential oil according to the plants parts collected and phenological stage of its development. In a study of EORO composition by Yosr et al. (2013), 1.8-cineol (35.8%) was the main component obtained from leaves extract, which is the most commonly used part for EORO extraction (Rašković et al., 2014). In the same study of Yosr et al. (2013), caryophyllene (16.7%) was the major compound present in the extracted oil from stems. Furthermore, flowers extracted essential oil had a high content of caryophyllene oxide (11,9%).

According a study by Mouahid et al. (2017), extraction of EORO by CO_2

supercritical fluid showed increased the concentration of some compounds, such as verbenone (34.16%) and bornil acetate (17.31%) compared with traditional hydrodistillation method (Napoli et al., 2010).

In essential oils, the terpenes − molecules formed by isoprene units − are usually the most abundant and diverse compounds. Being accountable for the characteristic smell and flavor of aromatic or spicy plants (Silveira e Sá et al., 2013). Napoli et al. (2015) surveyed the phytochemical components found in EORO from a 40 different plant samples. A total of 82 molecules were identified and classified in three categories: monoterpenes hydrocarbon, oxygenated monoterpenes, and sesquiterpenes. Components that did not fit either group were classified as "other."

The monoterpenes hydrocarbons constituted from 20.9 to 65.6% of the samples content. Among them, the most abundant were α-pinene (11.8 - 39.8%) and canfene (3.2 - 12.1%). Some other identified compounds were limonene, β-pinene, terpinolene, and β-myrcene. The components classified as oxygenated monoterpenes constituted from 27.7 to 74.3% of the EORO composition, represented majorly by 1,8-cineol (0.1- 62.7%) followed by camphor (2.6 - 30.5%). Borneol, linalool, and verbenone were also found in significant amount. In turn, sesquiterpenes constituted between 0.6 to 7.2% of the EORO composition and the highest amount compound was β-caryophyllene. No sesquiterpene exceeded 1%. The group classified as "others" constituted 0.2 to 2.2% of the total sample content. No compound exceeded 1%, with octan-3-one being the most representative.

In this present review was noticed that until now, about 150 different chemical compounds were identified in EORO (Table 1). Linear and oxygenated monoterpenes were the most frequently reported terpenes, such as 1,8-cineol, α-pinene, camphene, β-pinene, camphor, borneol, bornyl acetate, *p*-cymene, β-myrcene, limonene, α-terpinene, verbenone, β-terpineol, linalool and terpinen-4-ol (Figure 2). Many of these compounds are considered the most representative for EORO, as they are often present, and may act as biochemical markers of the oil characteristics.

The EORO samples can be classified in chemotypes according the most abundant chemical component, such as cineoliferous (high concentration of 1,8-cineol), camphoriferous (high concentration of camphor), and α-pinene chemotype (high concentration of α-pinene) (Napoli et al, 2015; Yosr et al., 2013; Afolous et. al., 2013; Tomi et al., 2016; Tucker and Maciarello, 1986). In most of the articles surveyed, the major components frequently associated with the different biological activities of EORO were 1,8-cineol, α-pinene, and camphor (Table 2).

These are monoterpenes (10 carbon atoms) formed by two units of isoprene

(Tawfik et al., 1998). In the plant metabolism, 1,8-cineol is an epoxidized monoterpene biosynthesized from αterpineol through formation of ether at C3 and C7 carbons followed by reduction. The bicyclic monoterpene α-pinene is formed by electrophilic addition, creating a four-membered ring through formation of a covalent bond with the C2 carbon. Camphor, an oxygenated monoterpene, is biosynthesized from oxidation of C4 carbon hydroxyl group from the bicyclic monoterpene borneol (Figure 3) (Wiart, 2014; Dewick, 2009).

The 1,8-cineol stands out as the most reported chemical compound. It is known also by the name of eucalyptol, being found in the genera *Eucalyptus*, *Rosmarinus*, *Psidium*, *Croton* and *Salvia* (Juergens, 2014). This molecule is relatively abundant in EORO and may reach up to 40% of EORO volume in cineoliferous chemotype (Napoli et al., 2015).

4.2 Biological activities related to major compounds from the EORO

Several studies show that biological activities of EORO are influenced by its chemical composition. The most reported compounds of EORO in the literature associated with different biological activities are 1,8-cineol, camphor, and α-pinene (Table 2).

To 1,8-cineol was attributed antidepressive, antimicrobial, antioxidant, anti-allergic and anti-inflammatory properties (Selmi et al., 2017; Bajalan et al., 2017; Takayama et al., 2016; Vilela et al., 2016; Machado et al., 2013; Faria et al., 2011). While α-pinene is associated with antioxidant, antifungal and antibacterial activities (Lin et al., 2016; Mekonnen et al., 2016; Takayama et al., 2016). On the other hand, studies relate camphor to antimutagenic, antioxidant, anti-allergic and antiinflammatory effect (Borges et al., 2018; Takayama et al., 2016; de Melo et al., 2011).

In a research conducted by Machado et al. (2013), orally administered EORO showed antidepressant effect on TST assay by reducing the immobilization time of treated rats compared to the control group. This activity was associated with 1,8 cineol, which consisted of 45.1% of the essential oil used.

Using cineoliferous type oil extracted from Portugal cultivated plants, EORO showed antimicrobian activity against *Enterobacteriaceae*, *Pseudomonas spp*., Fungi, mesophilic, psychrotrophic and Lactic acid bacteria, reducing microbial quantity in vacuum stored meat at 2°C.

High α-pinene concentration EORO was effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Shigella exneri*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Serratia marcescens* and *Escherichia coli* bacteria.

Showing also antifungal activity against *Trichophyton sp.* and *Aspergillus sp.* (Mekonnen et al., 2016).

In Iran cultivated *R. officinalis*, α-pinene chemotype essential oil showed more prominent antibacterial effect against *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* compared to cineoliferous and camphoriferous chemotypes. In DPPH antioxidant assay, all the samples showed inhibition of free radical activity, suggesting antioxidant potential, which was attributed to 1,8-cineol, camphor, and α-pinene (Bajalan et al., 2017).

The antioxidant effect of EORO was demonstrated by preventing superoxide anion formation and considerably decreasing myeloperoxidase activity in gastric injury induced by ethanol in rats. This antioxidant activity was attributed to the major compounds identified (1,8-cineol, camphor, and α-pinene), suggesting a synergistic effect (Takayama et al., 2016).

To 1,8-cineol was also attributed the antioxidant activity of EORO in liver and kidney oxidative stress studied in rats (Selmi et al., 2017) using oil extracted from plant samples from Tunisia. An additional study report antioxidant activity of EORO, evidenced by the significant reduction of mutagenicity induced by cyclophosphamide in rats. The antimutagenic affect in bone marrow cells was attributed to the antioxidant activity of EORO compounds, with camphor as one of the oil's major compounds (Fahim et al., 1999).

4.2.1 Anti-inflammatory activity of EORO and mechanisms of action described

Concerning the biological activities of EORO, its potential as an anti-inflammatory agent was reported in several pharmacological experiments. Great part of these studies are attributed the anti-inflammatory activity of EORO to the presence of 1,8-cineol, camphor, and α-pinene.

Among these components, 1,8-cineol has been the most associated with the anti-inflammatory activity. Santos and Rao (2000) demonstrated its ability to inhibit *in vivo* the vascular permeability increase and granuloma formation in carrageenan-induced rat paw edema assay. In addition to that, 1,8-cineol inhibited *in vitro* the production of cytokines and arachidonic acid metabolites in human blood monocytes (Juergens et al., 1998).

Anti-inflammatory action of 1,8-cineol involves at least three possible mechanisms. On the one hand, it may be due inhibition of 5-lipoxygenase pathway, on the other hand, could be due through inhibition of cyclooxygenase, consequently preventing arachidonic acid metabolites formation, such as LTB_4 and PGE_2. It is well established that these

pathways are associated with several biological processes, such as inflammation (Figure 4) (Juergens, 2014). Alternatively, its evidenced that 1,8-cineol can inhibit the NF-κB transcription, as demonstrated by Greiner et al. (2013). In the previously mentioned study of de Melo et al. (2011), 1,8-cineol was one of the major compounds of the tested OERO. The author discusses that the presence of terpenes, and their potential to inhibit NF-κB transcription may contribute to EORO anti-inflammatory potential (Figure 5).

It is an established fact that NF-κB plays a key role in the inflammation (Hoesel and Schmid, 2013; Baker et al., 2011; Zhang et al., 2017), since it acts as transcription factor of several molecules involved in this process, such as cytokines (TNF-α, IL-1, IL-6), metalloproteinases (MMP-9), inflammatory enzymes (ex: COX-2, iNOS), adhesion molecules of diapedesis (ICAM-1, VCAM1), chemokines (MIP-1, MIP-2) among others (Gilmore, 2018). Besides 1,8-cineol, other terpenes are known to act this way (Santana Souza et al., 2014; Silveira e Sá et al., 2013; Heras et al., 2009) (Figure 5).

A study performed with the rat paw edema assay, oral administration of EORO significantly inhibited the edema formation induced by carrageenan. This anti-edematogenic activity was associated to the presence of 1,8-cineol with possible synergistic action with myrcene. Antiinflammatory and antinociceptive effects were explained at least partially by inhibition of prostaglandin synthesis (Figure 4) or due release of other endogenous mediators (Takaki et al., 2008).

In another study, the anti-inflammatory and antinociceptive effects were also related to the presence of 1,8-cineol and camphor. Using carrageenan-induced rat paw edema and intraperitoneal acetic acid injection nociceptive models. EORO showed anti-inflammatory action in acute and chronic inflammation and peripheral analgesic activity, besides not causing damage to the gastric mucosa (Faria et al., 2011).

In the TNBS-induced colitis test, 1250 ppm concentration of cineoliferous EORO significantly decreased myeloperoxidase activity and IL-6 levels compared to control animals (Juhás et al., 2009). Borges et al. (2017) showed through a molecular modeling study (docking) that camphor has the highest number of interactions with therapeutic targets related to inflammation, such as PGI_2 and COX-2 (Figure 4).

Studies reports that EORO components with anti-inflammatory activity also shows antioxidant potential, which may contribute to its anti-inflammatory effect. β-pinene, limonene, γ-terpinene, linalool, terpin-4-ol, α-terpineol and β-caryophyllene show oxygen radical absorption capacity, while α-terpinene and γ-terpinene exhibit radical sequestering activity. Also, α-pinene, camphene, and 1,8-cineol, shows chelating capacity of Fe^{2+} ions, attributed to the possible interactions between these compounds (Cutillas et al., 2018).

Another study assigns the antioxidant activity mainly to the essential oil's myrcene of the used sample (Ojeda-Sana et al., 2013), which may be potentiated by other present compounds. EORO samples with high concentration of α-pinene, 1,8-cineole, and camphor, just as nanoemulsions obtained from these oils presented significant antioxidant action by inhibition of ROS formation in MRC5 fibroblasts, with values similar to the antioxidant standard of quercetin (Borges et al., 2018).

Bozin et al. (2007) evaluated the EORO free radical scavenging activity in DPPH and hydroxyl radicals scavenging in the deoxyribose assay. Furthermore, antioxidant activity was investigated through lipid peroxidation induced by the Fenton reaction with Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2. EORO reduced formation of DPPH in a dose-dependent manner. Moreover, showed scavenging activity of hydroxyl radicals and strongly inhibited lipid peroxidation. The authors suggest that these activities may be due result of synergistic effect of compounds identified in the oil, mainly oxygenated monoterpenes (bornyl acetate, camphor, and menthene), besides mono and sesquiterpene hydrocarbons (Figure 6).

In tested animals with ethanol-induced gastric injury, pre-treatment with EORO exhibited increased glutathione levels and low glutathione peroxidase activity. Since the oxidative stress promoted by ethanol induces formation of superoxide ion ($O_2^{\bullet-}$), which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and H_2O_2 is subsequently inactivated by the enzyme glutathione peroxidase (GTX). The results evidenced that EORO inhibited gastric damage through induction of increased glutathione levels and prevention of ROS formation (Takayama et al., 2016). The EORO sample used presented high concentration of 1,8-cineol, camphor, and α-pinene, but the authors attributed the antioxidant effect to the combined action of the compounds (Figure 6).

Considering that reactive oxygen species are some of the main molecules associated with the nocive potential of inflammation (Arulselvan et al., 2016). EORO can act additionally by suppressing ROS levels and scavenging reactive species (Hussain et al., 2010; Selmi et al., 2017; Rašković et al., 2014), mitigating its injurious activity.

Considering also the fact that the smooth muscle relaxant activity contributes to the reduction of airway inflammation, where limited flow of air causes the excessive contraction of smooth muscle cells, its plausible to consider that this activity could indirectly aids improvement in inflammatory condition of respiratory diseases. EORO inhibited tracheal smooth muscle contractions of rabbits and guinea pigs induced by acetylcholine, histamine and high concentrations of K^+ (Aqel, 1992), evidencing smooth muscle relaxant activity. The study suggests that EORO may perform an antagonistic

action to calcium (Figure 7). This activity was demonstrated by Coelho-de-Souza et al. (2005) which attributed to 1,8-cineol a potent inhibitory activity of K^+-induced tracheal smooth muscle contractions in guinea-pigs at 600 µg/mL concentration.

Overall, currently available data about the anti-inflammatory mechanism of action by *Rosmarinus officinalis* L. essential oil is mainly based on the inhibition of arachidonic acid metabolites formation, inhibition of NF-κB transcription, antioxidant acitivity and smooth muscle relaxant effects (in airway related inflammation). The combination of these activities makes EORO a potential anti-inflammatory agent.

4.2.1.1 Anti-inflammatory action of 1,8-cineol

The 1,8-cineol (also known by eucalyptol) has been identified in oils of various plants genera such as *Eucalyptus*, *Rosmarinus*, *Psidium*, *Croton* and *Salvia* (Juergens, 2014). Studies with isolated 1,8-cineol describe its action by inhibiting the synthesis of pro-inflammatory substances.

In *in vitro* assays, eucalyptol showed significant inhibition of leukotriene and prostaglandin production in monocytes from patients with bronchial asthma (Juergens et al., 1998). Eucalyptol, was able to decrease production of TNF-α, IL-1β, IL-4, IL-5 in lymphocytes and TNF-α IL-1β, IL-6, IL8 in monocytes (Juergens et al., 2004). In amyloid beta-induced inflammation of PC-12 cells, cineol-treated group showed decreased concentration of inflammatory cytokines TNF-α, IL-1β and IL-6. In addition, decreased expression levels of INOS, COX-2 and NF-κB (Khan et al., 2014).

According to Greiner et al. (2013), 1,8-cineol inhibition of NF-κB may occurs by impeding its translocation into nucleus, thus avoiding the transcription of its products. In LPS-stimulated alveolar macrophages, 1,8-cineol reduced IL-1 and IL-6 levels. In addition, a study reported that it inhibited activity of TREM-1 membrane receptors and the intracellular NLRP-3 of inflammasome, predictably reducing the activity of NF-κB, MAPKs and MKP-1, suggesting an alternative mechanism of action (Yadav and Chandra, 2017).

Orally administered cineol significantly reduced carrageenan-induced inflammatory edema in the rat paw test, reduced also the formation of granuloma induced by the cotton pellet. Additionally, decreased vascular permeability (Santos and Rao, 2000). In rats LPS-induced pleurisy, 1,8-cineol diminished formation of pulmonary edema and leukocyte infiltration inside the bronchoalveolar fluid; inhibited also activity of myeloperoxidase. In a study performed by Zhao et al. (2014) cineol inhibited production of TLR4 and p65 protein domains, a subunit of the NF-κB complex. Furthermore 1,8-cineol reduced concentration of TNF-α and IL-1β (potent pro-inflammatory cytokines) in bronchoalveolar fluid, and

reduced myeloperoxidase activity in LPS-stimulated guinea pigs airways, as reported by Bastos et al. (2011).

Eucalyptol was evaluated in a double-blind placebo-controlled clinical study. Groups of patients with bronchial asthma were treated with this molecule. Of a total of 16 patients treated with 1,8-cineol, 12 presented significant improvement of this inflammatory induced condition (Juergens et al., 2003). Currently, 1,8-cineol is the active principle of the Soledum®, used in the treatment of airways obstructive inflammatory diseases (Greiner et al., 2013).

4.2.1.2 Anti-inflammatory action of α-pinene

The α-pinene is a bioactive monoterpene abundantly found in essential oils. To this compound, anti-inflammatory activity was attributed in several essential oils.

In the rat paw edema test, essential oil of *Bupleurum fruticescens* showed inhibition of the edema formation. This activity was attributed to its major components, including α-pinene. Curiously, it was noticed that the anti-inflammatory effect depends on the integrity of the adrenal glands, since it was ineffective in adrenalectomized animals (Martin et al., 1993). *Pistacia vera* L. oil containing α-pinene as the major compound, also showed anti-inflammatory activity, reducing carrageenan-induced rat paw edema (Orhan et al. 2006).

In another study, *Senecio flammeus* essential oil reduced carrageenan-induced rat paw edema; reduced TPA-induced rats ear edema; decreased granuloma formation by cotton pellet and significantly reduced myeloperoxidase activity. The α-pinene was identified among the major compounds of this essential oil (Xiao et al., 2014). The α-pinene was also identified as an abundant compound of *Pinus pinaster* essential oil, which showed anti-inflammatory effect on carrageenan-induced rat paw edema test at 100 mg/kg dose (Tümen et al. 2018).

The α-pinene is a major component in essential oils from *Helichrysum dasyanthum*, *Helichrysum excisum* and *Helichrysum petiolare*. These oils demonstrated capacity to inhibit 5-lipoxygenase activity, an indicator of its possible anti-inflammatory mechanism (Lourens et al. 2004). The 5-lipoxygenase enzyme catalyzes formation of LTA_4 in arachidonic acid pathway. Inhibition of 5-lipoxygenase also occurred in a different assay with essential oil of *Boswellia dalzielii* (100 mg/L concentration) with α-pinene as major compound (Kohoude et al. 2017) (Figure 4).

Using ovalbumin-sensitized allergic rhinitis model, rats were posteriorly treated with intranasal α-pinene. The treatment decreased levels of IL-4, IgE, TNF-α, ICAM-1 and MIP-2. Administration of α-pinene also inhibited eosinophils infiltration, decreased mast cells

presence in nasal mucosa. *In vitro*, inhibited NF-κB activity in human mast cells (Nam et al., 2014).

In rats with cerulein-induced acute pancreatitis, α-pinene treatment reduced pancreatic mean weight and body weight, evidencing decreased organ edema. There was reduction of biochemical markers of pancreatic damage as well. Histologically, the treatment decreased observed organ damage severity. The mRNA analysis revealed decreased synthesis of proinflammatory cytokines TNF-α, IL-1β, and IL-6, both in *in vivo* and *in vitro* cultured cells. Animals treated with α-pinene showed also decreased activity of myeloperoxidase (Bae et al., 2012).

In human chondrocytes stimulated *in vitro* by IL-1β, α-pinene exerted both anti-inflammatory and anti-catabolic activity, inhibiting NF-κB signaling pathway and expression of iNOS (Rufino et al., 2014). In this same study, LPS-stimulated mice macrophages, treatment with α-pinene reduced synthesis of IL-6 and TNF-α in a dose-dependent manner. Also decreasing expression of iNOS and predictably the synthesis of nitric oxide (NO). In addition, in an experiment performed by Kim et al. (2015), α-pinene inhibited COX-2 gene expression; attenuated activation of MAPKs and suppressed the NF-κB pathway, evidencing its possible mechanisms of anti-inflammatory activity.

4.2.1.3 Anti-inflammatory action of camphor

Studies associate camphor with the anti-inflammatory activity of essential oils as well. The essential oil of the *Curcuma kwangsiensis* root significantly decreased expression of proinflammatory cytokine TNF-α, decreasing also the expression of COX-2 in TPA-induced mouse ear edema assay. In this study, camphor was a major component of the oil used (Zhang et al., 2017).

Essential oil of *Tetraclinis articulata* also shows high camphor concentration. The oil was reported to inhibit carrageenan-induced edema in rats (200 mg/kg dose). The results suggest that the anti-inflammatory effect of the essential oil may be due inhibition of cyclooxygenase, and therefore, the formation of its products, considering that arachidonic acid metabolites produced by COX plays a fundamental role in the inflammatory response (El Jemli et al., 2017).

Oral administration of *Ocimum kilimandscharicum* leaves essential oil (30 and 100 mg/kg doses) in mice promoted significant inhibition of leukocyte migration and plasma extravasation. Possibly due the presence of camphor, a major component of the oil used (de Lima et al., 2014).

Camphor is also a major compound of *Artemisia judaica* L. essential oil. Which

showed potential to reduce production of NO in LPS-induced macrophages (Abu-Darwish et al., 2016). In LPS-stimulated RAF264.7 macrophages, the camphor-rich *Artemisia argyi* essential oil suppressed production of inflammatory mediators such as NO, PGE-2, ROS, IL-6, TNF-α, IFN-β and MCP-1, by inhibiting the phosphorylation of JAK2 and STAT1/3. Oral administration of the oil decreased TPA-induced rat ear edema and also COX-2 levels (Chen et al., 2017).

Moreover in an experiment performed by Farghadan et al. (2016), *Artemisia Judaica* essential oil inhibited iNOS expression in RAW264.7 macrophages, without showing cytotoxicity at higher doses. As being one of the major compounds identified in the previously mentioned studies, camphor may possibly contribute to the anti-inflammatory activity.

5 Conclusion

In the present article, we reviewed studies of *Rosmarinus officinalis* essential oil L., it's major chemical components and main biological activities reported with emphasis on anti-inflammatory potential and possible mechanisms of action.

It is interesting to notice that EORO leaves have been used in folk medicine in several countries, this may have influenced the execution of phytochemical studies to elucidate its pharmacological properties. Researches provided so far, identification of 150 chemical compounds found in EORO samples from different geographical regions. However, some of these are more frequently reported, and at higher concentration, such as 1,8-cineole, camphor, and α-pinene. Pharmacological investigation reported antidepressive, anti-allergic, antimicrobial, antimutagenic, antioxidant, and mainly, anti-inflammatory activities to these molecules.

Anti-inflammatory activity of EORO incited interest of intense research, being evaluated both *in vitro* and *in vivo* assays, which in general associates its mechanism of action by suppression of arachidonic acid metabolites formation and inhibition of NF-κB transcription. Some studies associate reduction of airway inflammation by concomitant action of smooth muscle relaxant effects by the major found compounds.

EORO will most likely continue drawing attention of researchers for future pharmacological analyses, since additional research is needed to elucidate its different biological activities and possible mechanisms of action. Consolidating it as a potential anti-inflammatory agent, and valuable source of bioactive molecules with pharmacology importance.

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Table 1. Compounds identified in the essential oil of *Rosmarinus officinalis* L. (OERO)

Number	Compounds	References
1	1,8 cineole	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Takaki et al. 2008) (Juhás et al. 2009) (Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Faria et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Akbari et al. 2015) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
2	α-pinene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Graber et al. 2010) (Napoli et al. 2010) (Faria et al. 2011) (Melo et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Afoulous et al. 2013) (Akbari et al. 2015) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Takayama et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
3	Camphene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Graber et al. 2010) (Napoli et al. 2010) (Faria et al. 2011) (Melo et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Akbari et al. 2015) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
4	β-pinene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Graber et al. 2010) (Napoli et al. 2010) (Faria et al. 2011) (Afoulous et al. 2013) (Machado et al. 2013) (Yosr et al. 2013) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
5	Camphor	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Napoli et al. 2010) (Faria et al. 2011) (Melo et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Akbari et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Borges et al. 2017) (Selmi et al. 2017)
6	Borneol	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Napoli et al. 2010) (Machado et al. 2013) (Yosr et al. 2013) (Akbari et al. 2015) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017) (Vilela et al. 2016)
7	Bornyl acetate	(Tucker and Maciarello, 1986) (Fahim et al.

		1999) (Napoli et al. 2010) (Yosr et al. 2013) (Akbari et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
8	β-caryophyllene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Afoulous et al. 2013) (Yosr et al. 2013) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
9	*p*-cymene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Juhás et al. 2009) (Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Kfoury et al. 2015) (Mouahid et al. 2017) (Lin et al. 2016) (Takayama et al. 2016) (Vilela et al. 2016)
10	β-myrcene	(Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Machado et al. 2013) (Yosr et al. 2013) (Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017)
11	Limonene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Yosr et al. 2013) (Kfoury et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Borges et al. 2017) (Mouahid et al. 2017)
12	α-terpinene	(Tucker and Maciarello, 1986) (Graber et al. 201 0) (Napoli et al. 2010) (Yosr et al. 2013) (Lin et al. 2016) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017)
13	γ-terpinene	(Tucker and Maciarello, 1986) (Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013) (Kfoury et al. 2015) (Mekonnen et al. 2016) (Takayama et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017)
14	Verbenone	(Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Melo et al. 2011) (Akbari et al. 2015) (Lin et al. 2016) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017)
15	α-terpineol	(Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Machado et al. 2013) (Lin et al. 2016) (Mekonnen et al. 2016) (Vilela et al. 2016) (Tawfeeq et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017)

16	Linalool	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013) (Kfoury et al. 2015) (Mekonnen et al. 2016) (Tawfeeq et al. 2016) (Borges et al. 2017) (Mouahid et al. 2017)
17	Terpinen-4-ol	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013) (Lin et al. 2016) (Tawfeeq et al. 2016) (Vilela et al. 2016) (Bajalan et al. 2017)
18	Terpinolene	(Tucker and Maciarello, 1986) (Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013) (Lin et al. 2016) (Mekonnen et al. 2016) (Mouahid et al. 2017) (Borges et al. 2017) (Takayama et al. 2016)
19	α-thujene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Lin et al. 2016) (Tawfeeq et al. 2016) (Borges et al. 2017) (Mouahid et al. 2017)
20	α-phellandrene	(Tucker and Maciarello, 1986) (Napoli et al. 2010) (Yosr et al. 2013) (Lin et al. 2016) (Tawfeeq et al. 2016) (Bajalan et al. 2017) (Borges et al. 2017) (Mouahid et al. 2017)
21	Myrcene	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Takaki et al. 2008) (Napoli et al. 2010) (Kfoury et al. 2015) (Mouahid et al. 2017)
22	Myrtenol	(Graber et al. 2010) (Napoli et al. 2010) (Vilela et al. 2016) (Bajalan et al. 2017) (Mouahid et al. 2017)
23	Methyl eugenol	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Graber et al. 2010) (Bajalan et al. 2017) (Mouahid et al. 2017)
24	Chrysanthenone	(Graber et al. 2010) (Lin et al. 2016) (Bajalan et al. 2017) (Mouahid et al. 2017) (Selmi et al. 2017)
25	α-humulene	(Tucker and Maciarello, 1986) (Graber et al. 2010) (Napoli et al. 2010) (Mekonnen et al. 2016) (Bajalan et al. 2017)
26	α-terpineol	(Tucker and Maciarello, 1986) (Napoli et al. 2010) (Afoulous et al. 2013) (Takayama et al. 2016) (Mouahid et al. 2017)
27	Caryophyllene oxide	(Fahim et al. 1999) (Napoli et al. 2010) (Yosr et al. 2013) (Vilela et al. 2016) (Bajalan et al. 2017)
28	Geraniol	(Tucker and Maciarello, 1986) (Napoli et al. 2010) (Lin et al. 2016) (Mouahid et al. 2017)
29	α-bisabolol	(Graber et al. 2010) (Yosr et al. 2013) (Tawfeeq et al. 2016) (Bajalan et al. 2017)
30	α-copaene	(Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010) (Selmi et al. 2017)
31	Germacrene-D	(Graber et al. 2010) (Napoli et al. 2010) (Yosr

		et al. 2013) (Selmi et al. 2017)
32	δ-cadinene	(Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013) (Selmi et al. 2017)
33	Pinocarvone	(Graber et al. 2010) (Napoli et al. 2010) (Bajalan et al. 2017) (Mouahid et al. 2017)
34	Sabinene	(Tucker and Maciarello, 1986) (Graber et al. 2010) (Napoli et al. 2010) (Tawfeeq et al. 2016)
35	cis-β-ocimene	(Tucker and Maciarello, 1986) (Graber et al. 2010) (Napoli et al. 2010)
36	α-campholenal	(Napoli et al. 2010) (Borges et al. 2017) (Mouahid et al. 2017)
37	α-muurolene	(Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013)
38	Verbenene	(Graber et al. 2010) (Napoli et al. 2010) (Mouahid et al. 2017)
39	Carvacrol	(Fahim et al. 1999) (Graber et al. 2010) (Napoli et al. 2010)
40	β-linalool	(Machado et al. 2013) (Vilela et al. 2016) (Borges et al. 2017)
41	γ-muurolene	(Napoli et al. 2010) (Afoulous et al. 2013) (Yosr et al. 2013)
42	Nerol	(Tucker and Maciarello, 1986) (Vilela et al. 2016) (Mouahid et al. 2017)
43	α-cadinol	(Graber et al. 2010) (Napoli et al. 2010) (Yosr et al. 2013)
44	Isoborneol	(Tucker and Maciarello, 1986) (Fahim et al. 1999) (Bajalan et al. 2017)
45	3-octanone	(Graber et al. 2010) (Napoli et al. 2010) (Bajalan et al. 2017)
46	4-terpineol	(Fahim et al. 1999) (Machado et al. 2013) (Mouahid et al. 2017)
47	Isopulegol	(Graber et al. 2010) (Napoli et al. 2010)
48	trans-β-ocimene	(Tucker and Maciarello, 1986) (Graber et al. 2010)
49	Thymol	(Graber et al. 2010) (Napoli et al. 2010)
50	β- bisabolene	(Graber et al. 2010) (Napoli et al. 2010)
51	α-amorphene	(Graber et al. 2010) (Yosr et al. 2013)
52	Genanyl acetate	(Takaki et al. 2008) (Faria et al. 2011)

53	Calamenene	(Graber et al. 2010) (Yosr et al. 2013)
54	α-calacorene	(Graber et al. 2010) (Yosr et al. 2013)
55	Cadalene	(Graber et al. 2010) (Yosr et al. 2013)
56	o-cymene	(Borges et al. 2017) (Tawfeeq et al. 2016)
57	Eugenol	(Tucker and Maciarello, 1986) (Napoli et al. 2010)
58	γ-2-carene	(Tucker and Maciarello, 1986) (Tawfeeq et al. 2016)
59	Ferruginol	(Yosr et al. 2013) (Mouahid et al. 2017)
60	α-cubebene	(Napoli et al. 2010) (Yosr et al. 2013)
61	Thuja-2,4(10)-diene	(Napoli et al. 2010) (Mouahid et al. 2017)
62	Pinocarveol	(Napoli et al. 2010) (Mouahid et al. 2017)
63	E-caryophyllene	(Akbari et al. 2015) (Tawfeeq et al. 2016)
64	α-humulene	(Yosr et al. 2013) (Selmi et al. 2017)
65	Spathulenol	(Fahim et al. 1999) (Yosr et al. 2013)
66	Linalyl acetate	(Tucker and Maciarello, 1986) (Fahim et al. 1999)
67	Piperitone	(Graber et al. 2010) (Bajalan et al. 2017)
68	α-terpinolene	(Vilela et al. 2016) (Bajalan et al. 2017)
69	β-phellandrene	(Borges et al. 2017)
70	cis-verbenol	(Mouahid et al. 2017)
71	Camphre	(Mouahid et al. 2017)
72	Nopol	(Mouahid et al. 2017)
74	1-octan-3-ol	(Mouahid et al. 2017)
75	octan-3-ol	(Mouahid et al. 2017)
76	terpin-1-en-4-ol	(Mouahid et al. 2017)
77	α-campholenol	(Mouahid et al. 2017)
78	Widdrol	(Bajalan et al. 2017)
73	trans-sabinene hydrate	(Bajalan et al. 2017)
79	α-thujone	(Selmi et al. 2017)
80	β-thujone	(Selmi et al. 2017)
81	3-carene	(Vilela et al. 2016)
82	β-thujene	(Vilela et al. 2016)
83	2,4(10)-thujadiene	(Vilela et al. 2016)

84	Italicene	(Tawfeeq et al. 2016)
85	E-isocitral	(Tawfeeq et al. 2016)
86	Citronellal	(Kfoury et al. 2015)
87	(E)- β-farnesene	(Afoulous et al. 2013)
88	Epicedrol	(Afoulous et al. 2013)
89	Carарene	(Yosr et al. 2013)
90	Caryophylla-4.8-diene-5-ol	(Yosr et al. 2013)
91	T-cadinol	(Yosr et al. 2013)
92	β-selinenol	(Yosr et al. 2013)
93	2-ethyl-4,5-dimethylphenol	(Faria et al. 2011)
96	α-fenchene	(Graber et al. 2010)
139	p-cymenene	(Graber et al. 2010)
140	Citronellol	(Graber et al. 2010)
98	γ-terpineneol	(Graber et al. 2010)
99	(+)-pulegone	(Graber et al. 2010)
100	Isonocamphone	(Graber et al. 2010)
101	Trans-sabinen hydrate	(Graber et al. 2010)
102	β-sesquiphellandrene	(Graber et al. 2010)
103	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)	(Graber et al. 2010)
104	α-cadinene	(Graber et al. 2010)
105	α-cubenene	(Graber et al. 2010)
106	Epizonarene	(Graber et al. 2010)
107	1-octene 3-ol	(Graber et al. 2010)
108	4-(4´-methyl-3´-pentenyl)-3-ciclohexenil propyl ketone	(Graber et al. 2010)
94	trans-pinene hydrate	(Napoli et al. 2010)
95	*p*-cymenen-8-ol	(Napoli et al. 2010)
97	β-citronellol	(Napoli et al. 2010)
109	Tricyclene	(Napoli et al. 2010)
110	Trans-3-pinanone	(Napoli et al. 2010)
111	Cis-3-pinanone	(Napoli et al. 2010)
112	Geranyl acetone	(Napoli et al. 2010)
113	Eugenol methyl ether	(Napoli et al. 2010)
114	Estragole	(Napoli et al. 2010)
115	Benzene acethaldheyde	(Napoli et al. 2010)
116	14-hydroxy-9-epi-E-caryophyllene	(Napoli et al. 2010)
117	allo-aromandrene	(Napoli et al. 2010)
118	epi-α-muurolol	(Napoli et al. 2010)
119	τ-muurolol	(Napoli et al. 2010)
120	cis-cadinen-4-en-7-ol	(Napoli et al. 2010)
121	Bisabolol-4-ol	(Napoli et al. 2010)
122	Valencene	(Napoli et al. 2010)
123	γ-amorphene	(Napoli et al. 2010)
124	β-copaene	(Napoli et al. 2010)
125	Ylangene	(Napoli et al. 2010)
126	Piperitenone	(Napoli et al. 2010)
127	cis-pulegol	(Napoli et al. 2010)
128	Thymol methyl ether	(Napoli et al. 2010)
129	cis-carveol	(Napoli et al. 2010)

130	trans-carveol	(Napoli et al. 2010)
131	Myrtenal	(Napoli et al. 2010)
132	Cymen-8-ol	(Napoli et al. 2010)
133	Camphene hydrate	(Napoli et al. 2010)
134	Chrysantenone	(Napoli et al. 2010)
135	exo-fenchol	(Napoli et al. 2010)
136	endo-fenchol	(Napoli et al. 2010)
137	cis-sabinene hydrate	(Napoli et al. 2010)
138	*p*-mentha-7,8-diene	(Napoli et al. 2010)
141	1-O-menthen-8-ol	(Takaki et al. 2008)
142	phenylacetic acid	(Takaki et al. 2008)
143	2-ethyl-4,5-dimethylphenol	(Takaki et al. 2008)
144	Methyl chavicolf	(Fahim et al. 1999)
145	Terpinyl acetate	(Fahim et al. 1999)
146	Carveol	(Fahim et al. 1999)
147	Thymole	(Fahim et al. 1999)
148	Carvone	(Fahim et al. 1999)
149	β-thujone + 1-octen-3-ol	(Tucker and Maciarello, 1986)
150	Isobornyl acetate	(Tucker and Maciarello, 1986)

Table 2. Major *Rosmarinus officinalis* L. essential oil compounds with related biological activities

Compound	***Biological activity***	***References***
1,8 cineole	Anti-depressive Antialgic Antioxidant Anti-inflammatory	(Juhás et al. 2009) (Faria et al. 2011) (Machado et al. 2013) (Vilela et al. 2016) (Takayama et al. 2016) (Bajalan et al. 2017) (Selmi et al. 2017)
α- pinene	Antifungal Antioxidant Antibacterial	(Lin et al. 2016) (Mekonnen et al. 2016) (Takayama et. al, 2016)
Camphor	Anti-inflammatory Antialgic Anti-mutagenic Antioxidant	(Fahin et. al, 1999) (Faria et. al, 2011) (Melo et al. 2011) (Takayama et. al, 2016) (Bajalan et. al, 2017) (Borges et. al, 2017)

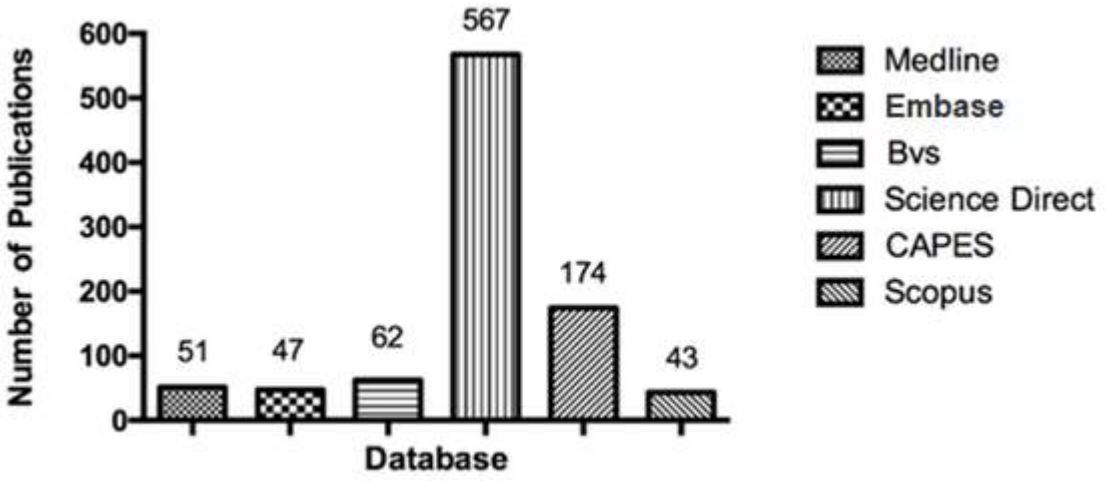

Figure 1. Publications containing selected terms in scientific databases.

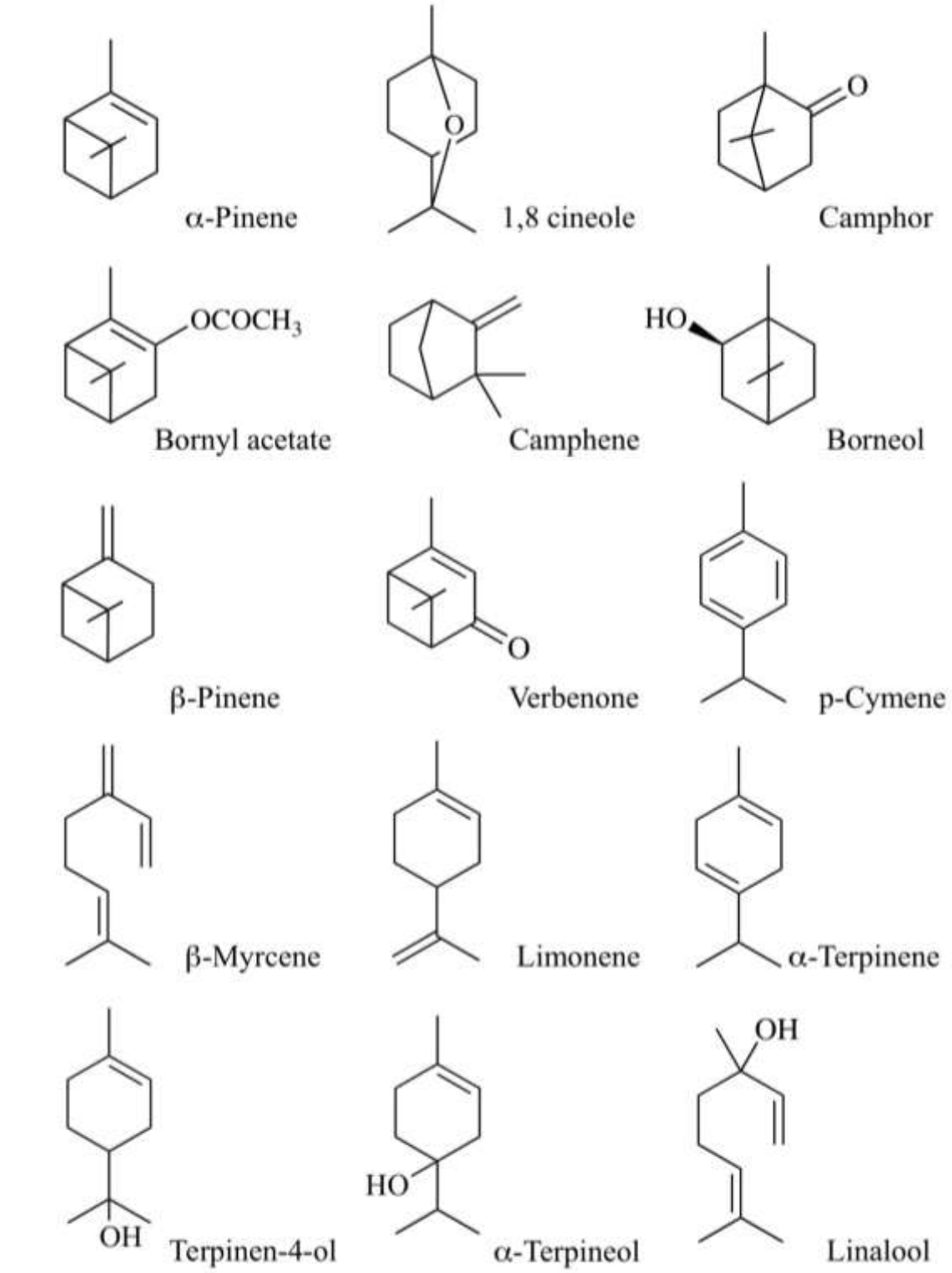

Figure 2. Compounds present in the essential oil of *Rosmarinus officinalis* L. (EORO).

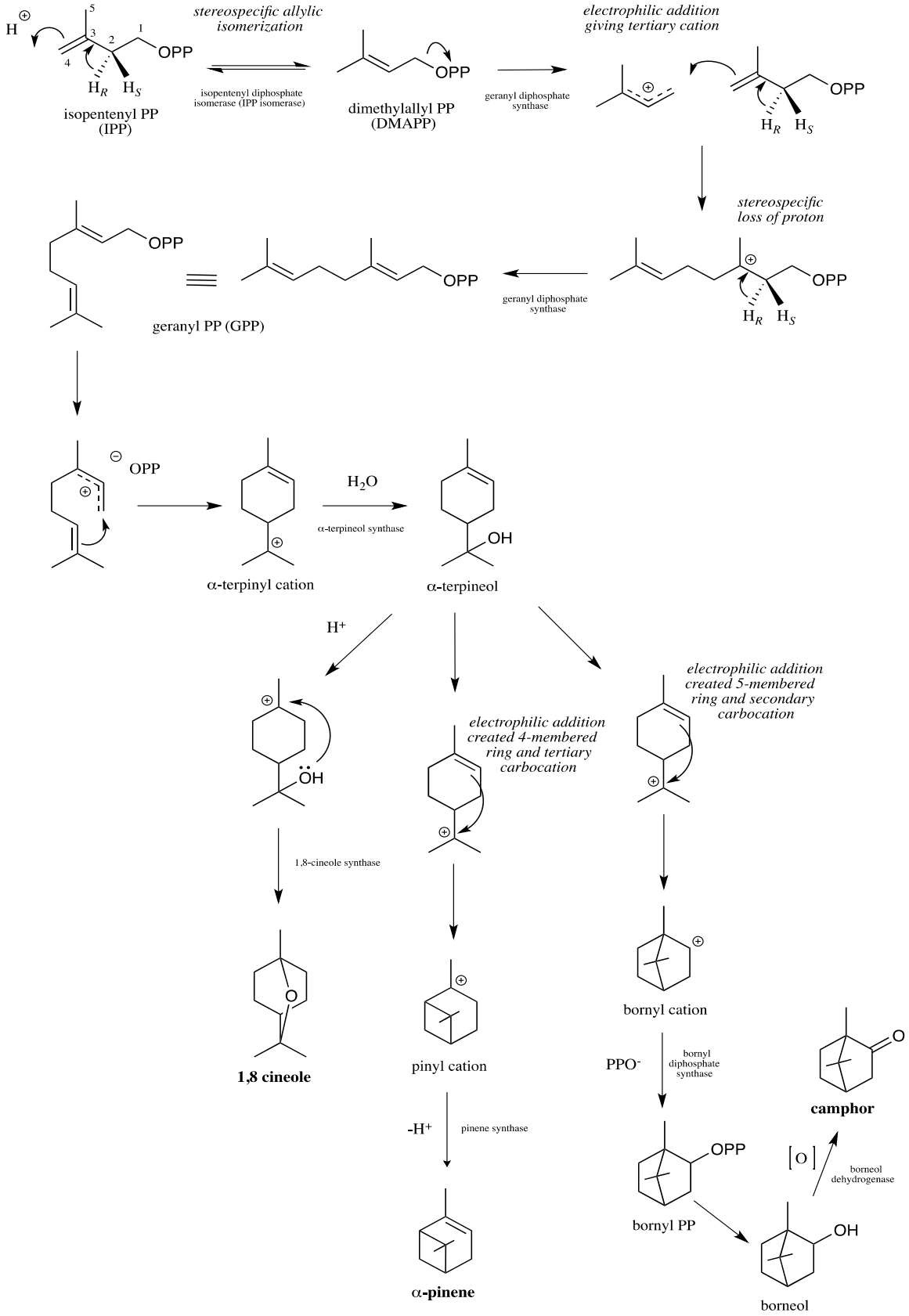

Figure 3. Biosynthesis of monoterpenes 1,8-cineol, α-pinene and camphor (mevalonate route), major compounds of EORO.

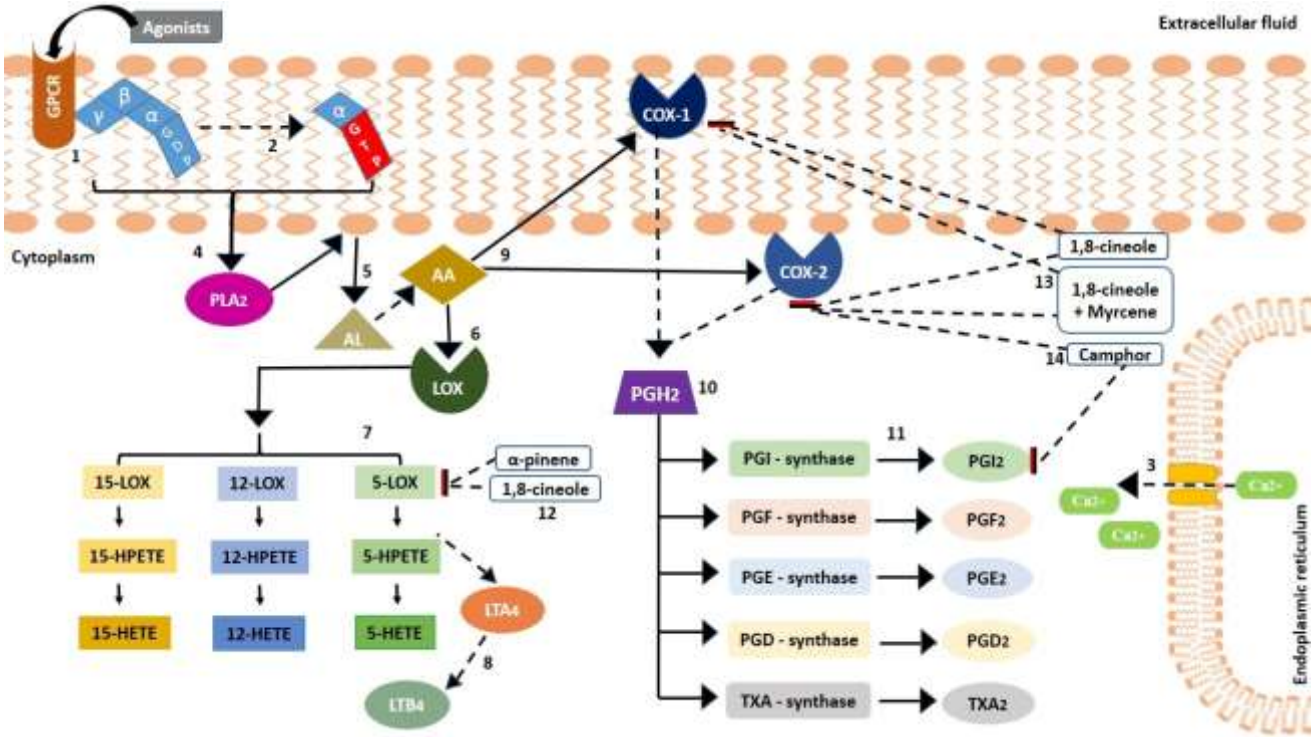

Figure 4. Arachidonic acid cascade pathway and mechanism of action of camphor, α-pinene, and 1,8-cineol isolated and combined with mircene. 1. G protein-coupled receptors (GPCR) are stimulated by agonists. 2. Guanosine diphosphate (GDP) is converted in guanosine triphosphate (GTP). 3. The concentration of calcium ions (Ca^{2+}) in the cytoplasm increases. 4. These processes activate phospholipase A_2 (PLA_2). 5. PLA_2 acts on membrane phospholipids releasing linoleic acid (AL), which is transformed into arachidonic acid (AA). 6. AA is oxidized by Lipoxygenase (LOX). 7. 5-lipoxygenase (5-LOX) converts hydroxyhexanoatetetraenoic acid (5-HPETE) to leukotriene A_4 (LTA_4); 8. LTA_4 is converted to leukotriene B_4 (LTB_4), acting on macrophages stimulating the production of proinflammatory cytokines; 9. AA is oxidized by cyclooxygenase (COX-1 and COX-2); 10. COXs form prostraglandin H_2 (PGH_2); 11. From PGH_2 the performance of enzymes results in prostraglandins (PGE_2, PGF_2, PGI_2, PGD_2) and thromboxane (TXA_2); 12. 1,8-cineol and α-pinene block the action of 5-LOX by preventing the formation of proinflammatory cytokines; 13. 1,8-cineol alone or combined with mircene acts on the blockade of COX-1 and COX-2; 14. Camphor has a high number of possible interactions with PGI_2 and COX-2.

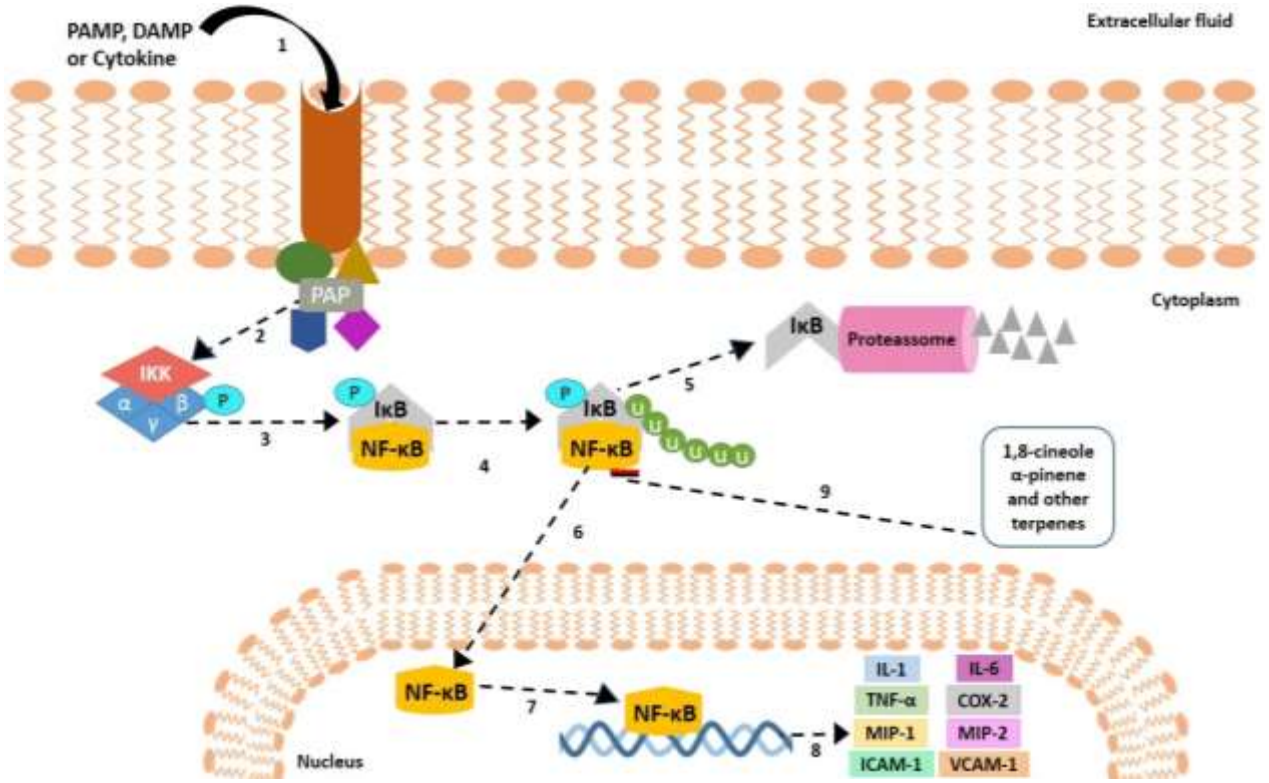

Figure 5: Mechanism of action of 1,8-cineol, α-pinene and other terpenes in NF-κB signaling pathway. 1: The pathogen-associated molecular pattern (PAMP), damage-associated molecular pattern (DAMP) or cytokine receptor is activated by its correspondent agonist molecule; 2: Receptor activation stimulus is transducted by coupled proximal adaptor protein (PAP), which in turn activates the IκB-kinase (IKK) by phosphorylation; 3: Activated IKK phosphorylates the inhibitor of κB (IκB), bounded to *nuclear factor-κB* (NF-κB); 4: Phosphorylation of IκB signalize its consequently polyubiquitination (u); 5: IκB polyubiquitination causes its degradation by proteasome; 6: Without its inhibitor, NF-κB is able to translocate into the nucleus; 7: In the nucleus, NF-κB binds to DNA, acting as transcription factor of several pro-inflammatory mediators; 8: NF-κB transcription products includes, among others, IL-1, IL-6, TNF-α, COX-2, MIP-1, MIP-2, ICAM-1 and VCAM-1; 9: The 1,8-cineole, α-pinene and other terpenes, act by preventing NF-κB translocation into nucleus, and the consequently formation of pro-inflammatory mediators.

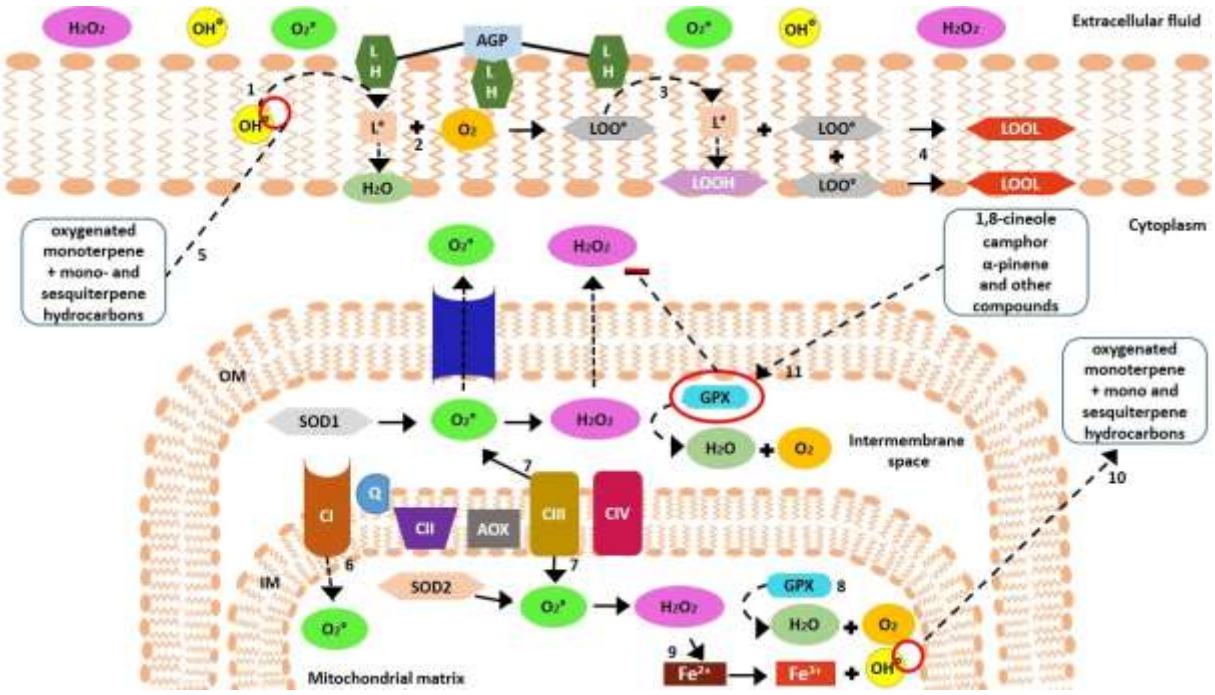

Figure 6. Mechanism of antioxidant action of EORO components in lipid peroxidation and ROS formation. 1. Hydroxyl ($OH^{\bullet}$) scavenge a hydrogen atom from the polyunsaturated fatty acids (AGP-LH) of the cell membrane, forming lipid radical ($L^{\bullet}$) and water (H_2O); 2. $L^{\bullet}$ reacts with oxygen (O_2) resulting in peroxyl radical ($LOO^{\bullet}$); 3. The $LOO^{\bullet}$ sequesters another hydrogen from the AGP-LH forming a new radical $L^{\bullet}$ and hydroperoxide (LOOH); 4. The L and the $LOO^{\bullet}$ form stable radical (LOOL), which is also formed by two $LOO^{\bullet}$; 5. EORO compounds (oxygenated monoterpenes and mono- and sesquiterpene hydrocarbons) have the ability to neutralize OH, avoiding membrane deterioration; 6. In the inner membrane (IM) of the mitochondria the I (CI) complex releases electrons generating superoxide radicals ($O_2^{\bullet}$) to the mitochondrial matrix; 7. Complex III (CIII) releases electrons generating $O_2^{\bullet}$ in the matrix and mitochondrial intermembrane space, the $O_2^{\bullet}$ undergo superoxide dismutase 1 (SOD_1) in the intermembrane space and superoxide dismutase 2 (SOD_2) in the matrix forming hydrogen peroxide (H_2O_2); 8. The enzyme glutathione peroxidase (GPX) reduces H_2O_2 to H_2O and O_2; 9. The Fe^{2+} ion reacts with H_2O_2 to form Fe^{3+} and OH (Fenton reaction); 10. EORO compounds (oxygenated monoterpenes and mono- and sesquiterpene hydrocarbons) react with OH, avoiding lipid peroxidation; 11. EORO compounds (1,8-cineole, camphor, α-pinene and other components) increase the amount of glutathione, resulting in the decrease of H_2O_2 levels by the action of GPX.

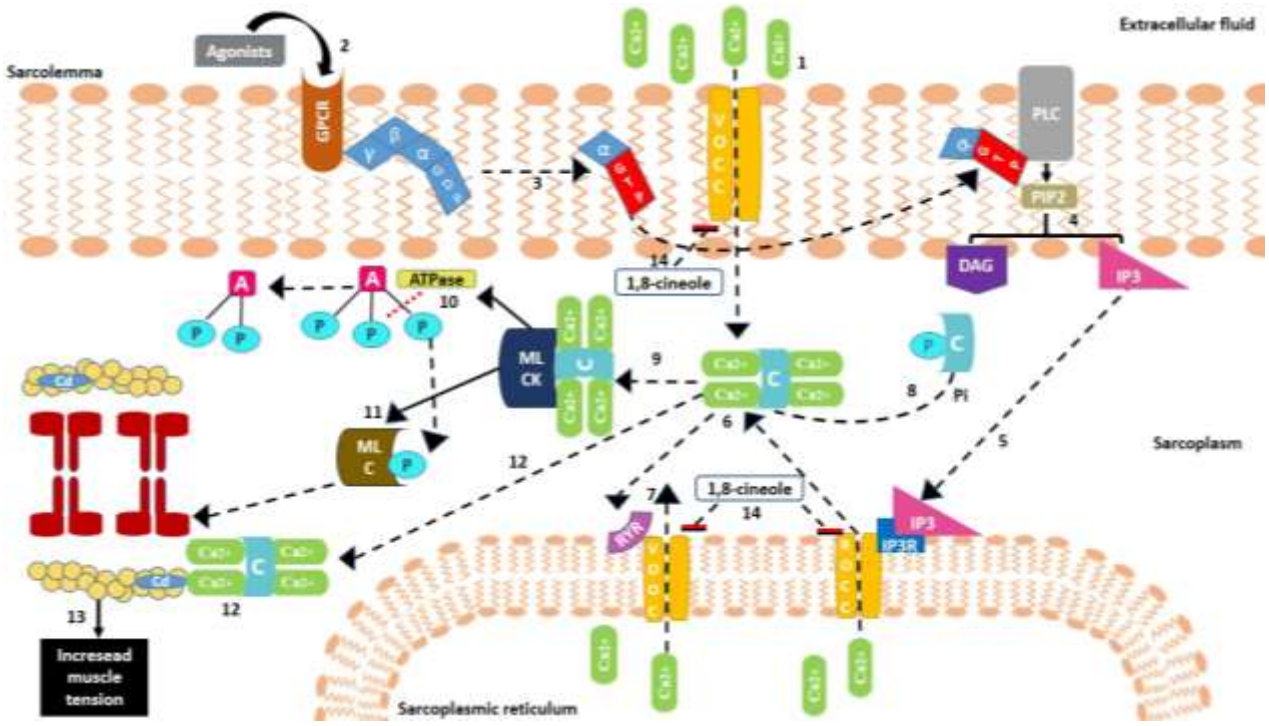

Figure 7. Mechanism of smooth muscle contraction and action of 1,8-cineol in calcium channel blockade. 1. The variation of the ion concentration between the intra and extracellular media causes membrane depolarization, which promotes the opening of voltage-operated calcium channels (VOCC) and calcium ions (Ca^{2+}) into the sarcoplasm; G protein coupled receptors (GPCR) are stimulated by agonists, activating guanosine diphosphate (GDP). 3. GDP is activated in guanosine triphosphate (GTP), which results in the release of the α-GTP moiety to activate Phospholipase C (PLC); 4. PLC cleaves Phosphatidylinositol biphosphate (PIP_2) to Diaglycerol (DAG) and Inositol triphosphate (IP_3); 5. IP_3 binds to the IP_3 receptor (IP_3R) of the sarcoplasmic reticulum membrane inducing the opening of the receptor-operated calcium channel (ROCC), which promotes the release of Ca^{2+} to the sarcoplasm; 6. Increased Ca^{2+} concentration in sarcoplasm induces the activation of the VOOC bound rianodine receptor (RYR); 7. VOOC-RYR release more Ca^{2+} into the sarcoplasm; 8. Four free calcium ions bind to Calmodulin forming the Ca^{2+}-calmodulin complex (CaCM); 9. CaCM activates myosin light chain kinase (MLCK); 10. MLCK activates the ATPase enzyme to hydrolyze the adenosine triphosphate (ATP) molecule to adenosine diphosphate (ADP) and inorganic phosphate (P), which will be assigned to the myosin light chain (MLC); 11. MLCK phosphorylates MLC, which promotes the phosphorylation of the globose region of Myosin (M); 12. The CaCM complex binds to Caldesmone (Cd), which liberates the binding site of Actin; 13. Muscle contraction; 14. 1,8-cineol acts as an antagonist to calcium by blocking the calcium channels, reducing the concentration of calcium ion in the sarcoplasm and preventing the process of muscle contraction.

4 CAPÍTULO 2

ANTI-INFLAMMATORY AND ANTIALGIC ACTIONS OF A NANOEMULSION OF *ROSMARINUS OFFICINALIS L.* ESSENTIAL OIL AND A MOLECULAR DOCKING STUDY OF ITS MAJOR CHEMICAL CONSTITUENTS

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Abstract

We evaluate the anti-inflammatory and antialgic potency of a nanoemulsion (NEORO) containing the essential oil of *Rosmarinus officinalis* L. (EORO), which is composed primarily of limonene, camphor and 1,8-cineole. The EORO and NEORO were administered orally 30 minutes prior to starting the experiments. In a test of rat paw edema induced by carrageenan, NEORO was effective in doses of 498 μg/kg, and it inhibited 46% of the maximum peak of the edema; in a dose of 300 mg/kg EORO inhibited 50% of the maximum peak of the edema. In an acetic acid-induced writhing test, NEORO yielded a dose-dependent effect, and a dose of 830 μg/kg inhibited 84% of the algesic process; a dose of 100 mg/kg of EORO inhibited 55%. In an assay for H_2S production in rat stomachs, a dose of 498 μg/kg of NEORO inhibited H_2S production in all of the measurement phases, and a dose of 100 mg/kg EORO inhibited 60% and influenced the effect of the ethanol significantly, reducing the production of H_2S. We suggest that NEORO potentiated the effect of EORO, demonstrating effectiveness in doses 600 times lower than those applied with EORO. Among the major compounds of EORO, the camphor molecule exhibited the largest number of interactions with the therapeutic targets related to the inflammatory process, suggesting that it is responsible for EORO's anti-inflammatory and antialgic effects. This work paves the way for future investigations related to the therapeutic role of NEORO in the inflammation process.

Keywords: *Rosmarinus officinalis*, nanoemulsions, H_2S, anti-inflammatory, antialgic, molecular docking.

Introduction

Nanotechnology is characterised by a multidisciplinary approach and involves the creation and usage of different systems a nanometric proportions (De Villiers et al. 2009). Nanoformulations have a wide variety of applications (e.g., the food industry, cosmetics, medicines and pesticides) (Assis et al. 2012; Patel and Velikov 2011; Duncan 2011; Brumfiel, 2006; Irache et al. 2011; Wang et al. 2007). Among nanoformulations, nanoemulsions are systems formed by two immiscible liquids and one or more stabilizing liquids, which enable the formation of small droplets (McClements 2012).

Nanoemulsions are characterised by their thermodynamic stability, and the possess drop sizes between 20 and 200 nm (Ostertag et al. 2012). Those formulations have a wide variety of industrial applications (Izquierdo et al. 2002; Tadros et al. 2004) (e.g., as an adjuvant in foods, medicines and agricultural products), and they exhibit a high economic potential.

Essentials oils are complex mixtures of volatile substances extracted from plants. They typically contain monoterpenes, sesquiterpenes and other low-molecular weight substances in addition to phenylpropanoids in some cases (plants used in drugs). The essentials oils are used in the food and pharmaceutical industries as flavoring agents and can be larval, antibacterial, antifungal, anticancer, antimutagenic, antidiabetic, antiviral, anti-inflammatory and antiprotozoal (Raut and Karuppayil 2014).

There is an active field research involving the anti-inflammatory drugs in nanoformulations. This field principally focuses on active compounds from natural products, which is the case for Z-ligustilide. This substance is isolated and purified from the essential oil of *Angelica sinensis*, and it attenuates inflammatory pain behavior in mice (Kuang et al. 2006; Yu et al. 2008; Du et al. 2008).

Rosmarinus officinalis L. is a medicinal plant of the *Lamiaceae* family (Lorenzi and Matos 2002) that is commonly used for medicinal purposes. It is ingested as a tea (Marchiori 2004). This species is a copious producer of essential oils, and it has been studied thoroughly. It possesses several biological activities, including spasmolytic (Ventura-Martínez et al. 2012), antioxidant (Raskovic et al. 2014; Ojeda-Sana et al. 2013), antibacterial (Ojeda-Sana et al. 2013), anti-inflammatory (Melo et al. 2011), antidepressive (Machado et al. 2013) and antifungal (Gauch et al. 2014; Cleff et al. 2012) properties. The chemical composition of the essential oil of *Rosmarinus officinalis* L. (EORO) can vary according to several factors, such as climate, soil, sun exposure and extraction procedure.

However, the chemotypes most commonly reported are cineoliferum, composed primarily of 1,8-cineole, and camphoric acid, where camphor prevails (Napoli et al. 2015).

EORO is mainly composed of terpenoids, more specifically monoterpenes. This group of terpenoids are biologically active, and many of of these terpenoids possess anti-inflammatory activity (Souza et al. 2014). One of the components of this essential oil, 1,8-cineol, had already been tested in a double-blind, placebo-controlled trial in patients with acute asthma and allergic inflammation. The results suggested that EORO exhibited anti-inflammatory activity in asthma and that it could be used as an mucolytic agent in upper and lower airway diseases (Juergens et al. 2003).

Here, we sought to obtain a nanoemulsion using EORO and to evaluate its anti-inflammatory potential in *in vivo* assays. We also evaluated the effects of the nanoemulsion on inflammation models and outlying pain in animals.

Methods and Materials

EORO acquisition

We acquired the EORO from the Florien Company (Sao Paulo, Brazil). It had been botanically authenticated as being *Rosmarinus officinalis* L., and it possessed organoleptic, physicochemical and microbiological characteristics. It also contained essential oil from the rosemary plant, which obtained from leaves collected in Brazil, lot 056757-LC02062016DA.

Analysis of EORO by GC-MS

Coupled gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Shimadzu system/GC 2010 coupled to a self-gun Shimadzu/AOC-5000 and mass detector (Shimadzu MS2010 Plus) with electron impact (70 eV) equipped with a fused silica column of DB-5MS (Agilent Advanced J & W; 30 m × 0.25 mm × 0.25 μm). The parameters of the X were as follows: split ratio, 1:30; helium as the carrier gas (65 kPa); injection volume, 1.0 μl; injector temperature, 250°C; detector temperature, 250°C; initial column temperature, 60°C for 1 min; heating rate, 3°C min-1 to 290°C. The total analysis time was 76,67 minutes, and we calculated retention indices (RI) via interpolation to the retention times for a mixture of aliphatic hydrocarbons (C9–C30) analysed under the same

conditions. The MS fragmentation pattern of the compounds was also compared with NIST mass spectrum libraries (National Institute of Standards and Technology).

Nanoemulsion preparation

The nanoemulsions were prepared using a low-energy load methodology that has been described by Fernandes et al. (2013). For a final mass of 50 g, we used 90% water, 5% EORO and 5% tween 20. Initially, an organic phase was prepared by adding EORO and the tensioactive tween into a beaker. The mixture was agitated using a magnetic agitator (750 rpm) for 30 minutes. Next, the aqueous phase was added at a flow rate of 0,5 mL/min with continuous agitation for 60 min. The stability of the emulsions was evaluated 1, 30 and 60 days after the preparation using macroscopic analysis (color, visual aspect, phase separation, creaming and sedimentation) (Falcão 2007). During this period, the emulsions were maintained at room temperature (25 ± 2°C) in screw-capped glass test tubes.

Droplet size analysis

We determined droplet size and polydispersity with photon correlation spectroscopy using a Zetasizer 5000 (Malvern Instruments, Malvern, UK). Each emulsion was diluted using ultrapure Milli-Q water (1:25), and the measurements were performed in triplicate. The average droplet size was expressed as the mean diameter (Orafidiya and Oladimeji 2002).

Evaluation of EORO and NEORO on rat paw edema, writhing test in mice and production of H_2S in rat stomachs

Experimental animals

Male Wistar rats (body weight: 180–200 g) were used, along with Swiss mice (body weight: 20–25 g) from the Central Biotério of the Department of Pharmacy of Facultad de Química da Universidad Autonoma de Mexico. The animals were housed in polyethylene boxes in groups of five, and their access to food was removed 12 hours prior to the experiments. They were given free access to water.

This research was approved by the Ethics Committee for the Use of Animals of Amapá Federal University (Authorisation nr. 0021/2015). After the experiments, the animals were euthanised according to the guidelines for the euthanasia of animals (AVMA, American Veterinary Medical Association 2013).

Rat paw edema induced by carrageenan

This test was carried out according to the method described by Winter et al. (1962). Groups of animals (n = 5) each received different doses of EORO (100 mg/kg and 300 mg/kg) and NEORO (498 µg/kg) 30 minutes prior to the application of the inflammatory agent (carrageenan 1000 mg/paw, 01 mL, kappa, Sigma Company, Sao Paulo, Brazil). We administered 0,1 mL of saline solution in the plantar space of the left hind paw and the same volume containing the inflammatory agent in the right paw. We measured paw volume using a plethysmometer (Model 7540; Ugo Basile, Italy). The paws were measured every hour prior to the administration of the inflammatory substance and four hours after the application of the carrageenan.

Acetic acid-induced writhing test

The acetic acid-induced writhing test was carried out in mice according to the method described by Koster et al. (1959). The different groups of animals were treated orally with EORO (100 mg/kg) and NEORO (166 µg/kg, 498 µg/kg and 830 µg/kg), and the control animals were treated with 0,5 mL of tween solution to 5%. Thirty minutes later, abdominal twitches (writhes) were induced intraperitoneal via (i.p.) administration of acetic acid 1% (0,25 mL). The writhing were observed, and we recorded the mean ± mean standard error of the number of writhes in an interval of 20 minutes.

Test of H_2S production in rat stomachs

This assay was based on the methods described by Khan et al. (1980) and Eto and Kimura (2002). Wistar rats were used (n = 6 per group), which were anesthetised with sodium pentobarbital (10 mg/kg, i.p, AnestesalTM, MSD, Mexico) and treated orally with EORO (100 mg/kg) and NEORO (498 µg/kg). After 30 minutes, L-cysteine was used as positive control (Sigma Company). The rats then underwent a laparotomy to reveal the pyloric region, and the H_2S microelectrode were attached to an analyser system (Micro

Hydrogen Sulfide Measurement System - microLazar Model ISM-146H2S-XS; Lazar, USA). We measured H_2S levels every 5 minutes, and after 30 minutes we injected 200 µL of ethyl alcohol (PA) directly in the animal's stomachs and recorded data for up to 60 minutes.

Molecular docking of the major chemical constituents of EORO

For the docking study, we downloaded files deposited in the Protein Data Bank (PDB) from the Research Collaboratory for Structural Bioinformatics (Li et al. 2008; Sandy and Butler 2009; Orlando and Malkowski 2016) with the coordinates of the crystallographic structures of the COX-1 therapeutic targets (PDB ID: 3N8X, resolution: 2.75 Å) complexed with the nimesulide inhibitor. COX-2 (PDB ID: 5IKQ, resolution: 2.41 Å) was complexed with meclofenamic acid and Prostacyclin syntase (PDB ID: 3B6H, resolution: 2.41 Å) was complexed with the minoxidil inhibitor.

To perform the molecular docking, we added hydrogen atoms and removed water molecules from the enzymes. The inhibitors that were complexed with each therapeutic target were extracted. Prior to performing the docking simulation, we validated our results by calculating the root-mean-square deviation (RMSD) between the experimental binder and the conformation of the binder that yielded the best posture after docking. In order to calculate the docking of the major phytochemical constituents of EORO, we used the following coordinates: Cyclooxygenase-1 (COX-1): x: -21.43; Y: -50.79 and z: 1.42; Cyclooxygenase-2 (COX-2): Î½: 22.83; Y: 51.56 and z: 17.81; and Prostacyclin (PGI-2): Î½: 72.25; Y: 54.20 and z: 42.19.

To identify the interactions between the compounds and the therapeutic targets, it was necessary to identify the amino acids that make up the catalytic site of the enzymes: COX-1 (ARG120, TYR355, SER530 and ILE523), COX-2 (TYR385 and SER530) PGI-2 (CYS441, TRY282, PHE483 and GLY482).

Statistical analysis

We applied analysis of variance (ANOVA) followed by the Tukey test. p values less than 0.05 were considered to be statistically significant. We plotted the data using GraphPad Prism 6.0.

Results and discussion

The chromatographic data indicated that the EORO used in this study to obtain the NEORO contained the following major compounds: 21.99% limonene, 33.70% 1,8-cineole and 27.68% camphor (Figure 1 and Table 1). These results are in accordance with those reported by Zaouali et al. (2010). These authors found that camphor and 1,8-cineol were the primary compounds of EORO from Tunisia. In studies conducted by Boix et al. (2010) and Fernandes et al. (2013), α-pinene, 1,8-cineol and camphor were the primary components of EORO samples from Brazil. Ribeiro et al. (2012) found α-pinene and 1,8 cineol to be among the major compounds of EORO from fresh rosemary leaves cultivated in northeastern Brazil.

The nanoemulsion, NEORO, prepared with EORO, presented a fluid appearance, a whitish coloration and a slightly bluish reflection, which are common macroscopic characteristics in this type of formulation. The mean droplet diameter remained below 200 nm in all emulsions, as described by Solans et al. (2005) and Solè et al. (2012). As shown in Figure 2, the NEORO presented a distribution of monomodal droplet sizes, but none of the samples exhibited signs of instability, such as cremation and phase separation, as described by Duarte et al. (2015).

The carrageenan-induced rat paw edema assay is widely used as a test for evaluating anti-inflammatory activity. This assay has become a standard model for experiments related to acute inflammation, that is described as a biphasic response because it has an initial phase in the first two hours after the injection of carrageenan, which is related to the release of histamine and serotonin and another phase that comprises the production of prostaglandins, bradykinin and proteases (Patgiri et al. 2014). Carrageenan was used in this assay because it is devoid of apparent systemic effects (Ganguly 2013).

In a study by De Faria et al. (2001) focusing on EORO, the authors noted ED50 values of 300 mg/kg and 261 mg/kg in the writhing test in mice and for carrageenan-induced paw edema in rats, respectively. Additionally, LD_{50} in the mice was greater than 2.0 g/kg.

Oral administration of NEORO at a dose of 498 μg/kg inhibited the maximum peak of edema by 46%, and EORO administered at a dose of 300 mg/kg inhibited the maximum peak of edema by 50%. This result demonstrates that EORO delivered as a nanoemulsion was much more effective on carrageenan edema, inhibiting this edema, with a dose six hundred times lower than that of EORO (Figure 3). In the acetic acid-induced writhing test in mice, the oral administration of NEORO yielded a dose-dependent effect, and a dose of

830 µg/kg inhibited the algogenic process by 84%. EORO alone at a dose of 100 mg/kg inhibited the algogenic process by 55% (Figure 4).

The physiological functions of hydrogen sulphide (H_2S) have been recognised and evidence is being sought that this endogenous, gaseous substance can modulate inflammatory processes. However, H_2S donors have been shown to reduce edema formation and the adhesion of leukocytes to vascular endothelium and inhibit the synthesis of proinflammatory cytokines (Wallace, 2007). In addition, H_2S donors can increase gastric mucosal resistance to injury and accelerate repair (Wallace, 2007). These observations and others suggest that anti-inflammatory drugs that are modified with the ability to release H_2S have improved anti-inflammatory efficacy and reduced toxicity (Wallace, 2007).

In our analysis of how EORO and NEORO affected L-cysteine-induced production of H_2S in rat stomachs, we observed that NEORO at a dose of 498 µg/kg inhibited production at all measurement times. Additionally, EORO at a dose of 100 mg/kg inhibited production of H_2S by 60% and potentiated the ethanol effect, thereby decreasing H_2S production (Figure 5).

The evidence suggests that H_2S is a mediator of several aspects of the gastrointestinal function and liver. In addition, changes in H_2S production may contribute to diseases of the gastrointestinal tract and liver, and non-steroidal anti-inflammatory drugs may reduce the production of H_2S in the stomach, and this fact has been shown to contribute to the generation of mucosal injury (Fiorucci et al. 2005; Fiorucci et al. 2006). However, Martínez et al. (2009) demonstrated the antinociceptive effect of EORO in arthritic pain in a rat model, suggesting the involvement of the serotonergic system via 5-HT1A receptors and endogenous opioids.

Furthermore, De Faria et al. (2011) described the anti-inflammatory effect of EORO via the inhibition of cyclooxygenase; this effect persisted without causing gastric lesions or increasing mucus production. This fact may explain the observed results regarding H_2S production: a consistent mucus layer could interfere with the release of this mediator and therefore not be detected by the electrode system, a hypothesis that should be explored further in future studies.

Molecular docking is a computational method currently widely used in the drug discovery process (Chandak et al. 2014). The benefit of docking is to identify the mode of interaction of the study molecules at the site of the enzyme or receptor through specific key interactions and to predict the binding affinity between the protein-binding complexes in this case, the chemical constituents of EORO. The Genetic Optimisation for Ligand Docking (GOLD) program uses a genetic algorithm to conduct flexible docking

experiments of ligands within protein-binding sites. The GOLD program has been used to investigate the modes of interaction between compounds and therapeutic targets (Chandak et al. 2014).

The RMSD value indicates the accuracy of the docking postures calculated by the GOLD docking algorithm compared with the experimentally determined poses of a compound bound to a biological target. An RMSD less than 2 Å is considered to be successful (i.e, to have justified validity) (Cole et al. 2005). In this study, the best RMSD values obtained with nimesulide, meclofenamic acid and minoxidil inhibitors were 0.87, 0.99 and 0.89 Å for the respective therapeutic targets COX-1, COX-2 and PGI_2.

We then performed docking between the therapeutic targets and the compounds limonene, 1,8-cineole and camphor. We selected the docking results that yielded the highest score for each therapeutic target limonene: 50.14 for COX-1, 45.85 for COX-2 and 38.21 for PGI-2; 1,8-cineole compound: 36.30 for COX-1, 37.76 for COX-2 and 33.71 for PGI-2; camphor compound: 33.49 for COX-1, 34.38 for COX-2 and 36.13 for PGI_2.

With the COX-1 therapeutic target, the docking of the limonene compound had two Alkil-type hydrophobic interactions with distances of 3.97 Å and 4.22 Å from the amino acid ILE523 (Figure 6a). Alkyl groups are defined as a predominantly aliphatic amino acid side chains, and they include alanine, valine, leucine, isoleucine, methionine, selenomethionine, cysteine, proline, CB, CG and CD atoms of lysine and CB and CG arginine atoms.

Hydrophobic groups in binders are contiguous sets of atoms that are not adjacent to charge concentrations (charged atoms or electronegative atoms). A group of atoms is considered to be hydrophobic if its surface area is equal to or greater than the area of a methyl group multiplied by the surface area scale factor, which corresponds to the surface area of a chlorine atom. The criteria for this type of interaction were met when the centre of the groups was within 5.5 Å of an alkyl centre (Wolber 2005).

It is important to note that the limonene compound also exhibited numerous interactions with amino acids close to the amino acids of the active site of COX-1. Therefore, this compound can potentially modify the local structure, which can result in a biological effect. For the amino acid LEU352, we noted a hydrophobic interaction of the alkyl type with a distance of 5.13 Å. For the amino acid LEU384, we measured a hydrophobic interaction of the alkyl type with a distance of 4.91 Å. For the amino acid TYR385, we noted a hydrophobic interaction of the Pi-alkyl type with a distance of 4.21 Å. For the amino acid LEU387, we observed a hydrophobic P-alkyl-type interaction with a distance of 4.72 Å. For the amino acid PHE518, we observed two hydrophobic

interactions, both of the Pi-Alkyl type, with distances of 4.84 Å and 5.38 Å. For the amino acid ALA527, we noted two hydrophobic interactions of the alkyl type with distances of 3.75 Å and 4.78 Å.

For the COX-2 therapeutic target, the limonene compound presented a hydrophobic Pi-alkil-like interaction with the amino acid residue TYR385 with a distance of 4.26 Å (Figure 6b). Pi-Sigma interactions are weak interactions between a hydrogen and a ring system Pi. Pi-Alkyl interactions exist where the centres of an aromatic ring and an alkyl group are within the alkyl centroid limit with a maximum distance of 5.5 Å, and they have at least one pair of atoms within the same atom closest to Pi-Pi. For this interaction to occur, the following conditions are necessary: a) Hydrogens that act as donors may be implicit or explicit hydrogens; they must be connected to a non-aromatic carbon atom; B) the distances between the hydrogen and the centre of the Pi ring must be within a maximum distance of approximately 4.0 Å; C) the centre angle CH can deviate from linear by a maximum of 20°; D) the angle between the C-centre and the normal plane of the ring must not exceed 45° (Fares et al. 2016).

Among the amino acids close to the site of action of COX-2, the limonene compound had the following interactions: a hydrophobic Pi-Alkyl-type interaction with the amino acid PHE381 with a distance of 5.27 Å, two hydrophobic interactions of the Alkyl type with the amino acid LEU352 with distances of 3.90 and 5.19 Å were presented, a hydrophobic interaction of the Alkyl type with the amino acid LEU384 with a distance of 4.48 Å, a hydrophobic interaction of the Pi-Alkyl type with the amino acid TRP387 with a distance of 4.85 Å, two hydrophobic Pi-Alkyl-type interactions with the amino acid PHE518 with distances of 4.87 Å and 5.31 Å, three hydrophobic interactions of the alkyl type with the amino acid VAL523 with distances of 3.92, 3,93 and 5,25 Å.

With the PGI-2 therapeutic target, the limonene compound exhibited two hydrophobic interactions with the amino acid CYS441 (both Alkyl type with distances of 4.6 and 5.2 Å) (Figure 1c). Regarding the amino acids close to the active site, the docking presented an Alkyl-type hydrophobic interaction with a distance of 4.46 Å with the amino acid LYS121.

Regarding the therapeutic target COX-1, the 1,8-cineole compound exhibited hydrophobic binding of the Pi-Alkyl type with the amino acid TYR355 with a distance of 4.46 Å. The 1,8-cineole compound also showed two hydrophobic bonds of the Alkyl type with distance of 5.37 Å and 4.43 Å with the amino acid ILE523 (Figure 7).

Compound-1,8-cineole docking resulted in amino acid linkages very close to the amino acids of the active site of COX-1: two hydrophobic interactions with the amino acid

LEU352 of the Alkyl type at distances of 4.81 Å and 5.01 Å and a hydrogen bridge-type interaction with a distance of 2.28 Å. There were also two hydrophobic interactions with the amino acid VAL349 of the Alkyl type with distances of 4.35 Å and 4.94 Å and four hydrophobic interactions with the amino acid ALA527 of the Alkyl type with distances of 3.71, 3.94, 4.14 and 4.68 Å.

The docking of compound 1,8-cineole did not present interaction results with the amino acids present in the active site of the COX-2 and PGI-2 therapeutic targets. However, it presented interactions with seven amino acids close to the active site of COX-2 and interactions with three amino acids close to the active site of PGE2.

In terms of the amino acids close to the active site of COX-2, the docking exhibited two hydrophobic alkyl-type interactions with the amino acid VAL349 with distances of 4.51 Å and 4.01 Å; three hydrophobic interactions of the Alkyl type with the amino acid LEU352 with distances of 5.40, 5.21 and 3.61 Å; and a conventional hydrogen-bonding interaction with a distance of 2.93 Å.

The amino acids close to the active site of PGI-2 that showed an interaction included LYS121 (with a hydrophobic interaction of Alkyl type with a distance of 5.17 Å), the amino acid HIS440, which exhibited a hydrogen-bonding interaction of the conventional type with a distance of 2.14 Å, and the amino acid LEU442 interaction of a conventional-type hydrogen bridge with a distance of 1.90 Å.

The compound that exhibited the largest number of interactions with the therapeutic targets was camphor. In the COX-1 therapeutic target, this compound exhibited an interaction of conventional hydrogen bonds with the ARG120 amino acid with a distance of 2.10 Å. Conventional hydrogen-bonding interactions may exist between a hydrogen-bonding donor atom and an acceptor atom such as N, O, P or S. The maximum accepted distance for this bond is 3.8 Å (Bissantz 2010). For the same target, a hydrophobic interaction with the amino acid ILE523 of the alkyl type with a distance of 4.52 Å was presented. We also noted a hydrophobic interaction of the Pi-Alkyl type with a distance of 4.36 Å with the amino acid TRY355 (Figure 8a).

With the amino acids present near the active site of COX-1, the docking presented three hydrophobic alkyl-type interactions with distances of 3.76, 4.18 and 4.80 Å with the amino acid VAL349; two hydrophobic interactions of the alkyl type with distances of 3.47 and 4.65 Å with the amino acid LEU352; and two hydrophobic alkyl-type interactions with distances of 3.33 Å and 4.94 Å with the amino acid ALA527.

In terms of the docking of the compound camphor with COX-2, this did not present interaction results with the amino acids present in the site. However, camphor presented

interactions with amino acids close to the active site: an alkyl-type hydrophobic interaction with a distance of 4.16 Å with the amino acid VAL349; three hydrophobic alkyl-type interactions with distances of 3.66, 4.08 and 4.64 Å with the amino acid LEU352; a hydrophobic interaction of the Pi-Alkyl type with a distance of 5.41 Å with the amino acid PHE518; three hydrophobic interactions of the Alkyl type with distances of 3.84, 4.30 and 4.41 Å with the amino acid VAL523; two alkyl-type hydrophobic interactions with distances of 4.81 and 3.65 Å; and a hydrogen-bonding interaction with the amino acid ALA527.

Camphor docking with the therapeutic target PGI-2 yielded two hydrogen bonding interactions: a conventional interaction with a distance of 2.09 Å and a carbon-hydrogen-type interaction with a distance of 2.09 Å. Both interactions occurred at amino acid CYS441 (Figure 8b).

The docking results yielded the following interactions with the amino acids close to the active site: two hydrophobic alkyl-type interactions with the amino acid MET124 with distances of 4.93 and 4.95 Å; a hydrophobic alkyl-type interaction with a distance of 4.15 Å with the amino acid ALA283; an alkyl-type hydrophobic interaction with a distance of 5.07 Å; and a conventional hydrogen-bonding interaction with a distance of 4.72 Å with the amino acid LEU 442; and a conventional hydrogen-bonding interaction with the amino acid GLY 443 with a distance of 2.08 Å and a metal-receptor type interaction with a distance of 2.61 Å (Figure 8c).

Conclusion

Our results demonstrate the importance of nanotechnology as an alternative for the delivery of drugs. Nanotechnology is capable of increasing the bioavailability of active principles from vegetable drugs and improving its action on certain target systems. The nanoemulsion (NEORO) obtained from EORO was able to reduce rat paw edema induced by carrageenan with a dose 600 times lower than the effective dose of EORO, and it produced a dose-response effect in the algesic test. NEORO accordingly demonstrated a potent antialgic effect. With the results obtained in the molecular docking study, we observed that among the primary compounds of EORO the camphor molecule presented the largest number of interactions with therapeutic targets related to the inflammatory process. This finding suggests that the camphor molecule is responsible for the anti-inflammatory and antialgic effects observed in the experimental results with NEORO and EORO. However, additional studies are necessary to elucidate the mechanism of H_2S production.

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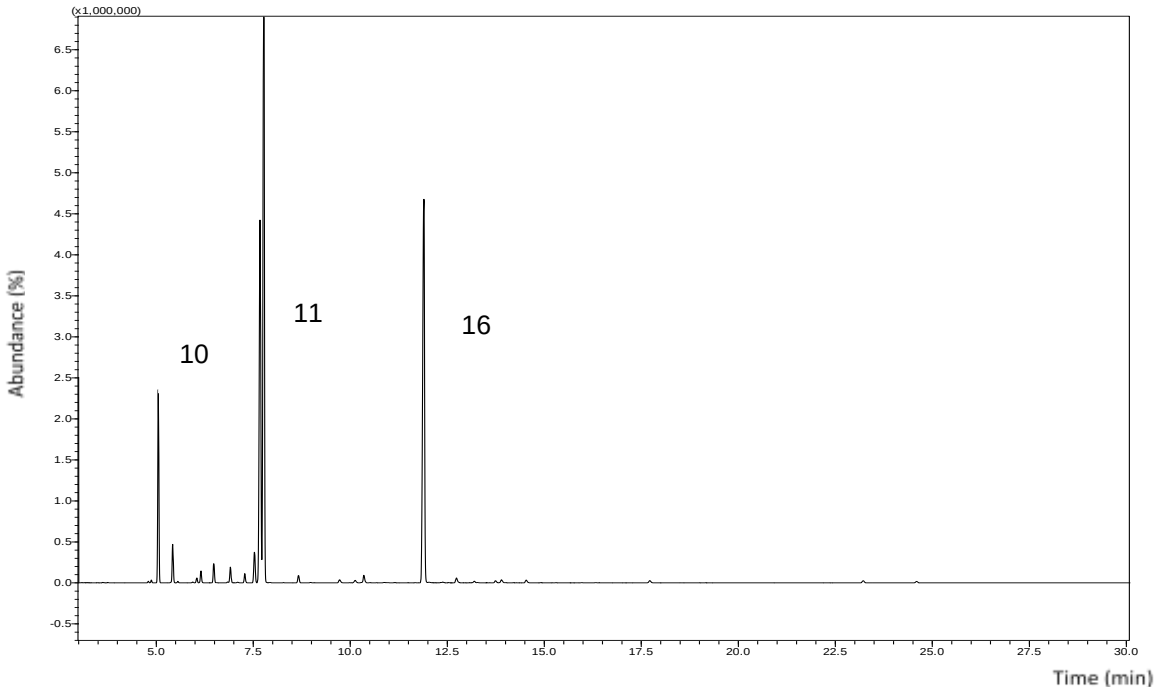

Figure 1. Chromatogram obtained by analysis of EORO by coupled gas chromatography-mass spectrometry (GC-MS) where: corresponding to **10 –** Limonene (21.99%), **11** - 1,8-cineole (33.70%) and **16** – Camphor (27.68%).

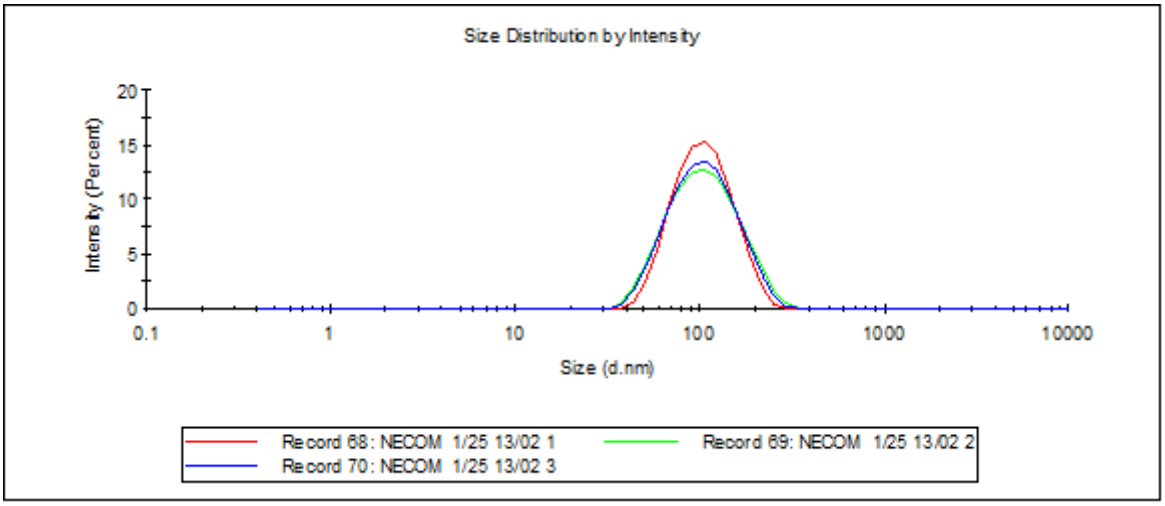

Figure 2. Particle size distribution of NEORO mean droplet – 89.87±0,083727 nm; polydispersity 0,193 ± 0,008 nm.

Table 1. Chemical constituents of *Rosmarinus officinalis* L. essential oil (EORO) determined by GC-MS analysis

Peak	RT (min)	Compound	(%) GC-MS	RI experimental*	RI literature**
1	4.872	α-thujene	0.11	928	926
2	5.054	α-pinene	8.13	935	939
3	5.424	Camphene	1.68	950	954
4	6.045	β-phellandrene	0.21	955	1031
5	6.152	β-pinene	0.58	979	979
6	6.482	β-myrcene	0.90	993	990
7	6.911	α-phellandrene	0.77	1007	1002
8	7.282	α-Terpinene	0.45	1018	1017
9	7.532	o-cymene	1.65	1026	1026
10	7.674	Limonene	21.99	1030	1031
11	7.773	1,8-cineole	33.70	1033	1033
12	8.666	γ -Terpinene	0.39	1059	1059
13	9.724	Terpinolene	0.20	1091	1088
14	10.128	β-linalool	0.16	1102	1098
15	10.350	***	0.44	1108	
16	11.897	Camphor	27.68	1147	1146
17	12.736	Borneol	0.32	1168	1169
18	13.739	α-terpineol	0.12	1193	1188
19	13.899	α-campholenal	0.20	1197	1125
20	14.532	Verbenone	0.18	1213	1205
21	23.220	β-caryophyllene	0.12	1421	1427

***RI experimental: Calculated RI**
****RI literature: RI tabulated for compound**

Table 2. Interactions between amino acids nearby and of the active site of the biological target and distances where interactions occur.

Compounds of EORO	Active Site Amino Acids	Atoms Involved	Type of interaction	Distance (Å)
Limonene	**COX-1**			
	ILE523	C9- CG1 C10- CG1	Hydrofobic Hydrofobic	3.97 4.22
	LEU352	Cyclohexane - CG	Hydrofobic	5.13
	LEU384	C8 – aromatic ring	Hydrofobic	4.91
	TYR385	C8 - aromatic ring	Hydrofobic	4.21
	LEU387	C8 - aromatic ring	Hydrofobic	4.72
	PHE518	C10 - aromatic ring Cyclohexane – aromatic rings	Hydrofobic Hydrofobic	4.84 5.38
	ALA527	C9 - CB Cyclohexane - CB	Hydrofobic Hydrofobic	3.75 4.78
	COX-2			
	TYR385	C8 - aromatic ring	Hydrofobic	4.26
	PHE381	C8 - aromatic ring	Hydrofobic	5.27
	LEU352	C9 - aromatic ring Cyclohexane - CG	Hydrofobic Hydrofobic	3.90 5.19
	LEU384	C8 – CG	Hydrofobic	4.48
	TRP387	C9 - aromatic ring	Hydrofobic	4.85
	PHE518	Cyclohexane - CG 	Hydrofobic Hydrofobic	4.87 5.31
	VAL523	C9 – CG2 C10 – CG1 Cyclohexane – CG1	Hydrofobic Hydrofobic Hydrofobic	3.92 3.93 5.25
	PGI-2			
	CYS441	C8 – SG Cyclohexane – SG	Hydrofobic Hydrofobic	4.60 5.20
	LYS121	C9 - CG	Hydrofobic	4.46
1,8 cineole	**COX-1**			
	TYR355	C10 - aromatic ring	Hydrofobic	4.46
	ILE523	C12 – CG1 H21 – CG1	Hydrofobic Hydrofobic	5.37 4.43
	LEU352	C11 – CG C4 – CG	Hydrofobic Hydrofobic	4.81 5.01
	VAL349	C11 – CG2	Hydrofobic	4.35

		C6 – CG2	Hydrofobic	4.94
	ALA527	C12 – CB	Hydrofobic	3.71
		C11 – CB	Hydrofobic	3.94
		C10 – CB	Hydrofobic	4.14
		C6 - CB	Hydrofobic	4.68
	COX-2			
	VAL349	C11 – CB	Hydrofobic	4.01
		C12 - CB	Hydrofobic	3.51
	LEU352	C12 – CG	Hydrofobic	5.40
		Cyclohexane – CD2	Hydrofobic	5.21
		H28 – CG	Hydrofobic	3.60
		H15 - O	Conventional H bond	2.93
	PGI-2			
	LYS121	Cyclohexane – CG	Hydrofobic	5.17
	HIS440	H15 – O	Conventional H bond	2.14
	LEU442	O2 - H	Conventional H bond	1.90
Camphor	**COX-1**			
	ARG120	01 – H22	Conventional H bond	2.10
	TYR355	H25 - aromatic ring	Hydrofobic	4.36
	ILE523	Cyclohexane – HB	Hydrofobic	4.52
	VAL349	C9 – CB	Hydrofobic	3.76
		C10 – CB	Hydrofobic	4.18
		C11 – CB	Hydrofobic	4.80
	LEU352	C9 – CG	Hydrofobic	3.47
		Cyclohexane – CG	Hydrofobic	4.65
	ALA527	C10 – CB	Hydrofobic	3.33
		Cyclohexane – CB	Hydrofobic	4.94
	COX-2			
	VAL349	H23 – CB	Hydrofobic	4.16
	LEU352	C10 – CG	Hydrofobic	3.66
		C9 – CG	Hydrofobic	4.08
		Cyclohexane – CG	Hydrofobic	4.64
	PHE518	C9 - aromatic ring	Hydrofobic	5.41
	VAL523	C9 – CB	Hydrofobic	3.84
		C11-CB	Hydrofobic	4.30
		Cyclohexane – CB	Hydrofobic	4.41
	ALA527	Cyclohexane – CB	Hydrofobic	4.81
		C11 – CB	Hydrofobic	3.65
		O1 - HA	Conventional H	2.58

		bond	
PGI-2			
CYS441	O1 – HG	Conventional H bond	2.09
	O1 – HA	Carbon H bond	2.09
MET124	Cyclohexane – SD	Hydrofobic	4.93
	C9 – SD	Hydrofobic	4.95
ALA283	C10 – CB	Hydrofobic	4.15
LEU442	Cyclohexane - CG	Hydrofobic	5.07
	O1 - H	Conventional H bond	4.72
GLY443	O1 – H	Conventional H bond	2.08

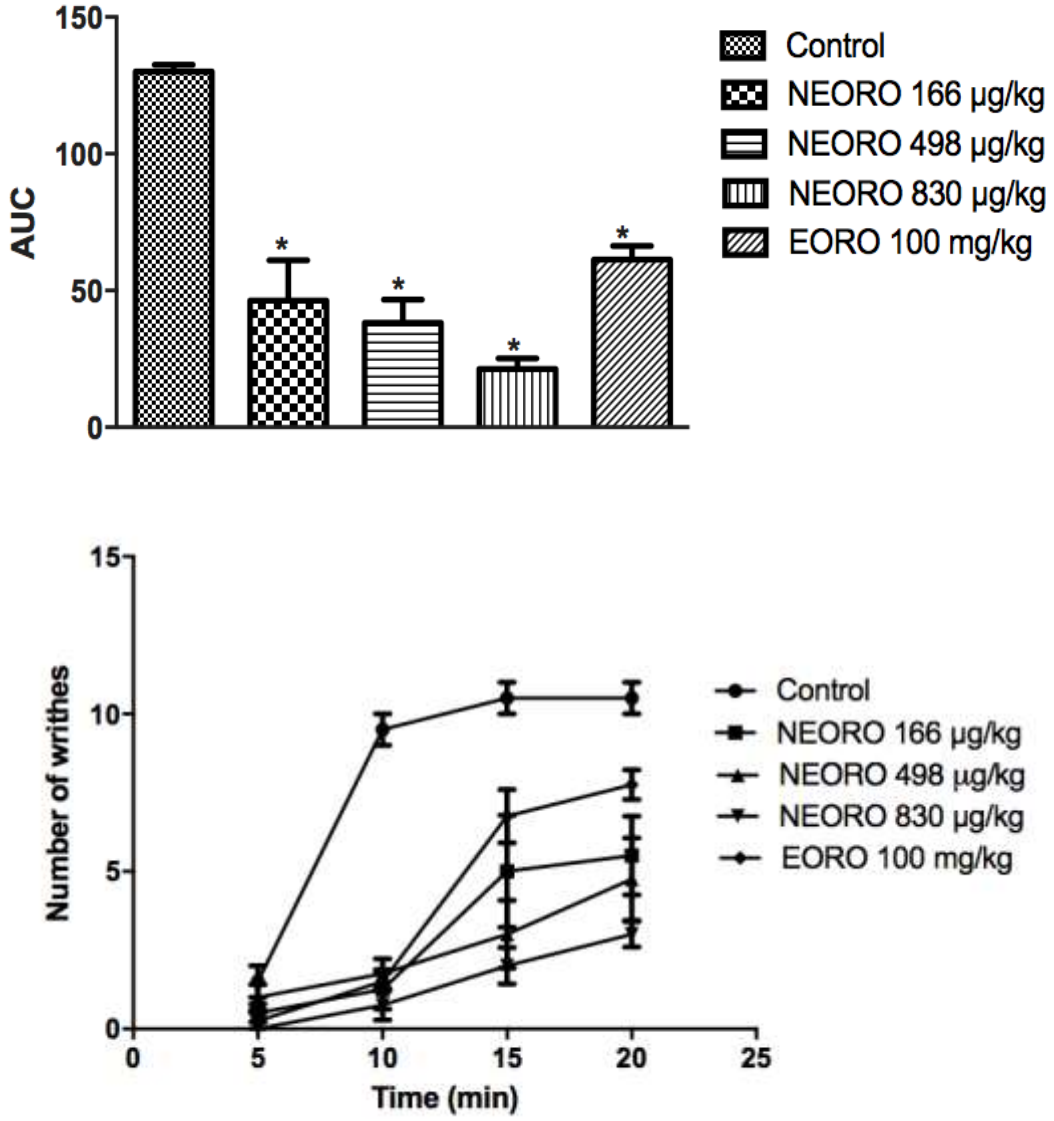

Figure 3. Effect of oral treatments with EORO (100 mg/kg) and NEORO (166, 498 and 830 µg/kg) on writhing in mouse induced by acetic acid. The points represent the mean ± SEM of n = 5/group. ∗ $p < 0.05$, ANOVA followed by the Tukey test.

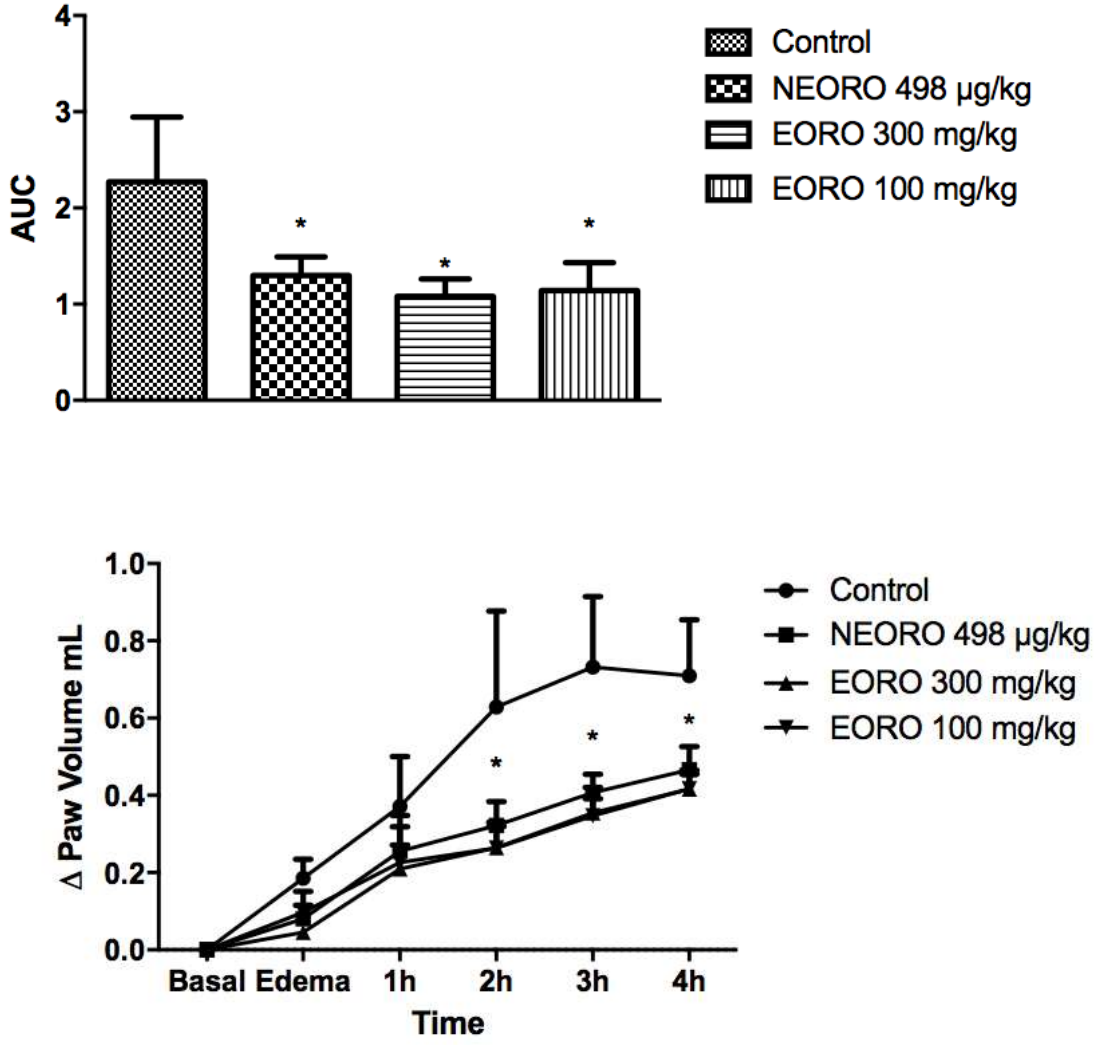

Figure 4. Effect of oral treatments with EORO (100 and 300 mg/kg) and NEORO (498 µg/kg) on rat paw edema induced by carrageenan. The points represent the mean ± SEM of n = 5/group. ∗ $p < 0.05$, ANOVA followed by the Tukey test.

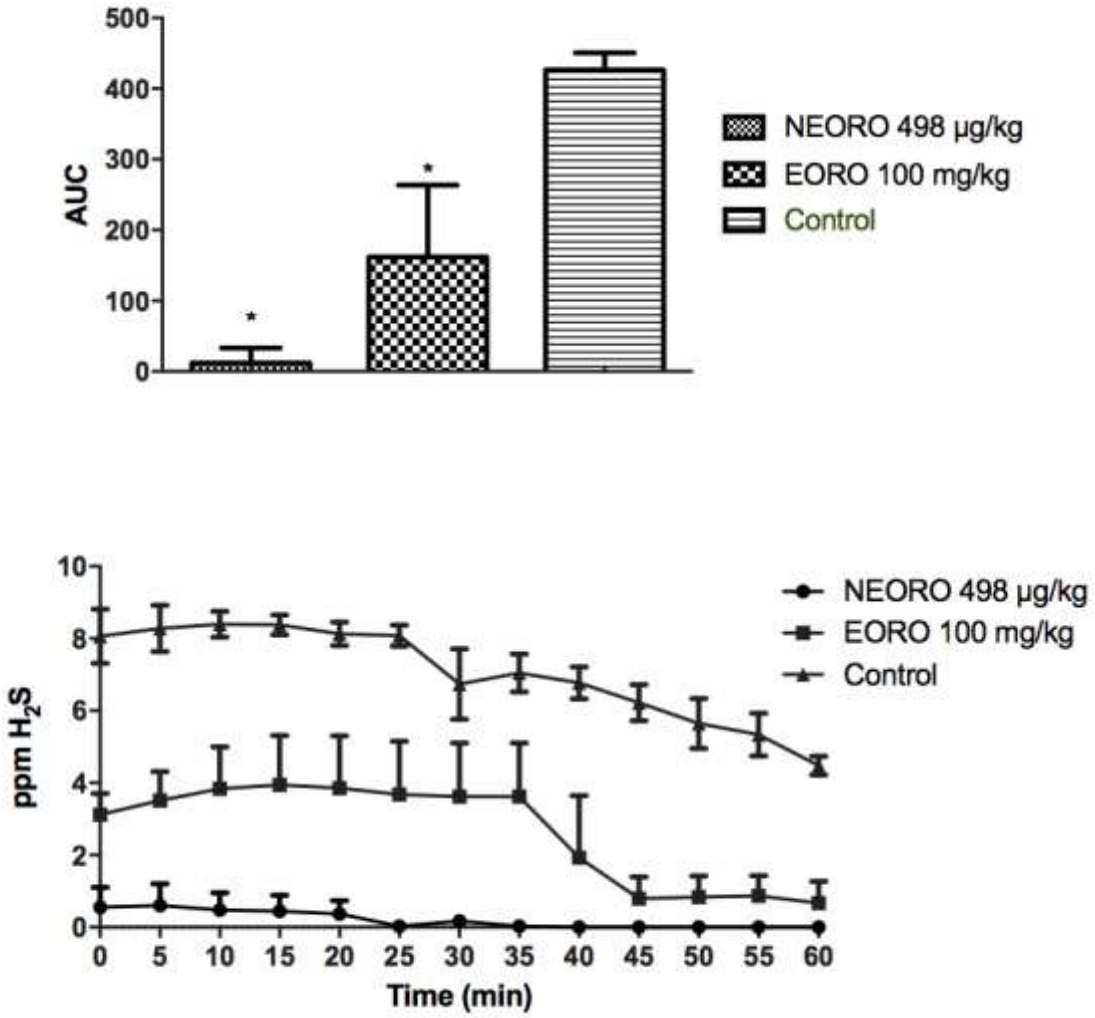

Figure 5. Effect of oral treatments with EORO (100 mg/kg) and NEORO (498 µg/kg) on the production of H_2S in the stomach of wistar rats. The points represent the mean ± SEM of n = 5/group. ∗ $p < 0.05$, ANOVA followed by the Tukey test.

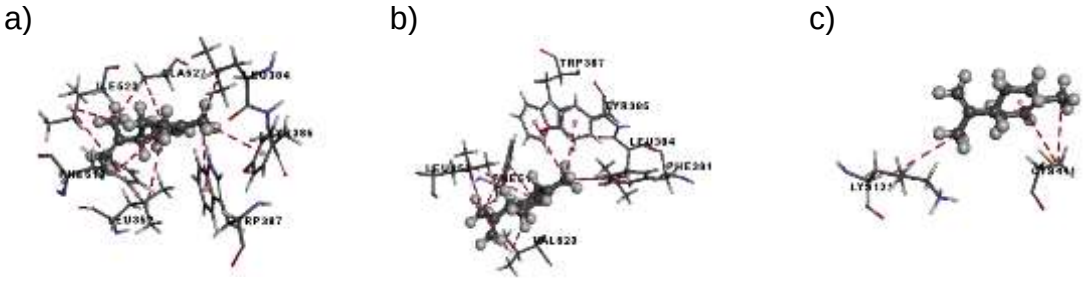

Figura 6. Docking of the compound Limonene performing interaction with a) COX-1 b) COX-2 e c) PGI-2.

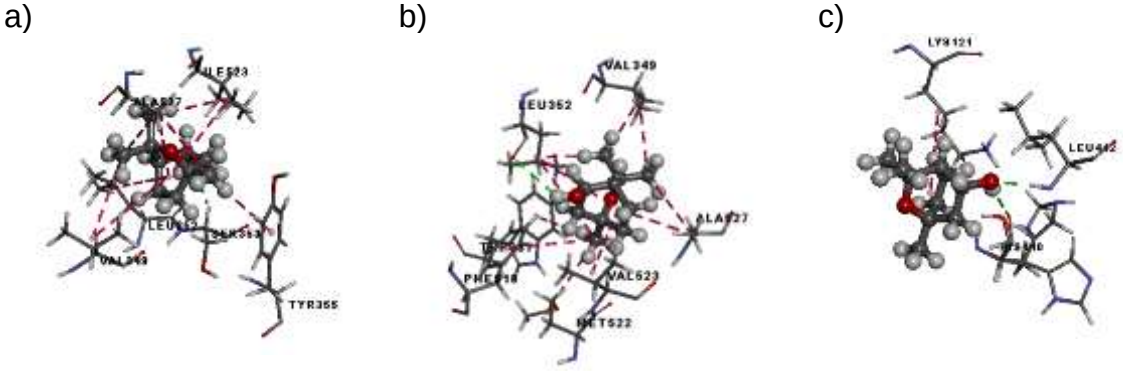

Figure 7. Docking of the compound 1,8-cineole performing interaction with a) COX-1 b) COX-2 and c) PGI-2.

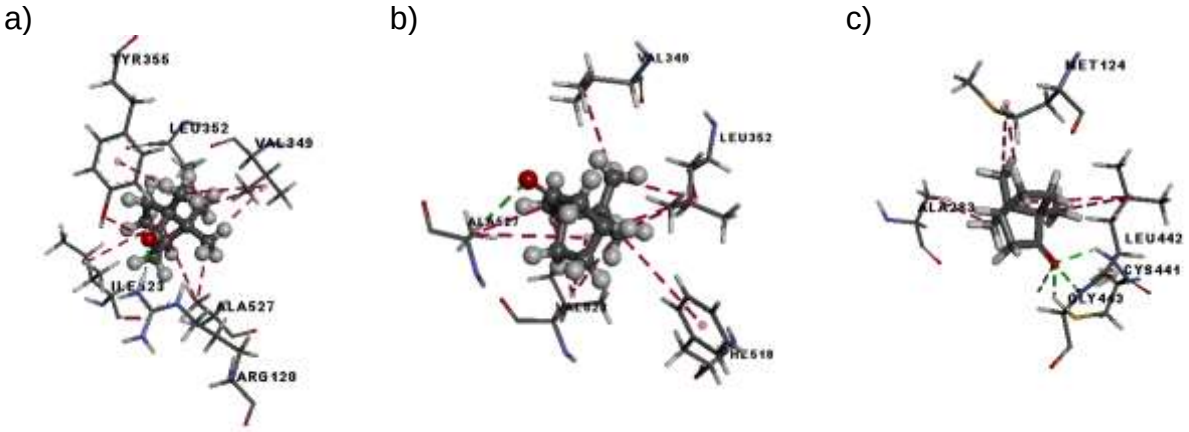

Figure 8. Docking of the camphor compound performing interaction with nearby amino acids and of the active site of COX-1, COX-2 and PGI-2.

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5 CAPÍTULO 3

ANTI-INFLAMMATORY ACTIVITY OF NANOEMULSIONS OF ESSENTIAL OIL FROM *Rosmarinus Officinalis* L.: IN VITRO AND IN ZEBRAFISH STUDIES

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Abstract

The essential oil from *Rosmarinus officinalis* L. (OERO) has bioactive compounds with anti-inflammatory activity. The objective of this study was to evaluate the anti-inflammatory potency of nanoemulsions containing essential oil of *Rosmarinus officinalis* L. (NOERO, NECHA, NECULT, and NECOM) *in vitro* and *in vivo*. This study was accomplished in a quantitative format through tests with diphenyl picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), cellular antioxidant activity (CCA), determination of nitric oxide production, cellular viability and anti-inflammatory activity in zebrafish. OERO's were submitted to the analysis-coupled gas chromatography–mass spectrometry (GC–MS), which highlighted 1,8-cineol and camphor as major compounds. NOEROs were obtained by a low-energy method and presenting the medium size smaller than 200 nm. The efficiency of encapsulation by spectrometry and gas chromatographic analysis was 67.61 and 75.38%, respectively. In the CCA assay, all of the samples presented percentage values of inhibition similar to the quercetin pattern, indicating antioxidant activity. In the test for determination of NO·, all of the samples inhibited the production of NO· when compared to LPS, and NOEROS were more effective than OEROS to 5 µg/mL. In the cell viability assay, the cells remained viable after contact with the samples, demonstrating an absence of cytotoxicity. This study showed that all nanoemulsions (NECHA, NECULT, and NECOM) showed no toxicity to macrophages, besides demonstrating antioxidant activity and potentiation of the essential oil effect in the proliferation of viable fibroblasts. Nanoemulsions has also shown the ability to potentiate the anti-inflammatory action of essential oils by exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide. The results obtained with NECHA in zebrafish confirm the hypothesis that prominent terpenic compounds, alpha-pinene, 1,8-cineole, and camphor, became more available at the target sites, inhibiting the inflammatory process in this animal species.

Keywords *Rosmarinus officinalis* · Essential oil · Nanoemulsions · Anti-inflammatory · *In vitro* · *In vivo* · Zebrafish

Introduction

The Rosemary, scientific name *Rosmarinus officinalis* L., is a medicinal plant of the Lamiaceae family, that presents erect subshrub feature with little branch and height of up 1.5 m (Lorenzi and Matos 2002). Their leaves, quite aromatic, and stem are used popularly for medicinal purposes, ingested as tea (Marchiori 2004). It has been widely used in traditional medicine and as a flavouring in food (Ohno et al. 2003).

The essential oil of *Rosmarinus officinalis* L. (EORO), from the leaves, has been related to the presence of chemical compounds with specific properties (Celiktas et al. 2007). The chemical profile of EORO may vary according to environmental conditions; however, the most known chemotypes in the literature are cineoliferous (higher concentration of 1,8-cineole), camphorliferous (high concentration of camphor), and verbenoniferous (predominance of verbenona) (Napoli et al. 2015). Studies report bactericidal and fungicidal properties (Mekonnen et al. 2016), larvicide (Duarte et al. 2015), insecticide (Badreddine et al. 2015), acaricide (Martinez-Velazquez et al. 2011), antioxidant (Rašković et al. 2014), antinociceptive (Faria et al. 2011), anti-caries (Freires et al. 2015), and antiinflammatory (Melo et al. 2011).

Among the bioactive compounds of EORO related to anti-inflammatory activity, the most reported is 1,8-cineole. This compound is a relatively abundant monoterpene and can reach a concentration of up to 40% by volume in the cineoliferous chemotype (Napoli et al. 2015).

Essential oils are lipophilic compounds that exhibit low interaction capacity with water. This issue has been considered a technological challenge. Pharmaceutical industries have used colloidal transport systems to encapsulate lipophilic compounds, enabling their dispersion in aqueous media (McClements and Rao 2011). Nanoemulsions are promising systems for drug delivery with little solubility in water and have already been proposed to be associated with the essential oil of *Rosmarinus officinalis* L. (Duarte et al. 2015). They consist of systems with thermodynamic stability, where oil/drugs, with lipophilic characteristics, is dispersed in an aqueous environment. They present a wide superficial area with low tension, thermodynamic stability, and solubilization capacity (Ostertag et al. 2012).

In studies for the screening of natural products, the zebrafish has stood out as an experimental model due to the increasing number of publications in recent years using the animal in biological assays for the isolation of bioactive molecules from plant extracts (Santos et al. 2016). For the evaluation of the anti-inflammatory activity of grape seed

extract, Kao et al. (2010) infected with *S. aureus* adult zebrafish pretreated with grape seed extract and observed a lower frequency of death and a reduced inflammatory response in the animals. In a study by Wang et al. (2013), the anti-inflammatory effects of *Gentiana dahurica* (Gentianaceae) ethanolic extract on zebrafish and RAW 264.7 cells were evaluated, with orotic acid and liriodendron exhibiting inflammatory inhibitory activity and none of the compounds being cytotoxic.

Yang et al. (2014) used lipopolysaccharide (LPS) to induce an inflammatory response in zebrafish larvae. The quantitative reverse transcription polymerase chain reaction (RT-PCR) showed elevated levels of IL-1β, IL-6 and TNFα. Cytokines were involved in the inflammatory process, caused by increased macrophages and neutrophils at the site where the LPS was injected, suggesting LPS as a possible new model of inflammation induction in zebrafish.

On the other hand, Huang et al. (2014) demonstrated the efficacy of adult zebrafish as an animal model for the carrageenan-induced abdominal oedema test. The substance administered intraperitoneally presents a direct induction of inflammatory oedema, making possible the evaluation of small volumes of drugs or compounds.

Due to the presence of very sensitive organs, zebrafish can be useful to evaluate the toxic effects of substances through the histopathology of damaged organs (Carvalho et al. 2017). Studies have been carried out with zebrafish as an experimental model for evaluating the anti-inflammatory activity of several compounds (Santos et al. 2016). With a relatively small size and body weight, the adult zebrafish requires a considerably small amount of the compounds to be tested in the carrageenan inflammation assay, which represents a significant advantage in its use in laboratories (Huang et al. 2014).

As nanoemulsions have the potential to enhance the penetration of lipophilic substances through cell membranes, these systems may be an appropriate vehicle for the transport of EORO, increasing its bioavailability and improving its anti-inflammatory effect. The effect of nanoemulsion containing essential oil of *Rosmarinus officinalis* L. (NEORO) on the inhibition of inflammation was evaluated by in vitro and in vivo (in zebrafish) assays.

Methods and Materials

Chemical products

All the reagents used in the *in vitro* tests were obtained from Sigma Chemical Company (ST. Louis, Millstone, USA). The purified water was obtained from the Millipore

Direct® Q3 equipment (Millipore Corp., MA, USA). Dulbecco´s Modified Eagle´s Medium (DMEM) low glucose and the fetal bovine serum (FBS) were obtained from the manufacturer GIBCO®, and trypan blue solution from Sigma-Aldrich.

Vegetal materials

Three samples of essential oil of *Rosmarinus officinalis* L. (EORO), denominated OECULT, OECHA, and OECOM, were used. The first two were extracted in the Laboratory of Phytopharmaceutical Nanobiotechnology of UNIFAP, and the other was acquired from a specialised company. The sample OECULT was obtained from the leaves of the rosemary cultivated in the field of cultivation of medicinal plants of the Goiana Agency of Technical Support, Rural Extension and Agricultural Research (EMATER) from Goiânia, GO, with exsiccate stocked in the Herbarium of the Federal University of Goiás, under nr. 49581, characteristics according to those described in British Pharmacopeia (2009).

The denominated sample OECHA was obtained from the dry leaves of rosemary sold for the preparation of tea by the company Native Natural Products Ltda, in a sealed package from Lot 209. The essential oil, OECOM, was acquired from the company, Florien, containing the decision of a botanical analysis, and showing organoleptic, physiochemical, and microbiological characteristics besides the following descriptions: essential oil of rosemary obtained from the leaves; lot 056757.

Extraction of the essential oil

The extractions of OERO's were accomplished according to Fernandes et al. (2013). The essential oil was obtained by hydrodistillation using the apparel Clevenger. The samples were triturated in a turbolizer and transferred to a 5 L volumetric balloon containing distilled water. The balloon was placed on a heating platform, and the vaporised water carried the vegetable oil through the coobation tube, promoting the separation of phases in the sifter tube (De Groot and Schmidt 2016). The collected sample was stored and cooled to 4 °C for subsequent analyses.

Gas Chromatography-mass spectrometry

Coupled gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Shimadzu system/GC 2010 coupled to a self-gun Shimadzu/AOC-5000 and mass detector (Shimadzu MS2010 Plus) with an electron impact of 70 eV, and equipped with a fused silica column of DB-5MS (Agilent Advanced J & W 30 m × 0.25 mm × 0.25 µm). The parameters were as follows: split ratio, 1:20; helium as carrier gas (65 kPa); injection volume, 1.0 µl; injector temperature, 250 °C; detector temperature, 250 °C; initial column temperature, 50 °C for 1 min; and heating rate, 5 °C min−1 to 250 °C. The total analysis time was 35 min, and the identification of compounds was performed using the NIST 5.0 equipment library.

Nanoemulsions preparation

The nanoemulsions were prepared through the low energy load methodology described by Fernandes et al. (2013). For a final mass of 50 g, 90% of water was used, 5% of OERO, and 5% of Tween 20. Initially, an organic phase was prepared, adding OERO and the tensioactive into a beaker. The mixture was agitated using a magnetic agitator (750 rpm) for 30 min. Next, the aqueous phase was added, with a flow of ≈ 0,5 mL/min with continuous agitation for 60 min. The stability of all of the emulsions was evaluated 1, 30, and 60 days after the preparation through macroscopic analysis (colour, visual aspect, phase separation, creaming, and sedimentation) (Falcão et al. 2007). During this period, all of the emulsions were maintained at room temperature (25 ± 2 °C) in glass test tubes (Borges et al. 2017).

Droplet size analysis

The droplet size and polydispersity were determined by photon correlation spectroscopy using a Zetasizer 5000 (Malvern Instruments, Malvern, UK). Each emulsion was diluted using ultra-pure Milli-Q water (1:25). Measures were performed in triplicate. An average droplet size was expressed as the mean diameter (Orafidiya and Oladimeji 2002).

Determination of encapsulation efficiency

The encapsulation efficiency (EE %) of NECHA nanoemulsion was determined according to the techniques described by Natrajan et al. (2015), Rivera et al. (2015),

Hosseini et al. (2013) and Ixtaina et al. (2015), based on the free (unencapsulated) oil content.

Hexane (2 mL) was first added to the nanoemulsion (2 mL) and the mixture vortexed. After that, centrifugation was performed at 4000 rpm for 30 min to separate the unencapsulated oil. The supernatant was collected and quantified in a UV–vis spectrometer (Model 1240, Shimadzu, Kyoto, Japan) at 306 nm, and also by mass spectrometry (GC–MS) coupled to gas chromatography (Shimadzu MS2010 Plus), following the technique previously described. The amount of free oil was evaluated in triplicate from a standard curve obtained with different concentrations of the essential oil of *R. officinalis* (OECHA), used to prepare the nanoemulsion, diluted in hexane (Natrajan et al. 2015; Rivera et al. 2015). For the curve, the peak area value of camphor, which is one of the major compounds of OECHA, was used.

The percentage of encapsulation efficiency of the nanoemulsion was calculated by the following equation:

$$\%EE = \frac{(\text{Total oil} - \text{Free oil})}{(\text{Total oil})},$$

where total oil corresponds to the total amount of oil present in the nanoemulsion, and free oil corresponds to the amount of unencapsulated oil.

Cultivation and maintenance of cells

Macrophages J774A1 were provided from the ascites of mice (*Mus musculus*) of the strain BALB/cN. The fibroblasts of the lineage MRC5 were obtained from healthy human lung (*Homo sapiens*). They were obtained from Fiocruz Manaus. The cells were maintained frozen in liquid nitrogen and stored in the Laboratory of Biological Activity (BIOPHAR) in the Federal University of Amazonas with DMSO + RPMI without serum (macrophages) or bovine fetal serum (fibroblasts).

The macrophages were cultivated according to the conditions adapted to the Laboratory of Biological Activity II of the Federal University of Amazon (UFAM), with cultivation mean from Dulbecco´s modified eagle's medium (DMEM)—GIBCO, low glucose, supplied with 10% bovine fetal serum (BFS)—GIBCO, penicillin (100 U/mL), and streptomycin (100 U/mL), and incubated with 5% CO_2 to 37 °C. After the formation of cellular confluence (monolayer), the trypsin was applied, and the mean DMEM, containing

10% BFS, was used for the resuspension. The cells were distributed among cell culture plates (96 wells) in density (nr of cells/mL) according to the necessity of each assay. All the procedures involving the cellular cultivation were accomplished in a vertical laminar flow chapel (VECO).

DPPH Chemical assays

The chemical test for the evaluation of the reducing potential of the stable free radical 2,2-diphenil-1-picrylhydrazyl (DPPH) was accomplished in agreement with the method described by Molyneux (2004) in a plate of 96 wells. The solution of DPPH (0.9 mmol/L) was prepared in Ethanol. The final volume of each well of the plate (300 µL) was obtained with 30 µL of the first dilution (sample of 10 mg + 1 mL of Dimethyl sulfoxide—DMSO) or 30 µL of the second dilution (100 µL of the first dilution + 900 µL of DMSO), this dilution resulted in the amount of 389 µg of nanoencapsulated oil (25 times less than oil), and 270 µL of DPPH was added in each well. The gallic acid was used as pattern and DMSO as a negative control. After the addition of the solution of DPPH, the plate was kept at rest in the dark at room temperature. The absorption was measured in microplates reader DTX800 (Beckman Coulter) to 517 nm. The results were obtained using the following formula:

% inhibition = 100 x [1 - (Abs dillution2 – Abs dillution1 / Abs control)].

ABTS Chemical assays

The assay for evaluation of the reducing potential with the radical preformed monocation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)—ABTS+ was accomplished as the method described by Re et al. (1998), using a plate of 96 wells. An oxidized solution was prepared ABTS (ABTS+) containing 10 mg of ABTS + 5 mL of deionised water MiliQ + 5 mL of potassium persulfate to 5 mM. The solution was stored in an amber flask and kept in a magnetic mixer (potency 1) for 16 h. The final volume of each well of the plate (300 µL) was obtained with 30 µL of the first dilution (10 mg of sample + 1 mL of DMSO) or 30 µL of the second dilution (100 µL of the first dilution + 900 µL of DMSO) added 270 µL of ABTS in each well. The gallic acid was used as a pattern and DMSO as the negative control. This dilution resulted in the amount of 389 µg of nanoencapsulated oil (25 times less than oil). After the addition of the solution of

ABTS, the plate was kept at rest, in the dark, at room temperature for 1 h. The absorption was measured in a microplates reader DTX800 (Beckman Coulter) at 714 nm. The results were calculated using the following formula:

% inhibition = 100 x [1 - (Abs dilution 2 – Abs dilution 1 / Abs control)].

Cellular Antioxidant Activity

Antioxidant activity in cells was evaluated in the MRC5 fibroblasts lineage obtained from a human lung (*Homo sapiens*), according to the method described by Wolfe and Liu (2007). 6×10^4 cells were cultivated by a well in a plate of 96 wells. After 24 h of incubation in a CO_2 chamber at 37 °C, the medium was removed, and the wells were washed with PBS. The cells were treated in triplicates with 100 µL of dichlorofluorescein diacetate (DCFH-DA) to 25 µM, followed by incubation for 1 h in a CO_2 chamber at 37 °C. After the incubation period, the solution was discarded, and the cells were washed with PBS. Afterwards, 50 µL of solution containing different concentrations of the samples were added (50, 5, 0.5 and 0.05 µg/mL, corresponding to the concentrations of 0.01621 µg, 0.1621 µg, 1.621 µg, and 16.21 µg of the nanoencapsulated oil), together with 50 µL 2,2′-azobis (amidinopropane) dihydrochloride (ABAP) to 60025 µM. The fluorescence readings were accomplished in the microplate reader (DTX 800, Beckman) with excitement 485 nm and emission 535 nm for 60 min. As a pattern for the test, quercetin was used in the concentrations of 50, 5, 0.5, and 0.05 µg/mL. The median inhibitory concentration was calculated for the time of 60 min according to the formula:

% inhibition = 100 - (F sample / F control) x100,
where F = fluorescence to the 60 minutes - fluorescence to the 0 minutes.

Cell viability assay (Alamar Blue)

Cytotoxicity was evaluated by the Alamar blue method according to Nakayama et al. (1997). Cultivated macrophages were used in the concentration of 5×10^3 cells by well in plates of 96 wells. After 24 h of incubation and adherence of the cells, those were treated with the samples in the concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56 µg/ mL, corresponding to the concentrations of 0.5065, 1.013, 2.026, 4.052, 8.105 and 16.21 µg of the nanoencapsulated oil, respectively, and triplicate for 72 h. For the positive control of

cell death, doxorubicin (5 µg/mL) was used (Sigma Aldrich 20, Germany) and as a negative control, DMSO, to evaluate the influence of the diluents in the cells. After the treatment period (72 h), 10 µL de Alamar Blue was added (resazurin 0.4%) (diluted 1:10 = 950 µL of DMEM + 50 µL of Alamar). After the metabolization of the resazurin, 3 h for J774A1, the reading of the fluorescence was accomplished in the apparel DTX800 (Beckman Coulter); 540 nm of excitement and 585 emission nm were applied. The viability was calculated according to the formula:

% Viability = Ft x 100 / ∆Fb
where Ft = (fluorescence of the cell + half + substance + resazurin) and ∆Fb = (fluorescence of the cell + half + resazurin).

Viability of fibroblasts test (Trypan blue)

The fibroblasts MRC5 (1×10^3 cells/mL) were seeded onto a plate. Then, the cells were treated with complete medium only (control cells), NECHA, OECHA, NECONTROL, or Ascorbic Acid (positive control) at 12.5 µg/mL (0.10 µg of nanoencapsulated oil) for 24, 48, and 72 h. After that, the cells were removed from the plate, and an aliquot of cell suspension was diluted with trypan blue solution (1:10) (Sigma-Aldrich, St. Louis, MO, USA). The cells were then observed under light microscopy (Nikon T1-SM, Eclipse, Konan, Tokyo, Japan) and the viability of the cells was estimated using a Neubauer Chamber.

NO• production assay

The assay was accomplished measuring the accumulation of nitric oxide (NO·) in a medium of culture, using the Griess reaction (Green et al. 1982). The macrophages were plated at a density of 1×10^6 cells/mL in plates of 96 wells, following the adhesion per 24 h at 37 °C, in an atmosphere containing 5% CO_2. After adherence, the mean was removed and added to the cultivation mean of DMEM, supplied with 10% of BFS with a volume of 100 µL/well. The cells were stimulated by the addition of lipopolysaccharide (LPS) of gram-negative bacteria *Escherichia coli* (final concentration 1 µg/ mL) and treated together with the samples in the concentration of 5, 10, 25, 50, and 100 µg/mL, corresponding to 8.11, 16.21, 40.52, 81.05, and 162.1 µg of the nanoencapsulated oil. For the experiment control, the cells were cultivated with and without LPS in the cultivation mean of DMEM containing

10% of BFS. After this, the cells were incubated for more than 24 h at 37 °C, 5% CO_2, and the cell supernatant was collected. To determine the production of NO·, 100 µL were removed from the cell supernatant, and 100 µL of the Griess reagent was added. Stock solutions of N-(1-naphthyl)ethylendiamine—Merck ($C_{12}H_{16}Cl_2N_2$) dissolved at 0.1% in H_3PO_4 (5%) and of sulfanilamide—sigma ($C_6H_8N_2O_2S$) dissolved at 1% in H_3PO_4 (5%) were used for the preparation of this reagent. Before the usage, the solutions were added in a proportion of 1:1, forming the Griess Reagent as known. After the 15-min incubation period, the samples were analysed in the microplates reader (DTX 800, Beckman) at 560 nm. The calculation of the concentrations of nitrite was accomplished based on pattern curves using different concentrations of $NaNO_3$ (15 µM up to 1000 µM).

Study in zebrafish with NECHA

Animals

Zebrafish (*Danio rerio*) males weighing 400–1000 mg were used, which were purchased from the company, Acqua New Aquarium and Fish LTDA, ME., located in Itagassu-PE-Brazil and kept quarantined on the Zebrafish Platform of the Laboratory of Research in Pharmaceuticals of the University Federal University of Amapá—UNIFAP, Macapá, Amapá, Brazil.

The animals were kept in tanks, in which the water conditions were controlled for 2 months before the trials, as described by Carvalho et al. (2017). The experiments were carried out according to the norms established for the care of animals, and the project was approved by the Ethics Committee on Animal Use—CEUA—UNIFAP, the Federal University of Amapá with protocol number 0021/2015.

Routes of administration and treatment groups

The treatments were performed in a double-blind manner, and the different test substances were coded. Diclofenac, dexamethasone, saline, NECONT, and NECHA were administered by gavage, as described by Collymore et al. (2013) 1 h before the application of carrageenan.

The animals were divided into six groups (n = 5/group): group A—control PBS, a substance used to solubilize carrageenan, was applied intraperitoneally, along with saline via gavage; group B—with the application of carrageenan via intraperitoneal and saline via

gavage; group C—Diclofenac (0.5 mg/kg, Sigma Co., São Paulo, Brazil) with the application of carrageenan intraperitoneally; group D—Dexamethasone (0.5 mg/kg, Sigma Co., São Paulo, Brazil) with the application of carrageenan intraperitoneally; group E—NECONT group (control nanoemulsion, Tween 20, and water) with application of carrageenan intraperitoneally; and group F—NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg, described by Borges et al. (2017)) with the application of carrageenan intraperitoneally.

Induction of inflammation with carrageenan and measurement of edema

The induction of inflammation in the adult zebrafish was performed according to the method described by Huang et al. (2014), and carrageenan (Carrageenan kappa, Sigma Co., Lot 16HO616) was injected intraperitoneally into a volume of 20 µL (300 µg) in PBS. The animals were individually anaesthetized in water (8–10 °C) for approximately 3 min before injection.

All animals were weighed individually at the beginning and at the end of the experiment (5 h after carrageenan injection) on an analytical balance (FA2104N, Bioprecisa Co., São Paulo, Brazil). They were photographed at the end of the experiment using a camera (Samsung WB150F, Brazil) and, after euthanasia, were immediately stored in Bouin's solution for histopathological study.

Behavioral analysis

The behaviour of the animals was evaluated over 1–5 h after the injection of carrageenan. The behavioural parameters observed were those described by Souza et al. (2016). The behaviour of zebrafish was classified into three stages: I with increased swimming activity and presence of tail tremors, II with circulatory movement in swimming and loss of balance, and III with loss of motility, rest at the bottom, and death.

Histopathological study

After the experiment, the animals were stored in identified cassettes and immersed for 24 h in Bouin's solution. Afterwards, they were descaled with EDTA (Ethylenediaminetetracetic acid, Sigma Co., São Paulo, Brazil) for 24 h. After decalcification, alcohol dehydration was performed at 70, 80, 90, and 100%, for 1 h each.

Then diaphanization with xylol and inclusion with paraffin was performed. The 5 µm sections were obtained on a microtome (Rotary Microtome Cut 6062, Slee Medical, Germany), transferred to glass slides and stained with Hematoxylin and Eosin (Souza et al. 2016). Histological analysis was performed under an optical microscope. Histological changes in gills, liver, kidneys, and gut were evaluated according to parameters presented by Meletti and Rocha (2003), Souza et al. (2016) and Carvalho et al. (2017).

To define the degree of tissue change, the Histopathological Change Index (HCI) for the gills, liver, kidneys and intestine was used, which classified the organs as normal when the HCI values ranged from 0 to 10, moderately altered when the values are from 11 to 20, changed in moderate to severe degree when the HCI is between 21 and 50, and severe with irreversible changes to values greater than 100 (Poleksic and Mitrovic-Tutundzic 1994; Rigolin-Sá 1998; Takashima and Hibiya 1995).

Statistical Analysis

In the results obtained with assays, the significance was determined through the ANOVA test to compare the means between the control and treated groups, considering significant values of $p < 0.05$ (Sokal and Rohlf, 1995), and highly significant values of $p < 0.01$. In the zebrafish study, one-way ANOVA (Analysis of variance) was used, followed by Dunnett's multiple comparison test, and to histopathological change index, Kruskal–Wallis one-way ANOVA and Student–Newman–Keuls test were used. Values of $p < 0.05$ were considered statistically significant. Data were expressed as a mean ± error standard mean. The graphs were recorded by the program GraphPad Prism 6.0.

Results

The essential oils of *Rosmarinus officinalis* L. (OECULT and OECHA) presented an average yield of 2.5 and 1.0%, respectively. In the characterization of these oils and the OECOM commercial essential oil, the major components identified were: 1,8-cineole (16.84, 50.82 e 33.70%), camphor (30.93, 19.16 and 27.68%) and α-pinene (14.24, 10.12 and 8.13%), respectively (Table 1 and Fig. 1). It should be noted that OECULT essential oil also presented 10.20% β-myrcene.

In the measurement of droplet size and polydispersity index (PDI), all nanoemulsions had a mean droplet size less than 100 nm and PDI less than 1. NECOM showed mean droplet of 89.87 ± 0.083727 nm and PDI of 0.193 ± 0.008 nm, while in

NECULT, the mean droplet value was 98.01 ± 0.302900 nm and polydispersity of 0.182 ± 0.001 nm, and NECHA showed mean droplet of 77.32 ± 1.192000 nm and PDI of 0.239 ± 0.006 nm (Fig. 2).

The efficiency of encapsulation by spectrometry determined the free oil content of 32.39 ± 0.069%, with 67.61 ± 0.069% of the encapsulated oil. In the gas chromatographic analysis (GC–MS) the free oil content was 24.62 ± 0.090% and 75.38 ± 0.090% encapsulated.

In the chemical assays, the nanoemulsions (NECHA, NECULT, and NECOM) demonstrated free radical reducing action in ABTS and DPPH. In the ABTS assay, it was observed that, in the concentration of 100 µg/mL, all the samples had values of reductive activity lower than 20%, indicating a low profile of this activity to the gallic acid standard (90.38 ± 0.13%). At the concentration of 1000 µg/mL, the oils had a low free radical reducing activity profile compared to gallic acid (91.99 ± 0.21), and the nanoemulsions showed an absence of this activity when compared to their respective essential oils, NECHA (20.09 ± 0.84) and OECHA (54.67 ± 1.98), NECULT (17.27 ± 0.75) and OECULT (46.64 ± 5.17), NECOM (10.67 ± 0.83) and OECOM (24.50 ± 3.95) (Fig. 3).

In the DPPH radical assay, at the concentrations tested, all nanoemulsions presented inexpressive values of reducing activity (< 10%, Fig. 3), while in the cellular antioxidant activity assay, the nanoemulsions presented antioxidant activity like that of the standard quercetin. There was no significant difference between the effects caused by the essential oils and their respective nanoemulsions, nor was there a dose–response effect, and the inhibitory responses to OECULT, NECULT, OECOM, OECHA, NECHA, and quercetin were 92, 82, 93, 91, 90, and 96%, respectively (Fig. 4).

In the cytotoxicity assay in J774 cells (cell viability), none of the samples tested showed a significant reduction in the viability of the macrophages compared to the diluent, DMSO, after 24 h of treatment (Fig. 5). The solubilization of DMSO samples presented 100% cell viability. When compared to DMSO, all essential oil samples showed an increase in the amount of cells at all concentrations tested (1.5, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL, respectively). Among the nanoemulsions, only in the concentration of 1.5 µg/mL, NECULT (56.3 ± 4.17) and NECOM (71.3 ± 1.0) presented a reduction in the number of cells to DMSO (100 ± 10.0). However, they presented a relevant quantity of viable cells in this concentration. In comparison to their respective oils, the NECHA nanoemulsion showed an increase in the number of cells at all concentrations, tested with values of cell viability similar to those demonstrated by OECHA. NECULT and NECOM

presented lower numbers of cells when compared to OECULT and OECOM, but maintained cellular viability (Fig. 5).

In the test of the viability of fibroblasts (Trypan blue), nanoemulsion (NECHA), after 72 h of treatment, showed higher values of 16, 24, and 48% of fibroblast proliferation compared to NECONTROL, OECHA, and the untreated cells, respectively (Fig. 6).

The action of the different essential oil samples of *Rosmarinus officinalis* at concentrations of 5, 25, and 50 μg/mL and their nanoemulsions corresponding to 1.621, 3.242, 8.105, 16.21, and 32.42 μg/mL of the nanoencapsulated oil on the production of NO· in μmol/L are shown in Fig. 7. The essential oils (OECHA, OECULT, and OECOM) inhibited the dose-dependent production of nitric oxide in the macrophages, with OECHA IC_{50} = 10.05 μg/mL, OECULT = 4.45 μg/mL, and OECOM = 11.81 μg/mL. With nanoemulsions, this inhibition occurred non-dose-dependent (Fig. 7).

In the NO· test, it is possible to observe that, in the lower concentrations, the nanoemulsions reduced or maintained the NO· production in the presence of LPS when compared to the essential oils. The NECHA nanoemulsion demonstrated a higher inhibitory effect of NO· than the OECHA essential oil at the concentrations of 25 μg/mL (3.0 ± 0.1, 5.6 ± 2.8) and 5 μg/mL (4.0 ± 2.4, 6.7 ± 3.5). The NECOM (4.5 ± 0.2) nanoemulsion also showed higher inhibition of NO· than OECOM (8.1 ± 6.5) and 5 μg/mL oil. At this same concentration, NECULT (5.9 ± 0.9) maintained an inhibitory activity similar to that of OECULT (5.5 ± 3.8) (Fig. 7).

In the zebrafish study, 40% of the animals in group C and 20% in groups B, D, and E showed an increase in swimming activity in the first hour after administration of carrageenan, and 20% in groups B, C, and E rested on the bottom of the beaker. In the second hour after the injection of carrageenan, 20% of the animals in group C and 40% of group D presented rest in the background. After 3 h, 20% of groups B and E and 60% of group C rested at the bottom, 20% of animals in groups A and B increased swimming activity, and 20% in groups D, E, and F showed loss of balance. Four hours after the application of carrageenan, 20% of the animals of groups A, B, D, E and 60% in group C rested in the background. In the last hour, 5 h after carrageenan, 60% in group C and 20% in groups A, B, D, E, and F rested on the bottom of the beaker. The animals that presented loss of balance corresponded to 40% in groups D and F. They presented loss of balance 20 and 40% of the animals in groups A and D, respectively. No death occurred in any of the groups (Table 2).

Group A (control, treated with saline solution by gavage) presented behavioural changes of level I only 3 h after the application of PBS, and level II from the fourth hour

and, there was no oedema formation (Fig. 8). While group B that received carrageenan, and was treated with saline presented the three levels of behavioural changes and increased in 272 mg (Fig. 8) when compared to the initial body weight, demonstrating the formation of inflammatory oedema.

The groups previously treated with diclofenac showed more behavioural changes than those treated with dexamethasone, in addition to losing 160 mg of initial body weight, while the dexamethasone group lost 32 mg of their initial body weight (Fig. 8).

Group E (NECONT) presented the three levels of behavioral changes with body weight gain of 304 mg (Fig. 8), and group F (NECHA) did not present behavioral changes in the first two hours after the injection of carrageenan, showing changes in levels II and III only after the third hour, with a body weight loss of 241 mg and inhibition of 77.99% of abdominal edema (Fig. 8 and Table 2).

In the histopathological study of the gills, group A (intraperitoneal PBS + saline via gavage) presented no histopathological alterations (Fig. 9a). The HCI value was zero and, consequently, the percentage of total tissue changes was zero (Table 3). Group B (intraperitoneal carrageenan + saline via gavage) showed the fusion of the secondary lamellae, removal of the primary lamella, and displacements of epithelial cells, such as level I alterations, epithelial rupture (level II), and aneurysm (level III) (Fig. 9b). The HCI value was 23, which indicated moderate to severe changes in the organ, with 71.4% of total alterations (Table 3).

Group C (diclofenac) showed only level I changes in gills, the fusion of secondary lamellae, the detachment of primary lamellae, and hyperplasia of epithelial cells (Fig. 9c). Its HCI (0) indicated that the organ remained normal and the total changes present in the tissue was 42.8% of changes in the gills (Table 4). The group treated with dexamethasone had an HCI of 22, indicating the presence of moderate to severe changes in the gills. The percentage of total changes was 57.1% (Table 3). In the histopathological analysis, the fusion of secondary lamellae and displacement of epithelial cells (Level I), epithelial rupture (Level II), and aneurysm (Level III) (Fig. 9d).

The group E (NECONT) presented the fusion of secondary lamellae, displacement of epithelial cells (Level I), HEC hyperplasia of epithelial cells (I), epithelial rupture (Level II), and aneurysm (III), with HCI of 22 indicating moderate alterations to severe (Fig. 9e, Table 4). The group F (NECHA) presented level I changes, such as fusion of secondary lamellae, the detachment of primary lamella and detachment of secondary lamella, and only epithelial rupture as level II alteration (Fig. 9f). The HCI was 3, demonstrating that the

organ was functionally normal and the alterations presented in the tissue corresponded to 57.1% (Tables 3 and 4).

In the hepatic tissue, group A (intraperitoneal PBS and saline solution by gavage) presented only level I changes, such as increased cell volume and loss of cell contour (Fig. 10a). The HCI was zero, indicating that the organ remained normal after the application of PBS. Histopathological changes were 15.4% (Table 3). The group B (treated with saline solution and intraperitoneal injection of carrageenan) presented histopathological alterations of level I and II: increased cell volume (I), loss of cell contour (I), nuclear degeneration (II), vessel rupture, hyperemia (II), and nuclear vacuolization (II) (Fig. 10b). He presented 53.8% of hepatic alterations with HCI of 10, with mild to moderate alterations (Table 4).

The animals of group C (Diclofenac) presented a normal liver (HCI of 4) with 30.7% of hepatic tissue alterations. The histopathological changes observed were loss of cell contour (I), loss of nucleus contour (I), cell disruption (II), and vessel rupture (II) (Fig. 10c). In the same way, group D, treated with dexamethasone, also presented organs with normal functionality (HCI of 3) and 38.4% of tissue alterations (Tables 3, 4). Only cell rupture was identified as level II alteration, and all others were level I: loss of cell contour, increased nuclear volume, decreased glycogen, and the decreased relative frequency of nucleus occurrence (Fig. 10d).

The animals of group E (NECONT) presented several level II hepatic alterations, such as nuclear degeneration, cell disruption, vessel rupture, hyperemia, nuclear vacuolization, and only decreased glycogen as level I alteration (Fig. 10e). Its HCI was 10, indicating mild to moderate changes and 46.1% of histopathological changes (Table 3 and 4). The F group (NECHA) presented functionally normal liver (HCI = 0), with increased nuclear volume (I), increased vessel volume (I), cytoplasmic degeneration (II), and cell disruption (II) (Fig. 10f) and 30.7% liver changes (Table 3). As for the kidneys, group A did not present any histopathological alteration, with HCI equal to zero. Group B presented histopathological changes of the three levels: hypertrophy of tubular cells (I), dilation of glomerular capillaries (I), increase in tubular lumen (II), tubular degeneration (II), cytoplasmic degeneration of tubular cells (II), and necrosis (III), HCI of 28, with moderate to severe alterations and with 53.8% renal tissue changes.

Groups C and D (diclofenac and dexamethasone) presented similar histopathological changes, such as vessel dilatation (I) and tubular degeneration (II). Group C also showed hyperemia, increased space of Bowman's capsule, and glomerular degeneration, and animals of group D tubular disorganization (Fig. 11c, d). Both

maintained the functionally normal organs with HCIs of 6 and 2, respectively (Table 4). Of the total histopathological changes in the kidneys, diclofenac had 38.4% and dexamethasone 23% (Table 4).

The animals that were treated with NECONT (Group E) presented the three levels of histopathological changes: vessel dilatation (I), tubular disorganization (I), tubular degeneration (I), cytoplasmic degeneration of tubular cells (II), hyperemia (II), and necrosis (III) (Fig. 11e), with an HCI of 27, with moderate to severe alterations, and 53.8% of tissue alterations (Tables 3, 4). In animals treated with NECHA (Group F), histopathological changes were observed, such as vessel dilatation (I), tubular disorganization (I), increased space of Bowman's capsule (I), tubular degeneration (II), and cytoplasmic degeneration of tubular cells (II), with an HCI of 7 and 38.4% of tissue changes (Fig. 11f, Tables 3, 4).

The intestinal tissue group A (PBS and treated with saline solution) presented cell degeneration (I), degeneration of muscle layer (I) and necrosis (III) (Fig. 12a), with 20% of total histopathological changes (Table 3) and HCI of 20, indicating mild to moderate changes (Table 4). Group B (injection of carrageenan and treated with saline) presented several histopathological changes: increased leukocyte infiltration (I), detachment of epithelial lining (I), hypertrophy of epithelial cells (I), hypertrophy of globet cells (I), cell degeneration (I), detachment of lamina propria (II), fusion of villous (II), villous degeneration (II), hemorrhage in the lamina propria (II), and necrosis (III) (Fig. 12b), with HCI of 29, alterations moderate to severe, and 66.6% total alterations (Tables 3, 4).

The animals treated with diclofenac and dexamethasone (Groups C and D) presented several changes in intestinal tissue, such as detachment of epithelial lining (I), increased leukocyte infiltration (I), villous atrophy (I), vacuolization of enterocytes (I), cell degeneration (I), villous degeneration (II), detachment of lamina propria (II), fusion of villous (II), and necrosis (III) (Fig. 12c, d). These changes were classified as moderate to severe, with HCI of 24.8 and 25, respectively (Table 4). Tissue changes were 46.6 and 53.3% (Table 3).

Group E (treated with NECONTROL) presented several changes. Among them were detachment of lamina propria (II), villous degeneration (II), leukocyte infiltration (I), vacuolization of enterocytes (I), hemorrhage in the lamina propria (II), partial or (II), necrosis (III) (Fig. 12e), with HCI of 30 (Table 4), indicating moderate to severe changes, and 60% changes in intestinal tissue (Tables 2, 3) The group, F (NECHA), presented leukocyte infiltration (I), vacuolization of enterocytes (I), hyperplasia of goblet cells (I), cell degeneration (I), degeneration of muscle layer (I), hypertrophy of epithelial cell hemorrhage in the lamina propria (II), and necrosis (III) (Fig. 12f). These changes were

classified as moderate to severe with an HCI of 23 and with 53.3% tissue changes (Tables 3 and 4).

Discussion

The chemical composition found in the OERO samples is as described by Takayama et al. (2016) where 1,8-cineole and camphor were found as major compounds of *Rosmarinus officinalis* essential oil collected in the Brazilian Midwest region. The same major compounds were found in the essential oil obtained from samples of *Rosmarinus officinalis* L. collected from three different geographical origins: Beja, Sidi Bouzid, and Gabes, Tunisia (Hcini et al. 2013). In samples of Rosmarinus officinalis L. collected in Turkey, 1,8-cineole and camphor were among the major components of essential oil (Türkmen et al. 2014). In a study by Salido et al. (2003), the chemical composition of twelve essential oil samples of *Rosmarinus officinalis* L., harvested at four different sites in Southern Spain at different periods, was analyzed, and it was observed that 1,8-cineole remained constant among the compounds regardless of the collection period while the other components varied according to the life cycle of the plant.

The nanoemulsions containing the essential oil of *Rosmarinus officinalis* L. (NEORO) presented a translucent and bluish-like appearance, as described by Forgiarini et al. (2000), with droplet sizes smaller than 200 nm and polydispersity indexes below 1 (Table 3). Similar results were obtained by Solans et al. (2005) and Solè et al. (2012).

NECHA maintained a higher amount of the encapsulated OERO than the essential oil oregano nanoparticles with EE of 21–47% (Hosseini et al. 2013), and than the essential oil of *Lippia sidoides* with EE of 55% (Oliveira et al. 2014).

The results obtained in this study can be compared to the study carried out with the essential oils of *Mentha piperita*, *Eucalyptus globulus* encapsulated in chitosan nanoparticles and analyzed by UV vis spectrometry, with EE values higher than 70% (Pijpers 2017) and oil of saffron showed 71.1% (Natrajan et al. 2015). According to Pijpers (2017) encapsulation efficiency values higher than 70%, it suggests that the encapsulation technique was successful. Although the encapsulation techniques of essential oils are different among the studies cited here, the results of encapsulation efficiency show values similar to those found in the present study (67–75%), suggesting that NECHA was effective in keeping most of OERO effectively encapsulated.

The low reductant activity of the nanoemulsions observed in the DPPH assay (Fig. 3) may be related to the fact that the antioxidant compounds present in the essential

oil were encapsulated in the nanoemulsions, which possibly hindered their release and the consequent interaction with the chemical agent employed. As the tests with ABTS and DPPH show the reduction activity and not the antioxidant activity, it can be stated that the nanoemulsions did not present reductive activity, but this does not exclude the possibility of their presenting antioxidant activity.

These results can be compared to those described by Ha et al. (2015), which tested lycopene-containing nanoemulsions with different particle sizes in DPPH and ABTS assays. This study demonstrated that the incubation period of the sample with the reagent might have influenced the release of the encapsulated compounds in some nanoemulsions. The authors stated that it would take a longer time for reactions with free radicals to occur in the nanoemulsions that did not exhibit reductive activity because the release of the molecules may have occurred slowly as they were protected by the structures of the droplets. Thus, it is understood that the relatively short incubation period used in the present study may have influenced the non-release of the active principles present in the nanoemulsions, implied by the expression values for the reductive activity.

The samples that presented the most relevant results in the ABTS test (NECHA, NECULT, OECHA, OECULT, and OECOM) were used in the cellular antioxidant assay (CAA). In this assay, at the three concentrations tested (0.05, 0.5, 5, and 50 μg/mL), they presented high values of antioxidant activity, with no significant difference to the quercetin standard (Fig. 4). It should be noted that the concentrations of the nanoencapsulated oils in the nanoemulsions corresponded three times less than those of the pure essential oils (0.01621, 0.1621, 1.621, and 16.21 μg), and these had the same antioxidant effect as pure oils. Possibly, this result can be explained by the fact that the lipophilicity of the cell membranes allowed the penetration of the micelles, present in the nanoemulsions, in the intracellular medium, promoting the decapsulation of the active antioxidant principles and, consequently, their ability to act.

A study by Sessa et al. (2013) involved polyphenols extracted from grape marc, which were encapsulated in the form of nanoemulsions and evaluated for the protective role of these formulations in preventing degradation and, in improving the distribution through biological membranes, preserved a high antioxidant activity. Two chemical tests were carried out to measure the radical-reducing activity, and, in one of them, one of the nanoemulsions presented low values of this activity. In the antioxidant assay with the cells, the nanoemulsions presented values relevant to the antioxidant activity, and these results were significantly higher for the encapsulated grape marc polyphenols than for the non-

encapsulated polyphenols, suggesting the fundamental role of the nanoemulsions in the release through biological membranes.

In the J774 cell viability assay, it can be seen that none of the samples tested showed cytotoxicity. All the results presented different profiles of cellular viability to the antineoplastic doxorubicin curve, indicating that the cell viability profiles of the oils and nanoemulsions were not concentration-dependent (Fig. 5).

The absence of cellular toxicity presented by nanoemulsions was similar to the study by Teixeira et al. (2017), who tested various nanoemulsions containing d-α-tocopherol and tween 80 surfactants at different concentrations, and observed that none showed toxicity to the cells.

To evaluate the action of nanoemulsification on cell proliferation, the viability of a fibroblast assay was performed in the presence of trypan blue, which is a diazo dye used to measure the viability of the cells through the penetration of the dye into the intracellular medium.

In the mechanism of this assay, one has to consider that the cell membrane is formed by a highly selective lipid bilayer; therefore, in cells considered viable, the dye does not penetrate. Cells with damaged membranes show distinct blue staining in their cytoplasm. In this way, this dye allows the discrimination between viable cells and cells with damaged membranes, considered as dead cells (Tran et al. 2011). MRC5 fibroblasts were used for this assay. Fibroblasts are common connective tissue cells with high protein synthesis capacity to maintain the fibres and fundamental substance of the extracellular matrix in the tissues. They also produce growth factors that control cell proliferation and differentiation, acting on healing processes (Tran et al. 2011).

The NECHA nanoemulsion, the essential oil of *Rosmarinus officinalis* (OECHA), and the control nanoemulsion (NECONTROL) were evaluated in the cell viability test, and it was possible to observe that NECONTROL did not significantly interfere, when compared to untreated cells (negative control), on cell proliferation, thus evidencing that the surfactant Tween 20, used in the preparation of nanoemulsions, did not influence this process. After 72 h of treatment, NECHA presented 48%, and ascorbic acid 54% increased cell proliferation.

In this essay, it should be noted that the essential oil of *Rosmarinus officinalis* (OECHA) delivered in the form of nanoemulsion (NECHA) was used at a concentration 125 times lower than the OECHA. Thus, it is possible to suggest that this nanoencapsulated oil potentiated the effect on fibroblast proliferation (Fig. 6). Anuchapreeda et al. (2012) evaluated the cytotoxicity of a nanoemulsion containing

curcumin in several cell lines and stated that control nanoemulsion (containing Tween 80) did not influence on cell viability. Rampersad (2012) reported that in metabolism assays, there is the drawback of no differentiation between cells that are actively dividing and those that are quiescent, which may result in overestimation of cell numbers. However, he claims that an increase in cell proliferation can be considered indicative of cell viability.

In the inhibition test of nitric oxide production (NO·) in vitro, LPS was used as a positive control of this production. NO· plays an important role in various inflammatory conditions, being produced in the form of nitric oxide synthase (iNOS) from the amino acid L-arginine (Abdelwahab et al. 2012). Several stimuli, such as LPS, can significantly increase the production of NO·by macrophages, which are cells derived from monocytes, capable of acting as antigen presenting to lymphocytes, in addition to, in some cases, phagocytosis. They play an important role in acute and chronic inflammatory reactions (Verma et al. 2009). In aerobiosis, NO· reacts with oxygen to produce stable nitrate (NO_3) and nitrite (NO_2·) whose quantities can be determined using Griess's reagent (Islam et al. 2016).

In the evaluation of NO· production in macrophages, in general, all samples of *Rosmarinus officinalis* oils and nanoemulsions inhibited production in macrophages when compared to cells receiving only LPS. Among them, the NECHA and NECOM nanoemulsions were characterised by a reduction in this production when compared to the nonencapsulated oils at the lowest concentration tested. This shows that while being distributed at only 5 µg/mL, nanoemulsions were able to potentiate the effects of essential oil in reducing the production of the pro-inflammatory mediator, nitric oxide (Fig. 7). Justo et al. (2015) observed that rosemary extract obtained by supercritical CO_2 extraction inhibited the release of NO· by peritoneal macrophages and J774 cells; maintaining reduced cytotoxicity when dispersed in DMSO at a concentration lower than 2.79 mg/mL.

In the study with zebrafish, it should be considered that when in contact with foreign substances, zebrafish may present altered behaviour (Mathur et al. 2011), including derivatives of plant species, as described by Ribeiro (2013) for the ethanolic extract composition of *Spilanthes acmella*. In evaluating the toxicity of nanoemulsion with perillyl alcohol, Souza et al. (2016) observed that behavioural changes in zebrafish begin with increased excitability, leading to loss of balance, rest in the background, and probably death.

The application of carrageenan (300 µg) in the abdomen of the animals produced oedema that was very visible in the control groups (B and E), with maximum peak at the 5th hour after the application (Fig. 8). The reactions observed in this study were described

by Huang et al. (2014) as typical symptoms of zebrafish inflammation, with the presence of oedema, leukocyte marker (MPO), proinflammatory proteins such as TNF-α and iNOS.

The different previous treatments of the animals with diclofenac (Group C), dexamethasone (Group D) and NECHA (Group F) inhibited the formation of oedema in 49.5, 10.35, and 77.99%, respectively (Fig. 8). Borges et al. (2017) demonstrated the anti-inflammatory action of NECHA in rats at the dose of 498 μg/kg orally and demonstrated the involvement of the camphor compound in this action. In the in vitro assays, this nanoemulsion was highlighted concerning activity. It should be emphasised that in the composition of this essential oil were found alphapinene, 1,8-cineole, and camphor compounds that are strictly related to the anti-inflammatory and anti-allergic actions of essential oils (Borges et al. 2017). These results in zebrafish confirm the hypothesis described by Huang et al. (2014) that compounds with anti-inflammatory properties can modulate the responses induced by carrageenan in adult zebrafish.

According to Petrillo (2012), dexamethasone can be up to thirty times more potent than cortisol, its natural analogue, whose mechanism of action occurs from its accumulation in the cellular cytoplasm. Dexamethasone was used as a positive control for the inhibition of the inflammatory process in zebrafish since it inhibits the recruitment of macrophages and neutrophils to the site of inflammation and decreases fish mortality (Yang et al. 2014). In this study NECHA was more effective when compared to diclofenac and dexamethasone. This fact demonstrates that the nanoencapsulation of the essential oil, OECHA, whose composition directs it to the anti-inflammatory activity, was potentiated because at the dose of 498 μg/kg per oral administration, was highly effective (Fig. 8).

Carvalho et al. (2017) have shown that because zebrafish is a small animal, injection of an inflammatory agent in the abdominal region, such as carrageenan, can provoke reactions in other vital organs such as gills, liver, intestine, and kidneys. This fact was detected in groups B and E (negative controls) that presented histopathological changes in all these organs (Table 3). Gills are structures that allow fish to efficiently extract oxygen from water for use in metabolic reactions, regulating the acid–base balance and allowing the excretion of toxic waste (Holden et al. 2012; Houlihan et al. 1982).

According to Mazon et al. (2002), epithelial cell distension promotes haemorrhages and aneurysms in the gill tissue, as observed in groups B, D, and E. Other histological alterations observed, such as lamellar epithelial cells (present in C and E) and epithelial cells identified in B, D, and E), indicate the attempt to adapt the tissue to the new pathophysiological conditions (Carvalho et al. 2017). One of the ways of tissue adaptation

is to reduce the passage of water and blood into the secondary lamellae (Souza et al. 2016).

In this zebrafish study, diclofenac treatment was more effective than dexamethasone in preventing histopathological changes caused by carrageenan in the gills, liver, intestine, and kidneys. This fact can be explained by the time it was evaluated, 5 h after the application of carrageenan, whereas in the study of Huang et al. (2014), with methylprednisolone intraperitoneally, it was 24 h after application.

The liver is the vital organ for the detoxification process of substances, and any dysfunction in its tissue can be harmful to the animal and even cause death (Carvalho et al. 2017). Hepatocytes are the most common cell type in zebrafish. Its cytoplasm contains abundant glycogen and stores lipids and iron, produces proteins and amino acids, and aids in the detoxification of several compounds (Holden et al. 2012). The groups of diclofenac, dexamethasone, and NECHA presented livers with normal functionality (Table 3).

The changes observed in this study are common in cases of inflammation. Changes in tissue may lead to hepatic dysfunction (Carvalho et al. 2017). None of the zebrafish groups presented serious alterations, such as biliary stagnation, excretion of bile pigments, and metabolic insufficiency. The hepatocyte vacuolization observed in groups B and E (Negative controls), which received carrageenan with saline via gavage and carrageenan with NECONT via gavage, respectively, may be related to the reduction of glycogen accumulation in the cells (Rodrigues 1994). When intense, it can change the functioning of the liver. According to Carvalho et al. (2017), the fact that zebrafish are very agile and have accelerated metabolism may justify the presence of vacuolization in the hepatocytes, indicating dysfunction in their metabolism. Groups B and E also presented hyperemia. This condition can be considered a defence mechanism, which aims to increase the number of blood cells in the tissue and, consequently, increases oxygenation and the arrival of nutrients (Takashima and Hibiya 1984).

The adult zebrafish's kidney contains the nephrons responsible for the filtration of blood residues and the uptake of salt and water. It presents regions with lymphoid, hematopoietic, steroidogenic, and endocrine cells. Therefore, the main role of the kidneys in Teleostei freshwater fish is to eliminate the large volume of water entering the fish through the mouth, not having to store it (Holden et al. 2012). All treatments maintained functionally normal organs; however, the NECHA-treated group presented a response equal to that of the animals treated with diclofenac (38.4%), suggesting that the

nanoemulsion may have a similar action as that of the nonsteroidal anti-inflammatory for the inhibition of the effects of carrageenan-induced inflammation in the kidneys (Table 3).

Zebrafish do not have stomachs, so the intestinal bulb, which precedes the oesophagus has an absorption function and acts as a food reservoir. The mucosal layer is formed by goblet cells, dispersed inflammatory cells, and by enterocytes, which probably absorb lipids. Through these cells, the intestinal epithelium performs the absorption of nutrients and acts on the immune response (Holden et al. 2012). The results show that anti-inflammatories were ineffective in reducing the histopathological changes observed in intestinal tissue; however, animals treated with NECHA showed moderate to severe changes.

All groups studied had many histopathological changes in the intestine. They may be related to the invasive technique of intraperitoneal injection, which can damage the intestine. Invasive procedures in fish can generate inflammation in the intestinal lamina propria and cause leukocyte infiltration in the epithelial tissue of the intestine (Carvalho et al. 2017). In addition to causing increased defence cells (Roberts and Ellis 2012). Exposure of zebrafish to toxic substances can cause damage to the intestinal mucosa, impairing organ physiology (Carvalho et al. 2017). Epithelial cell hypertrophy, observed in groups B and E (negative controls), can be considered a defence mechanism, serving as a barrier to reduce the entry of these substances into the intestinal epithelium (Takashima and Hibiya 1984). This process can lead to necrosis, change observed in all groups, and to vacuolation, present in groups C, E, and F. These changes may compromise the body's ability to absorb nutrients (Carvalho et al. 2017).

In a study carried out by Juerges et al. (2003), 1,8-cineol was effective in reducing airway inflammation caused by acute asthma in humans. The anti-inflammatory property of 1,8-cineol has also been demonstrated by Santos and Rao (2000), through the significant reduction of carrageenaninduced rat paw inflammation oedema, a decrease in cotton fibre-induced granuloma formation, and the reduction of Evan Blue dye induced by intraperitoneal acetic acid. Juerges (2014) demonstrated that 1,8-cineol was able to dose-dependently reduce arachidonic acid metabolism, inhibiting the formation of Leukotriene-B_4 (LTB_4), Prostaglandin-E_2 (PGE_2), and Interleukin-1β (IL-1β) and, consequently, the production of Tumor Necrosis Factor α (TNFα), interrupting the continuity of inflammatory activity.

By comparing the in vitro results with the zebrafish results with the NECHA nanoemulsion, it is possible to state that, in the form of the OECHA essential oil, it has

potentiated the involvement of the terpenoid compounds on the inflammatory pathways involved in the carrageenantriggered pathophysiology of this animal species.

Conclusion

This study showed that all nanoemulsions (NECHA, NECULT, and NECOM) showed no toxicity to macrophages, cells with a relevant role in the inflammatory response, besides demonstrating antioxidant activity and potentiation of the essential oil effect in the proliferation of viable fibroblasts, which are connective tissue cells acting in the healing processes. These results demonstrate that the essential oil, in the form of nanoemulsions, increased the bioavailability of the active principles since they require a lower concentration of the essential oil to present antioxidant effect, similar to that of the unencapsulated oil. Nanoemulsions have also demonstrated the ability to potentiate the anti-inflammatory action of essential oils by exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide. The results obtained with NECHA in zebrafish confirm the hypothesis that prominent terpenic compounds, alpha-pinene, 1,8-cineole, and camphor, became more available at the target sites, inhibiting the inflammatory process in this animal species.

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Table 1. Chemical constituents of *Rosmarinus officinalis* L. essential oil (EORO) determined by GC-MS analysis (IRexp: IR calculated, ** IRlit: IR table for compound, * not identified)

Peak	RT (min)	Compound	(%) GC-MS OECOM	(%) GC-MS OECULT	(%) GC-MS OECHA	IR exp.	IR lit.*
1.	4.872	α-thujene	0.11	-	-	928	926
2.	5.054	α-pinene	8.13	14.24	10.12	935	939
3.	5.546	β-thujene	-	0.35	-	955	971
4.	5.424	Camphene	1.68	4.48	3.26	950	954
5.	6.045	Sabine	0.21	-	-	955	976
6.	6.152	β-pinene	0.58	1.27	1.10	979	979
7.	6.347	3- octanone	-	0.24	-	987	983
8.	6.482	β-myrcene	0.90	10.20	0.65	993	990
9.	6.911	α-phellandrene	0.77	0.37	0.13	1007	1002
10	7.282	α-terpinene	0.45	0.87	0.33	1018	1017
11	7.532	o-cymene	1.65	1.67	1.92	1026	1026
12	7.674	Limonene	21.99	4.49	2.14	1030	1031
13	7.773	1,8-cineole	33.70	16.84	50.82	1033	1033
14	8.666	γ -terpinene	0.39	1.18	0.16	1059	1059
15	9.724	Terpinolene	0.20	0.93	0.22	1091	1088
16	10.128	β-linalool	0.16	1.67	1.01	1102	1098
17	10.350	***	0.44	0.36	-	1108	***
18	10.662		-	-	0.10	1216	
19	11.897	Camphor	27.68	30.93	19.16	1147	1146
20	12.620	Isopinocamphone	-	0.20	-	1165	1160
21	12.736	Borneol	0.32	1.61	4.32	1168	1169
22	13.194	Isopinocamphone	-	1.06	0.83	1179	1173
23	13.739	α-terpineol	0.12	2.23	2.98	1193	1188
24	13.899	α-campholenal	0.20	-	-	1197	1125
25	14.005	Myrtenol	-	0.19	-	1199	1194
26	14.532	Verbenone	0.18	3.71	-	1213	1205
27	17.723	Bornyl acetate	-	0.38	0.26	1288	1288
28	23.220	β-caryophyllene	0.12	0.54	0.29	1421	1427
29	29.850		-	-	0.19	1960	

(IRexp: IR calculated, ** IRlit: IR table for compound, * not identified)

Table 2: Behavioral alterations after treatment with **A** control PBS, **B** application of carrageenan via intraperitoneal and saline via gavage, **C** diclofenac (0.5 mg/ kg) with the application of carrageenan intraperitoneally, **D** Dexamethasone (0.5 mg/ kg) with the application of carrageenan intraperitoneally, **E** NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and **F** NECHA (OECHA essential oil nanoemulsion, 498 µg/kg) on *D. rerio* at different observation time.

Behavioural parameters	Zebrafish with behavioral parameter changes per group (%)					
1 hour	A	B	C	D	E	F
Stage I		20%	40%	20%	20%	
Stage II						
Stage III		20%	20%		20%	
2 hour	A	B	C	D	E	F
Stage I						
Stage II						
Stage III			20%	40%		
3 hour	A	B	C	D	E	F
Stage I	20%	20%				
Stage II				20%	20%	20%
Stage III		20%	60%		20%	
4 hour	A	B	C	D	E	F
Stage I						
Stage II				40%		40%
Stage III	20%	20%	60%	20%	20%	
5 hour	A	B	C	D	E	F
Stage I						
Stage II	20%			40%		
Stage III	20%		60%	20%	20%	20%

Stage I: (1) increase swimming activity, (2) tail tremors; Stage II: (1) Circular swimming movement, (2) loss of posture; Stage III: (1) Loss of motility; (2) Animal deposition in the base of the beaker, (3) Death

Table 3: Percentage of alteration presented by each tissue after treatment with A control PBS, B application of carrageenan via intraperitoneal and saline via gavage, C diclofenac (0.5 mg/kg) with the application of carrageenan intraperitoneally, D dexamethasone (0.5 mg/ kg) with the application of carrageenan intraperitoneally, E NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and F NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg) on *D. rerio*

Group/tissue	Stage I	Stage II	Stage III	Total	%
Gills					
A	0/5	0/1	0/1	0/7	0
B	3/5	1/1	1/1	5/7	71,4
C	3/5	0/1	0/1	3/7	42,8
D	2/5	1/1	1/1	4/7	57,1
E	3/5	1/1	1/1	5/7	71,4
F	3/5	1/1	0/1	4/7	57,1
Liver					
A	2/7	0/6	0/0	2/13	15,4
B	2/7	5/6	0/0	7/13	53,8
C	2/7	2/6	0/0	4/13	30,7
D	4/7	1/6	0/0	5/13	38,4
E	1/7	5/6	0/0	6/13	46,1
F	2/7	2/6	0/0	4/13	30,7
Kidney					
A	0/5	0/7	0/1	0/13	0
B	2/5	4/7	1/1	7/13	53,8
C	2/5	3/7	0/1	5/13	38,4
D	2/5	1/7	0/1	3/13	23,0
E	3/5	3/7	1/1	7/13	53,8
F	3/5	2/7	0/1	5/13	38,4
Intestine					
A	2/8	0/6	1/1	2/15	20
B	5/8	4/6	1/1	10/15	66,6
C	4/8	2/6	1/1	7/15	46,6
D	5/8	2/6	1/1	8/15	53,3
E	3/8	5/6	1/1	9/15	60
F	6/8	1/6	1/1	8/15	53,3

It is shown in every tissue the percentage of alteration observed on to alteration total in each stage. The percentage were determined about fish number per group (n = 5). According to Poleksic and Mitrovic-Tutundzic (1994), Rigolin-Sa´ (1998) and Takashima and Hibiya (1995)

Table 4: Histopathological Change Index (HCI) presented by groups of animals after treatment with **A** control PBS, **B** application of carrageenan via intraperitoneal and saline via gavage, **C** diclofenac (0.5 mg/kg) with the application of carrageenan intraperitoneally, **D** dexamethasone (0.5 mg/kg) with the application of carrageenan intraperitoneally, **E** NECONT (control nanoemulsion) with application of carrageenan intraperitoneally and **F** NECHA (OECHA essential oil nanoemulsion at a dose of 498 µg/kg) on *D. rerio.*

Organs	A	B	C	D	E	F
Gills	0.2 ± 0.03	22.6±0.33	0.6±0.00*	22.4±0.20	22.4±0.43	2.6±0.07*a
Liver	0.4±0.03	10.4±0.40	4.4±0.06*	3.0±0.09*	10.2±0.32	4.0±0.16*a
Kidney	0.0±0.00	28.4±0.46	6.4±0.07*	2.4±0.16*	26.6±0.25	4.6±0.05*a
Intestine	20.4±0.45	29.0±0.51	24.8±0.32*	25.0±0.23*	30.6±0.72	23.2±0.36*a

Values are expressed as the mean ± SEM (n = 5/group). Kruskal–Wallis one-way Analysis of Variance (ANOVA) $p < 0.001$. *$p < 0.05$ versus B group and $p < 0.05$ versus E group (Student–Newman–Keuls test)

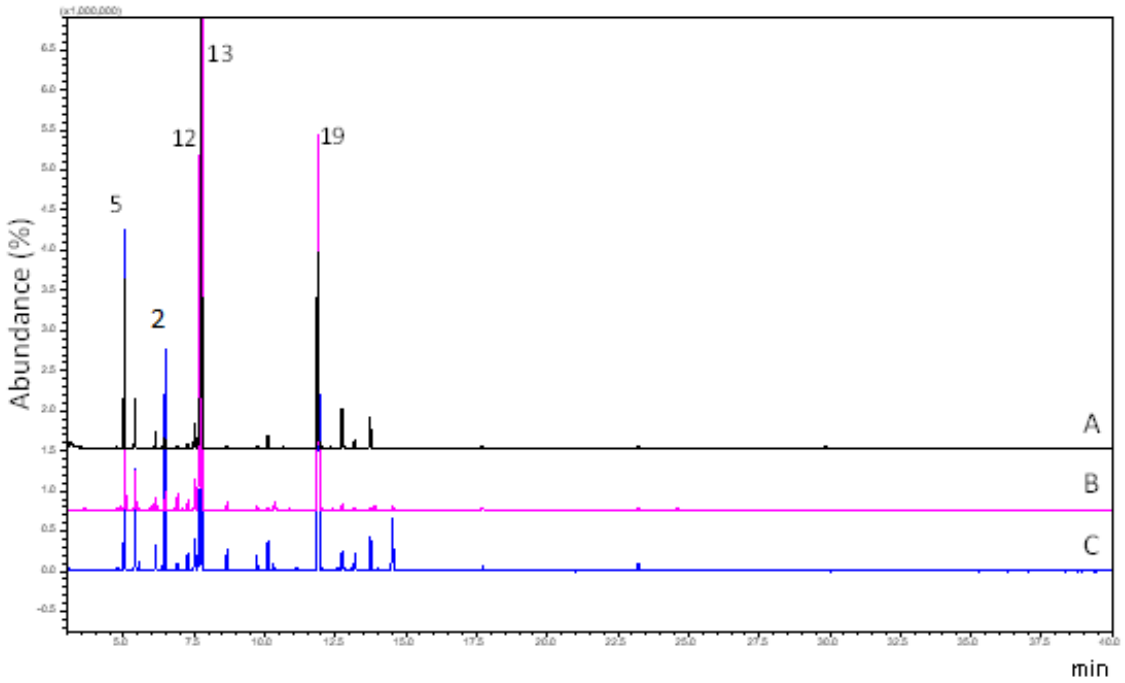

Fig 1. Chromatogram obtained by analysis of essential oils by coupled gas chromatography-mass spectrometry (GC-MS) where: A = OECOM corresponding to **12** – Limonene (21,99%), **13** – 1,8-cienole (33,70%), **19** – Camphor (27,58%); B = OECULT: corresponding to **2** – α-pinene (14,24%), **13** – 1,8-cienole (16,84%), **19** – Camphor (30,93%) and C = OECHA: corresponding to **2** – α-pinene (10,12%), **13** – 1,8-cienole (50,82%), **19** – Camphor (19,16%).

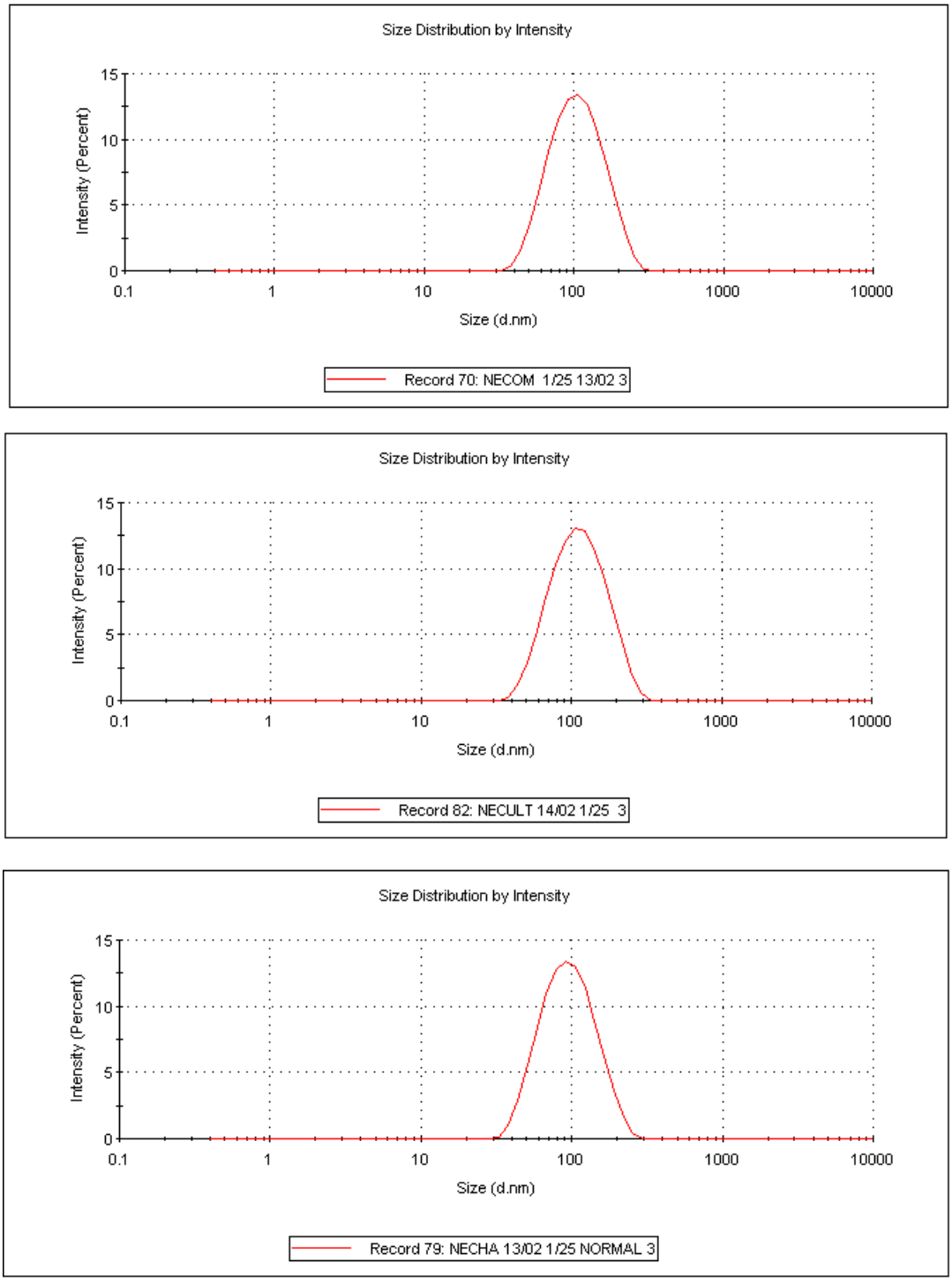

Fig 2. Particle size distribution of NECOM (mean droplet – 89.87±0,083727 nm; polydispersity 0,193 ± 0,008 nm), NECULT (mean droplet – 98.01±0,302900 nm; polydispersity 0,182 ± 0,001 nm) and NECHA (mean droplet – 77.32±1,192000 nm; polydispersity 0,239 ± 0,006 nm).

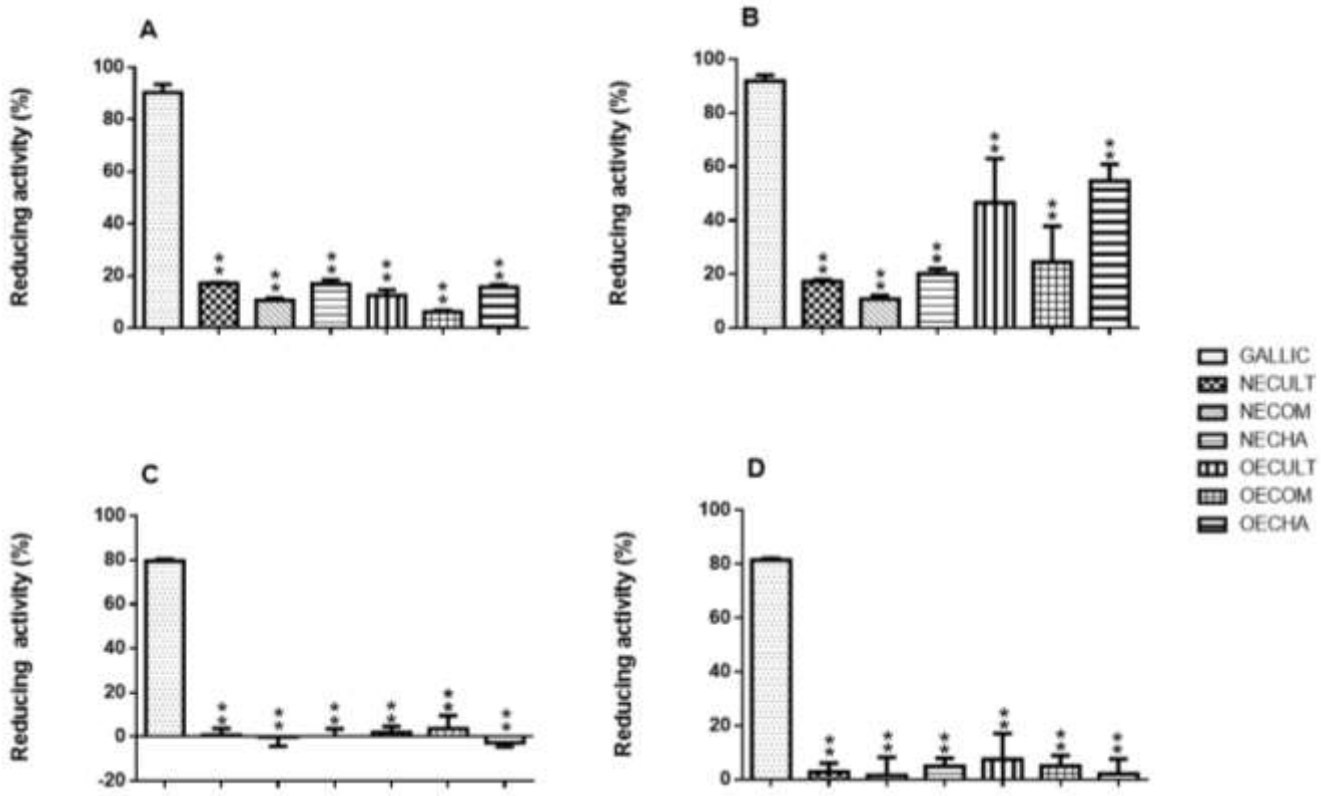

Fig 3. Reducing activity (%) of NECULT, NECOM, NECHA, OECULT, OECOM and OECHA in ABTS and DPPH assay. **A**: Reducing activity at 100 µg/mL in ABTS; Mean ± S.D, triplicate. **B**: Reducing activity at 1000 µg/mL in ABTS; Mean ± S.D, triplicate. **C**: Reducing activity at 100 µg/mL in DPPH; Mean ± S.D, triplicate. **D**: Reducing activity at 1000 µg/mL in DPPH; Mean ± S.D, triplicate. Significance was determined using ANOVA Test (∗ $p < 0.05$; ∗∗$p < 0.01$ compared to gallic acid).

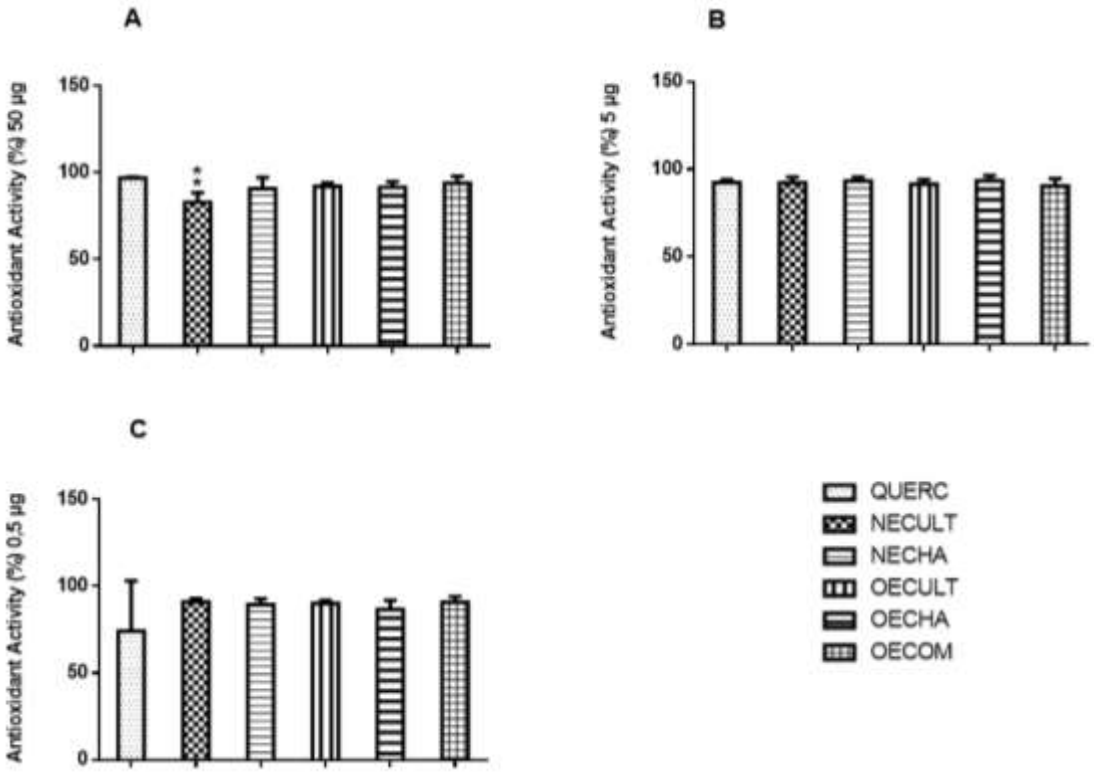

Fig 4. Antioxidant activity (%) of NECULT, NECHA, OECULT, OECHA and OECOM in Cellular Antioxidant Activity (CAA) Assay. **A**: Antioxidant activity (%) at 50 µg/mL in CAA; Mean ± S.D, triplicate. **B**: Antioxidant activity (%) at 5µg/mL in CAA; Mean ± S.D, triplicate. **C**: Antioxidant activity (%) at 0.5µg/mL in CAA; Mean ± S.D, triplicate. Significance was determined using ANOVA Test ($* p < 0.05$; $** p < 0.01$ compared to Quercetin).

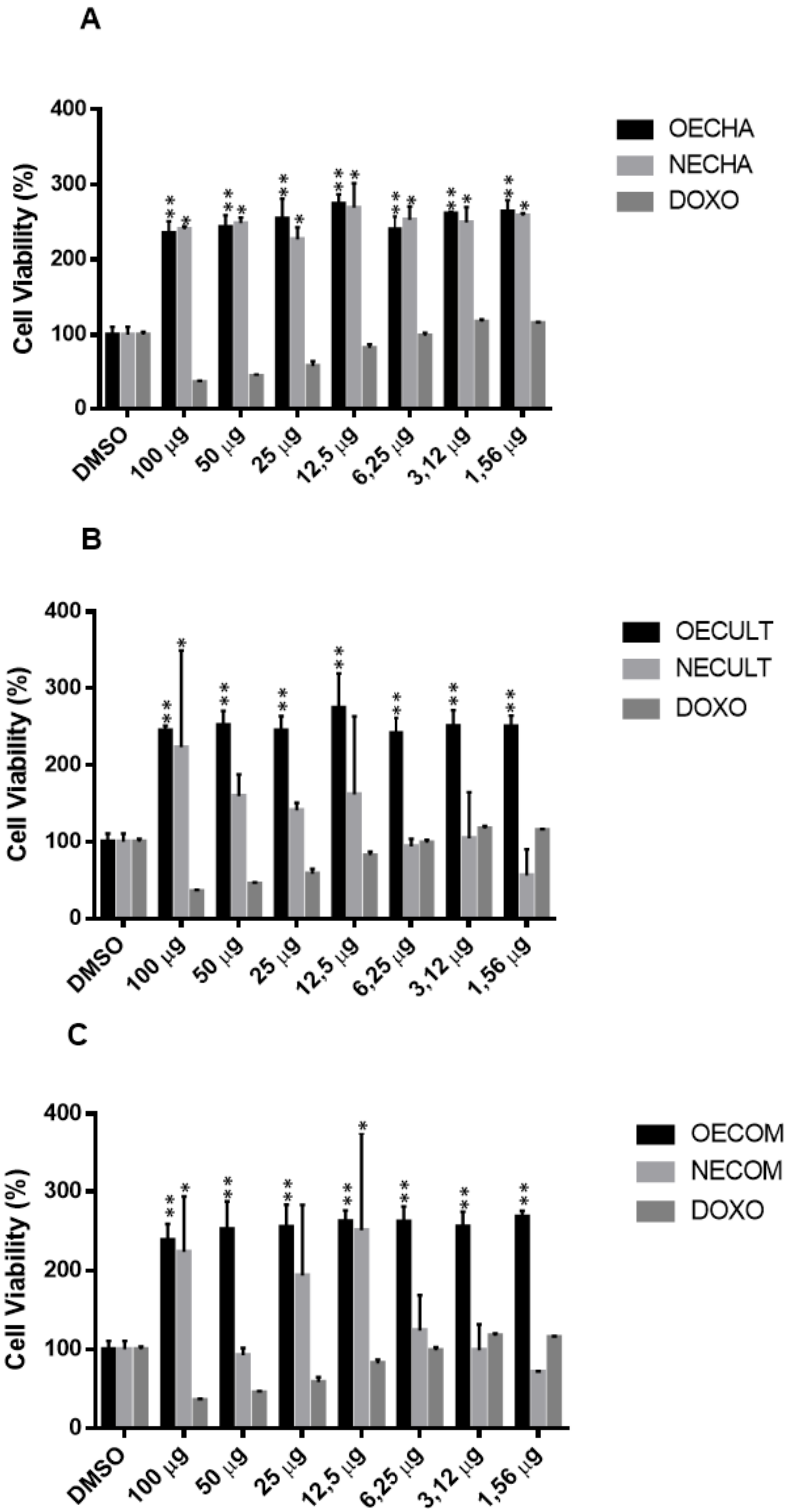

Fig 5. Viability (%) of J774 cells with OECHA and NECHA (A), OECULT and NECULT (B), OECOM and NECOM (C) at 1.56 – 100 μg/mL compared to doxorubicin curve and DMSO diluent. The values are the mean ± SD from three independent experiments. Significance was determined using ANOVA Test ($*$ $p < 0.05$; $**p < 0.01$ compared to DMSO).

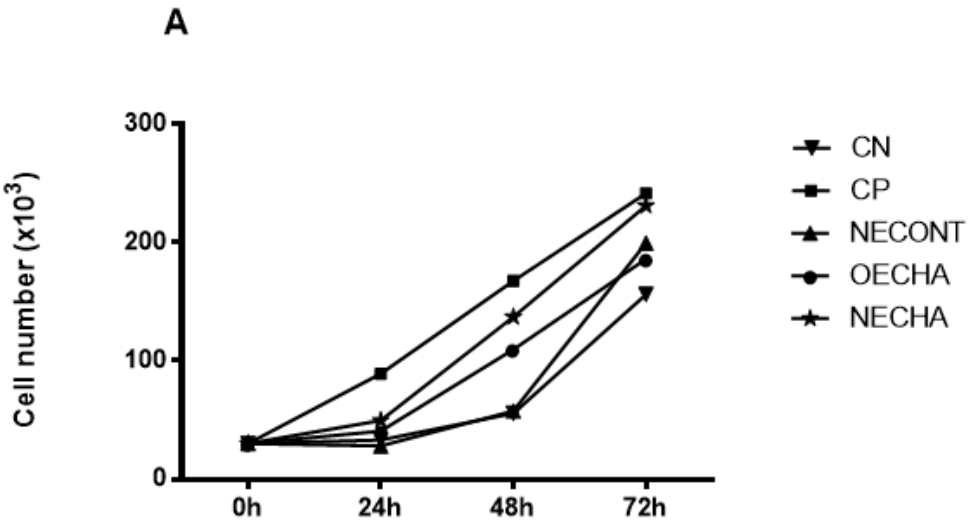

Fig 6. Effect of OECHA, NECHA and NECONTROL on viability of fibroblasts in the trypan blue test at 12.5 μg/mL. CN: cells without any treatment. CP: ascorbic acid. Data are the mean ± standard error (SE) of three independent experiments.

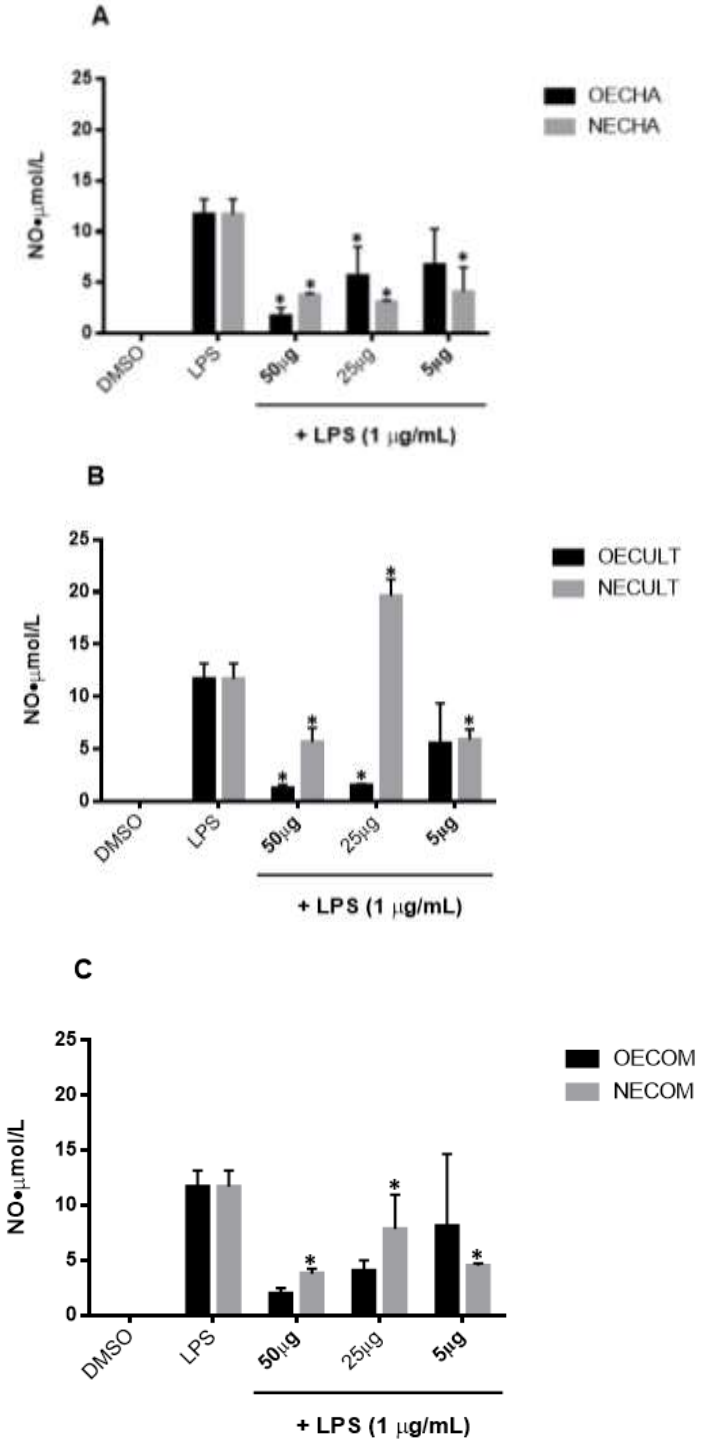

Fig 7. Effect of the OECHA and NECHA (A), OECOM and NECOM (B), OECULT and NECULT (C) on NO• production in LPS-stimulated J774 cells. Production of NO· was assayed in culture supernatants of macrophages stimulated with LPS (1 μg/mL) for 24 h in the presence of the compounds (5, 25, 50 μg/mL). At 0 the control whit the DMSO diluent. The essential oils presented the following IC_{50} values: OECHA = 10.05 µg/mL, OECULT = 4.45 µg/mL and OECOM = 11.81 µg/mL. The NO· values are the mean ± SD from three independent experiments. Significance was determined using ANOVA Test (∗ $p < 0.05$; ∗∗$p < 0.01$ compared to LPS).

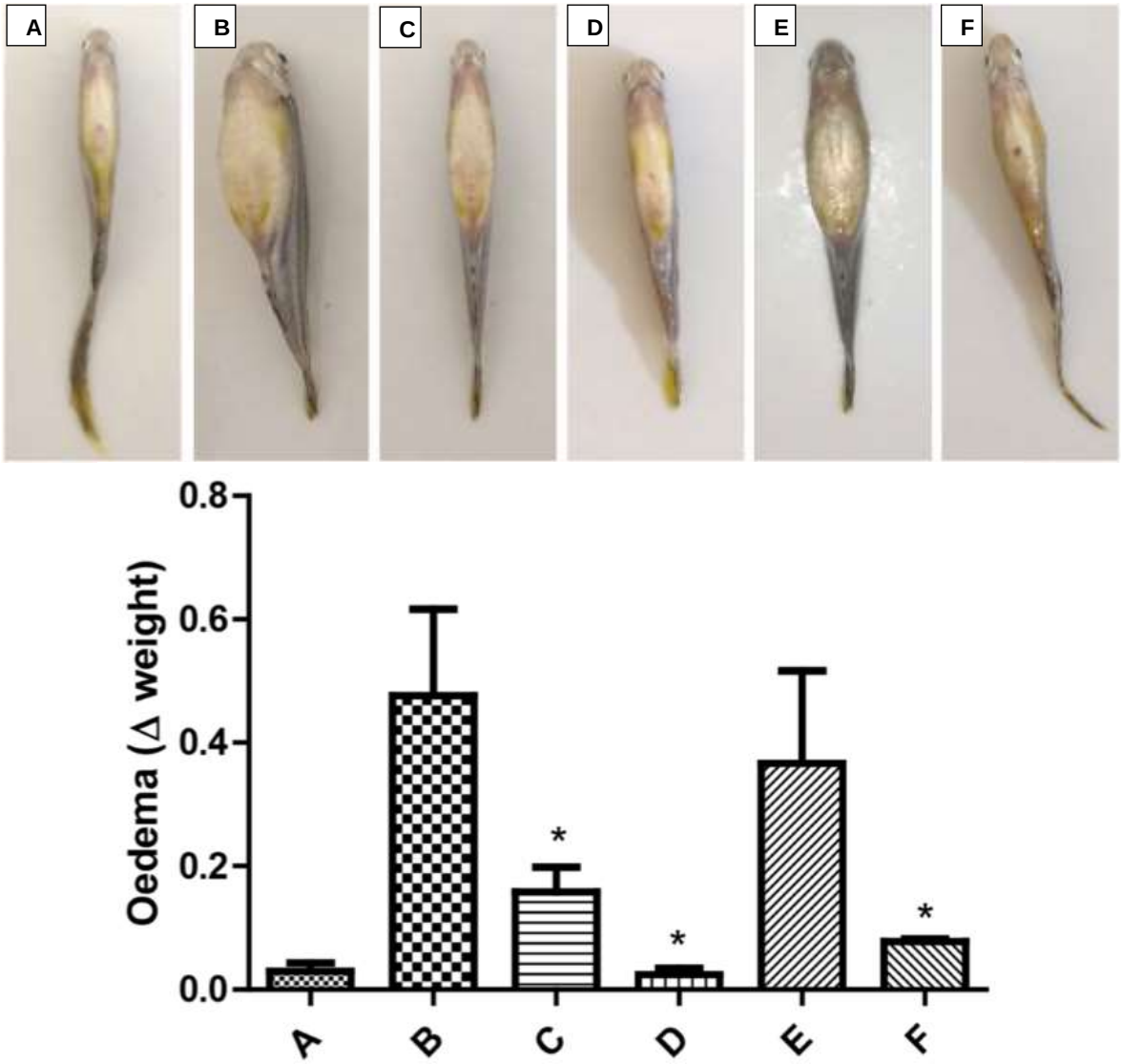

Fig 8. Effect of the oral treatment with **a** PBS, **b** saline, **c** Diclofenac (0.5 mg/kg), **d** Dexamethasone (0.5 mg/kg), **e** NECONT (control nanoemulsion) and **f** NECHA (OECHA essential oil nanoemulsion, 498 µg/kg) on oedema induced by carrageenan (300 µg, intraperitoneal) in *D. rerio*. *p < 0.05, ANOVA, Dunnett's Multiple Comparison Test.

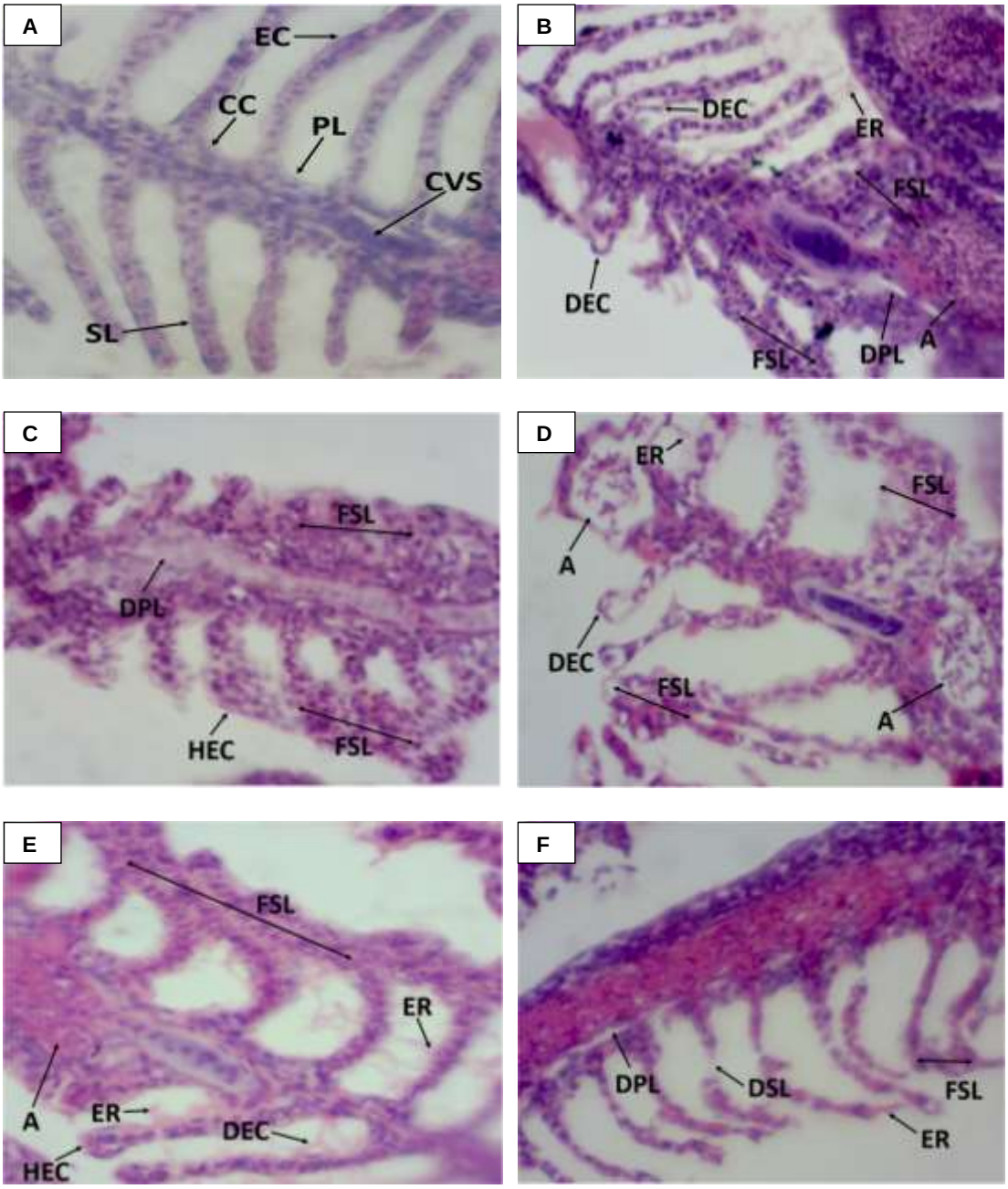

Fig 9 Longitudinal crosssection of Danio rerio gills, all figures were magnified by 9400, H & E. A: PBS intraperitoneal + saline by gavage showing EC epithelial cells, CC chloride cells, PL primary lamellae, CVS central venous sinusoids, SL secondary lamellae; B: carrageenan intraperitoneal + saline by gavage showing FSL fusion of secondary lamellae, DPL detachment of primary lamella, DEC displacement of epithelial cells, FSL fusion of secondary lamellae, ER epithelial rupture, A aneurism; C: carrageenan intraperitoneal + diclofenac by gavage showing DPL detachment of primary lamella, HEC hyperplasia of epithelial cells, FSL fusion of secondary lamellae; D: carrageenan intraperitoneal + dexamethasone by gavage showing epithelial rupture, A aneurism, DEC displacement of epithelial cells, FSL fusion of secondary lamellae; E: carrageenan intraperitoneal + NECONT by gavage showing DEC displacement of epithelial cells, HEC hyperplasia of epithelial cells, A aneurism, FSL fusion of secondary lamellae, ER epithelial rupture, DEC displacement of epithelial cells; F: carrageenan intraperitoneal + NECHA by gavage showing DPL detachment of primary lamella, DSL detachment of secondary lamella, ER epithelial rupture, FSL fusion of secondary lamellae.

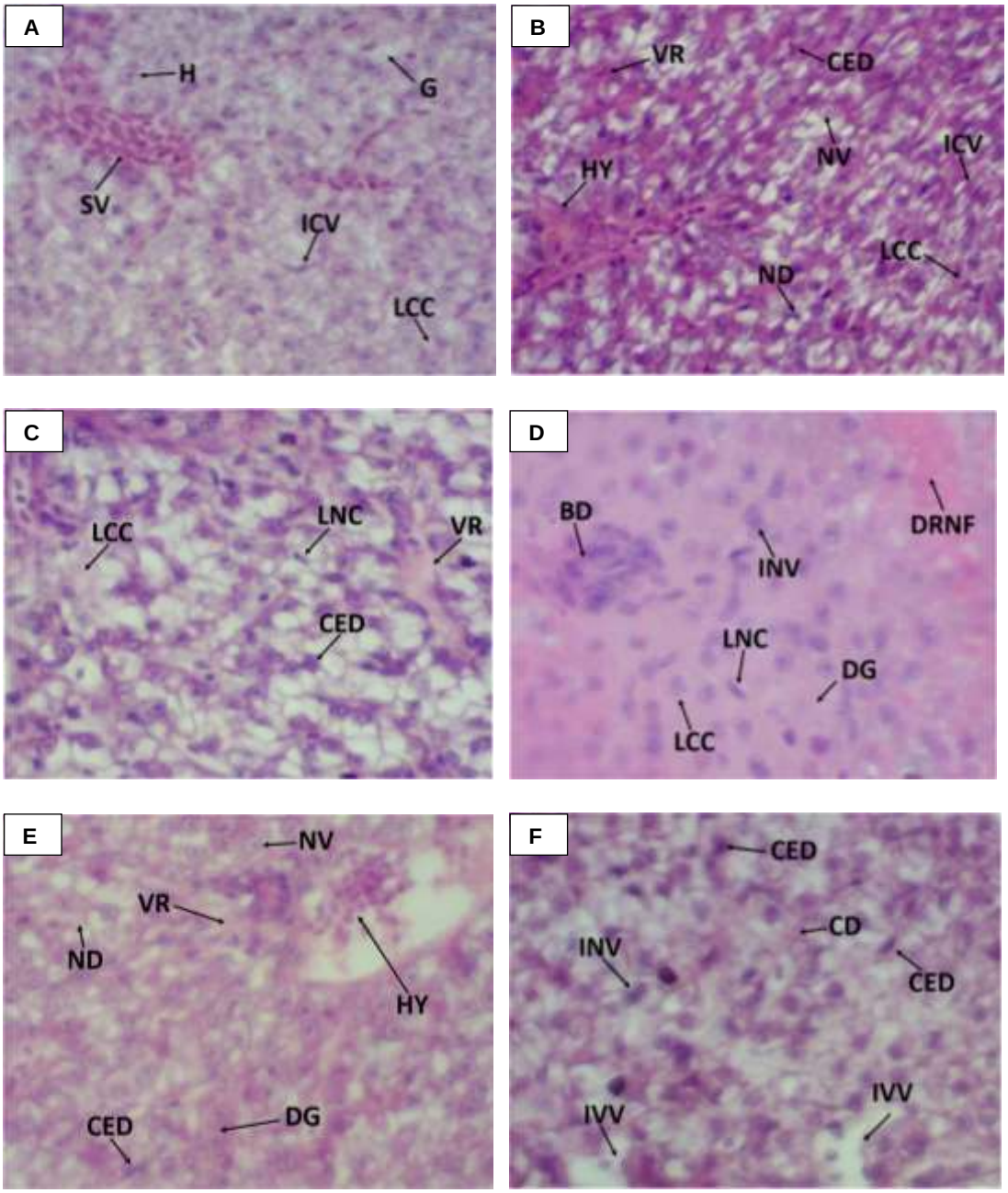

Fig 10. Longitudinal crosssection of Danio rerio liver, all figures were magnified by 9400, H & E. A: PBS intraperitoneal + saline by gavage showing H hepatocytes, SV sinusoids vessels, G glycogen, ICV increased cell volume, LCC loss of cell contour; B: carrageenan intraperitoneal + saline by gavage showing VR vessels rupture, HY hyperemia, CED cell disruption, NV vacuolization nuclear, ND nuclear degeneration, LCC loss of cell contour, ICV increased cell volume; C: carrageenan intraperitoneal + diclofenac by gavage showing LCC loss of cell contour, LNC loss of nucleus contour, CED cell disruption, VR vessels rupture; D: carrageenan intraperitoneal + dexamethasone by gavage showing BD bile ducts, LCC loss of cell contour, LNC loss of nucleus contour, INV increased nuclear volume, DG decreased glycogen, DRNF decreased relative frequency of nucleus occurrence; E: carrageenan intraperitoneal + NECONT by gavage showing DG decreased glycogen, CED cell disruption, ND nuclear degeneration, VR vessels rupture, NV vacuolization nuclear and HY hyperemia; F: carrageenan intraperitoneal + NECHA by gavage showing INV increased nuclear volume, IVV increased vessels volume, CED cell disruption, CD cytoplasmic degeneration.

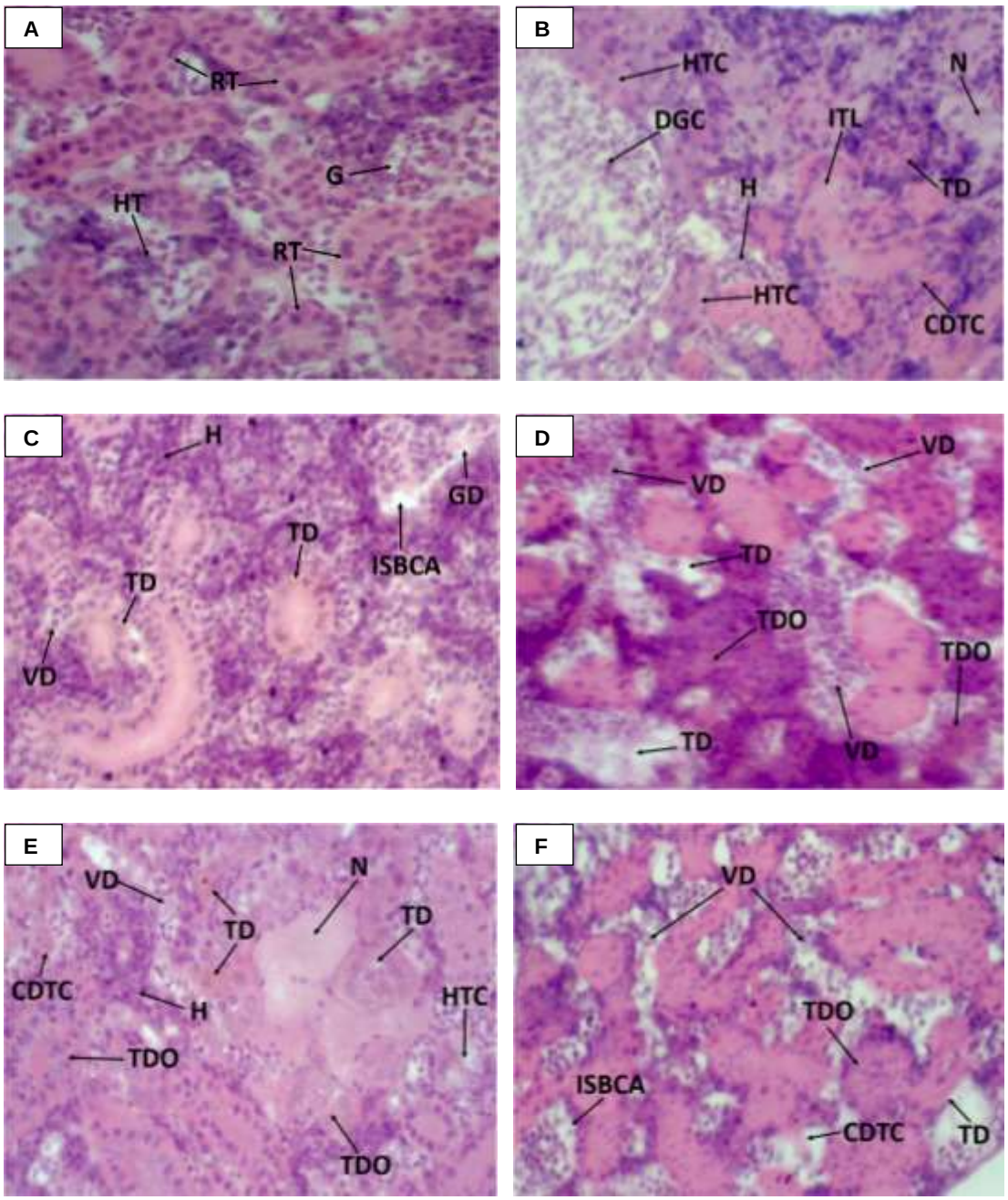

Fig 11. Longitudinal crosssection of Danio rerio kidney, all figures were magnified by 9400, H & E. A: PBS intraperitoneal + saline by gavage showing RT renal tubules , HT hematopoietic tissue , G glomerulus; B: carrageenan intraperitoneal + saline by gavage showing HTC hypertrophy of tubular cells, DGC dilation of Glomerular capillaries, ITL increase in tubular lumen, TD tubular degeneration, CDTC cytoplasmic degeneration of tubular cells, N necrosis, H hyperemia; C: carrageenan intraperitoneal + diclofenac by gavage showing VD vessels dilatation, H hyperemia, TD tubular degeneration, ISBCA increased space of Bowman's capsule, GD glomerular degeneration; D: carrageenan intraperitoneal + dexamethasone by gavage showing VD vessels dilatation, TD tubular degeneration, TDO tubular disorganization; E: carrageenan intraperitoneal + NECONT by gavage showing VD vessels dilatation, TD tubular degeneration, TDO tubular disorganization, HTC hypertrophy of tubular cells, CDTC cytoplasmic degeneration of tubular cells, H hyperemia, N necrosis; F: carrageenan intraperitoneal + NECHA by gavage showing VD vessels dilatation, TD tubular degeneration, TDO tubular disorganization, ISBCA increased space of Bowman's capsule, CDTC cytoplasmic degeneration of tubular cells.

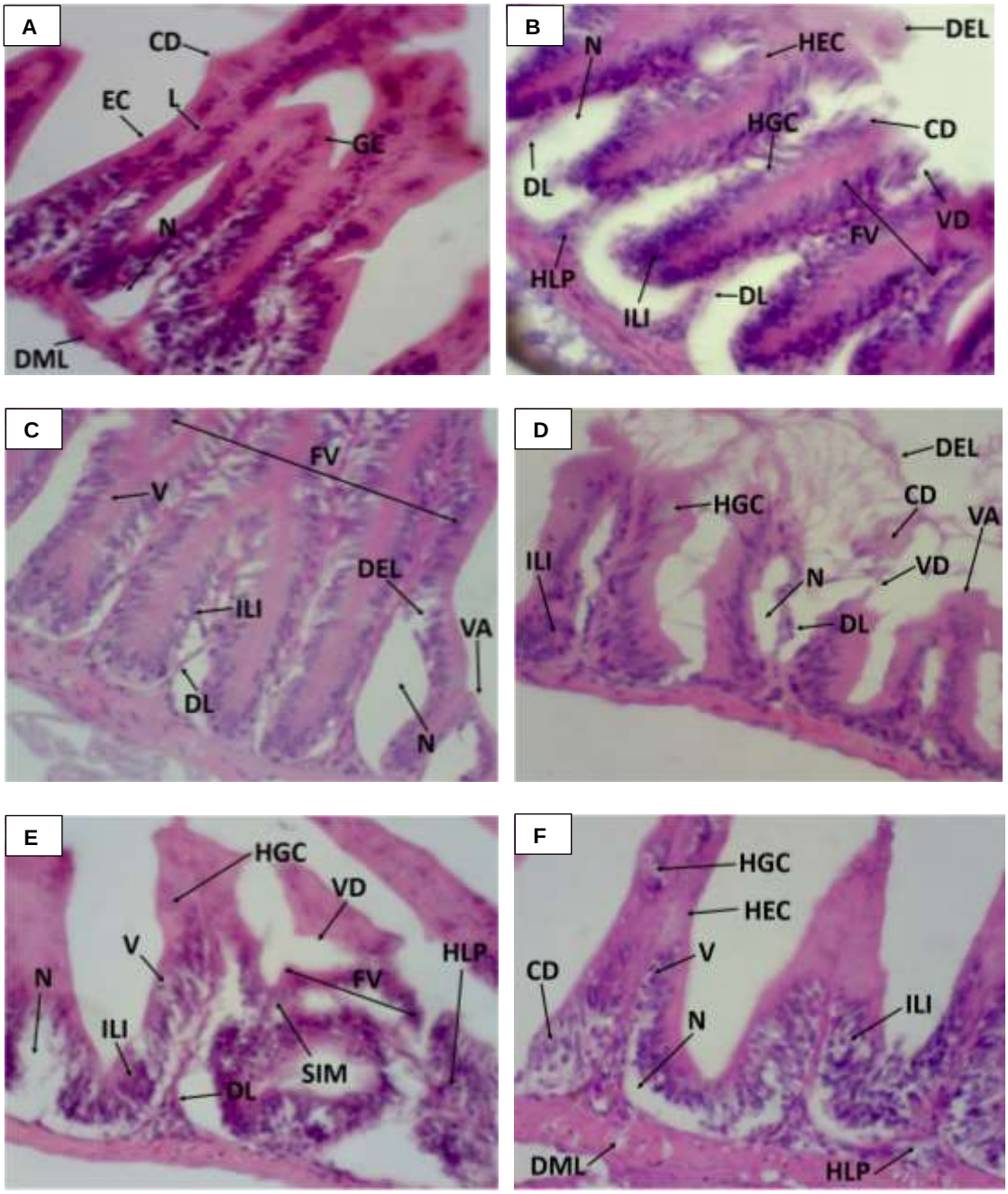

Fig 12. Longitudinal crosssection of Danio rerio intestine, all figures were magnified by 9400, H & E. A: PBS intraperitoneal + saline by gavage showing L lamina propria, DML degeneration of muscular layer, GC goblet cell, EC enterocyte cell, CD cell degeneration, N necrosis; B: carrageenan intraperitoneal + saline by gavage showing ILI leukocyte infiltration, DEL detachment of the epithelial lining of the apex of the intestinal villous, CD cell degeneration, DL detachment of the lamina propria, FV partial or complete fusion of villous, VD villous degeneration, HLP hemorrhage in the lamina propria, HEC hypertrophy of epithelial cells of the lamina propria, HGC hyperplasia of globet cells, N necrosis; C: carrageenan intraperitoneal + diclofenac by gavage showing V vacuolization of enterocytes, ILI leukocyte infiltration, DL detachmentof the lamina propria, FV partial or complete fusion of villous, DEL detachment of the epithelial lining of the apex of the intestinal villous, VA villous atrophy, N necrosis; D: carrageenan intraperitoneal + dexamethasone by gavage showing ILI leukocyte infiltration, HGC hyperplasia of globet cells, N necrosis, DL detachmentof the lamina propria, VD villous degeneration, DEL detachment of the epithelial lining of the apex of the intestinal villous, VA villous atrophy; E: carrageenan intraperitoneal + NECONT by gavage showing N necrosis, ILI leukocyte infiltration, V vacuolization of enterocytes, DL detachmentof the lamina propria, SIM

sloughing of the intestinal mucosa, HGC hyperplasia of globet cells, VD villous degeneration, FV partial or complete fusion of villous, HLP hemorrhage in the lamina propria; F: carrageenan intraperitoneal + NECHA by gavage showing CD cell degeneration, DML degeneration of muscular layer, N necrosis, V vacuolization of enterocytes, HGC hyperplasia of globet cells, HEC hypertrophy of epithelial cells of the lamina propria, ILI leukocyte infiltration, HLP hemorrhage in the lamina propria.

6 CAPÍTULO 4

TOXICIDADE DE NANOEMULSÕES CONTENDO ÓLEO ESSENCIAL DE *Rosmarinus officinalis L.* EM ZEBRAFISH

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Resumo

O óleo essencial de *Rosmarinus officinalis* L. (OERO) tem sido historicamente utilizado para fins medicinais em diversas regiões o mundo, o que impulsionou muitas pesquisas para a avaliação de suas atividades biológicas. Em geral, apresenta 1,8-cineol, cânfora e α-pineno entre os componentes químicos de destaque. Uma metodologia de encapsulação do OERO em nanoemulsão foi padronizada anteriormente. Esta nanoformulação tem demonstrado capacidade de aumentar a disponibilidade do óleo em sistemas biológicos. Entretanto, seus possíveis efeitos tóxicos ainda não foram reportados. Neste contexto, o presente estudo objetivou avaliar a toxicidade de nanoemulsões obtidas com o OERO de diferentes procedências (NECHA, NECULT e NECOM) em zebrafish (*Danio rerio*). A DL_{50} foi obtida, sendo avaliados os parâmetros comportamentais até 24h após a exposição e as alterações histopatológicas de brânquias, fígado, rins e intestino. A exposição de zebrafish a diferentes doses (1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg e 5 mg/kg) de NECHA, NECULT e NECOM permitiu a obtenção de valores dos DL_{50} de 3.6, 2.8 e 2.4 mg/kg, respectivamente, indicando menor toxicidade da NECHA. Nenhuma das nanoemulsões provocou 100% de morte nos animais, apresentando valores de taxa de mortalidade de até 90% nas doses mais elevadas. A histopatologia dos órgãos mais alterados, rins e intestino, demonstraram menor frequência de alterações em animais tratados com a NECHA. Estudos anteriores relatam que essa nanoemulsão mantém 75% do óleo encapsulado e melhora suas atividades biológicas. Isso pode ser devido a elevada quantidade de 1,8-cineol em sua composição, que pode atuar como estabilizador de nanoformulações.

Introdução

Conhecida popularmente como alecrim (português) e rosemary (inglês), *Rosmarinus officinalis* L. é uma planta arbustiva da família Lamiaceae, nativa da região do Mediterrâneo, amplamente cultivada em diversas regiões do mundo (Ribeiro-Santos et al., 2015). Tem sido utilizada para fins medicinais na forma de infusão e óleo essencial.

Estudos realizados com o óleo essencial de *Rosmarinus officinalis* L. (OERO), descrevem as propriedades antioxidante, anti-inflamatória, anti-mutagênica, antimicrobiana, antidepressiva, entre outras (Selmi et al., 2017; Borges et al., 2018; Ghasemian et al., 2016; Ali et al., 2015; Machado et al., 2013; Fahim et al., 1999). Os componentes químicos de destaque no OERO, como 1,8-cineol, borneol, pineno, limoneno, canfeno, cânfora e mirceno, têm sido bastante relacionados às atividades biológicas descritas (Borges et al., 2017; Selmi et al., 2017; Bajalan et al., 2017; Vilela et al., 2016; Takayama et al., 2016; Chávez-González et al., 2016; Akbari et al., 2015; Machado et al., 2013; Faria et al., 2011; Juhás et al., 2009).

Devido à fraca capacidade de absorção dos compostos lipofílicos, vários sistemas de encapsulamento são atualmente desenvolvidos a fim de aumentar o índice de absorção dessas subtâncias (McClements and Rao, 2011). Nanoemulsões são sistemas que podem ser obtidos a partir da encapsulação de substâncias lipolíficas em gotículas de

escala nanomérica (d < 200), ampliando sua capacidade de solubilização (Ostertag et al., 2012). O OERO pode ser encapsulado em nanomulsão a partir de um método de baixa energia anteriormente descrito (Duarte et al., 2015). Em sistemas biológicos, essa nanoemulsão aumentou a absorção do óleo essencial, desencadeando efeitos antiinflamatório e antiálgico relacionados ao OERO (Borges et. al, 2017; Borges et. al, 2018).

Em ensaios utilizando animais de experimentação, os efeitos de biodistribuição e bioatividade de sistemas nanoencapsulados são pouco conhecidos (Asharani et al., 2008), tendo crescido nos últimos anos. Por apresentarem diferença de tamanho e área superficial, ao serem aplicadas em organismos vivos, as substâncias em escala nano podem provocar propriedades diferentes daquelas exibidas pelo mesmo material em macroescala. É evidente que a variação da estrutura e composição das nanopartículas pode causar toxicidade diferencial (Martinez et al., 2017).

O efeito de nanopartículas em organismos aquáticos depende de diversos fatores, tais como a solubilidade, a composição química, a estrutura da superficie, entre outros (Brundo et al., 2016). O zebrafish (*Danio rerio*) tem sido apontado como um modelo animal adequado para ensaios com sistemas nanoencapsulados (Chakraborty et al., 2016; Martinez et. al, 2017). Alguns estudos o têm utilizado para a avaliação dos efeitos e da toxicidade de nanoformulações (Mukherjee et al., 2016; Vibe et al., 2016; Calienni et al., 2017; Borges et al., 2018). Entretanto, as informações disponíveis ainda são limitadas.

Embora grande parte dos estudos toxicológicos utilizem o zebrafish na fase embrionária, os peixes na fase adulta são igualmente aptos (Qin et al., 2014; Chakraborty et al., 2016). Muitas são as abordagens utilizadas nos ensaios de toxicidade em zebrafish, como a avaliação da taxa de sobrevivência, a indução de teratogênese, e a observação de alterações histológicas e comportamentais (Zhang et al., 2013; Ramsden et al., 2013; Fang et al., 2014; Dai et al., 2014). Alterações no comportamento normal, como aumento da atividade de natação, perda de equilíbrio, natação circular e descanso no fundo são importantes indicadores de estresse, que pode ser causado por substâncias tóxicas ao animal (Souza et al., 2016).

Ao avaliar a toxicidade induzida por indometacina, diclofenaco e metotrexato em intestino de ratos e de zebrafish, foram detectadas alterações histológicas equivalentes entre os modelos animais, o que indica que o zebrafish pode ser um modelo animal confiável para a análise histopatológica (Ryu et al., 2018).

Em estudos de alterações histológicas induzidas por toxicidade, diferentes órgãos de zebrafish podem ser analisados de acordo com o tipo de substância aplicada, como

brânquias, intestino, coração, fígado, bexiga natatória ou a combinação de mais de um órgão (Griffitt et al., 2013; Vliegenthart et al., 2014; Ryu et. al, 2018).

O zebrafish apresenta sistemas digestivo, cardiovascular e nervoso similares aos dos mamíferos e, elevado grau de genes homólogos com os seres humanos (Vliegenthart et al., 2014; Mueller and Wullimann, 2002; Hsu et al., 2008; Aillon et al., 2010). Baseado nessas descrições este estudo teve como objetivo avaliar a toxicidade de nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. em zebrafish dando ênfase aos parâmetros histopatológicos das brânquias, fígado, rins e intestine.

Material e Métodos

Material vegetal

De acordo com Borges et. al (2018), foram utilizadas duas amostras de folhas de *Rosmarinus officinalis* L. de diferentes procedências (Nativa Produtos Naturais pertencentes ao lote 209 e da Agência Goiana de Assistência Técnica, Extensão Rural e Pesquisa Agropecuária (EMATER) em Goiânia-GO-Brasil. Exsicata depositada no Herbário da Universidade Federal de Goiás, sob o nº 49581). As amostras foram submetidas ao processo de extração do óleo essencial, obtendo-se as amostras de óleo essencial OECHA e OECULT, respectivamente. Também foi utilizada uma amostra de óleo essencial obtida da empresa Florien (OECOM), pertencente ao lote 056757 (Borges et al., 2018).

Cromatografia Gasosa acoplada ao Espectrômetro de Massa (CG-MS)

As análises foram realizadas por Borges et. al (2018), utilizando um sistema Shimadzu / GC 2010 acoplado a um self-gun Shimadzu / AOC-5000 e detector de massa (Shimadzu MS2010 Plus) com um impacto de elétrons de 70 eV e equipado com uma coluna de sílica fundida de DB-5MS (Agilent Advanced J & W 30 m × 0,25 mm × 0,25 µm).

Obtenção das nanoemulsões

As nanoemulsões foram obtidas anteriormente por Borges et. al (2018), através de método de baixa energia, anteriormente descrito por Duarte et al. (2015). O método consiste na mistura de 2,5 g de surfactante (Tween 20) adicionados a 2,5 g de OERO através de agitador magnético (800 rpm) durante 30 min. Em seguida, adiciona-se 45 mL de água destilada a 3.5 ml/min, agitando por 60 min.

Nanoemulsões com óleo essencial de *Rosmarinus officinalis* L. **(NOERO)**

Foram obtidas três nanoemulsões (NECHA, NECULT e NECOM) a partir das amostras de OERO. A NECHA foi obtida a partir do óleo essencial OECHA, a NECULT foi obtida com o óleo essencial OECULT, e para a NECOM foi utilizada amostra de óleo essencial comercializado pela empresa Florien (OECOM) (Borges et al., 2018).

Caracterização das nanoemulsões

As nanoemulsões foram caracterizadas em estudo anterior (Borges et. al, 2018), em triplicata e diluídas em água (1:25) através da espectroscopia de correlação de fótons (Zetasizer ZS, Malvern, UK), que indica o tamanho das gotículas e o índice de polidispersão das nanoemulsões. O tamanho médio das gotas foi expresso como o diâmetro médio ± DP. A eficiência de encapsulação para a nanoemulsão NECHA foi descrita por Borges et al., (2018).

Animais

Foram utilizados zebrafish, *Danio rerio*, da linhagem wild (225 animais), adquiridos da Aqua New Aquários e Peixes Ltda. ME (PE, Brasil), e mantidos na Plataforma de Zebrafish do Laboratorio de Pesquisa em Farmacos da Universidade Federal do Amapá – UNIFAP, Amapá, Brasil. Os animais foram mantidos em água sob condições controladas de temperatura, alimentação e ciclo claro/escuro, seguindo os padrões descritos por Souza et. al (2016) e Borges et. al (2018). Este estudo foi aprovado pelo Comitê de Ética em Experimentação Animal da Universidade Federal do Amapá (Brasil) sob o número de registro 0021/2015.

Determinação de DL_{50}

Para a obtenção da dose letal média (DL_{50}), os animais foram coletados aleatoriamente e privados de alimentação por 24h antes do início dos experimentos. Após esse período foram submetidos a uma administração por via oral (n = 5/grupo), sendo utilizadas as doses de: 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg e 5 mg/kg, que corresponderam aos volumes de 1 µL, 2 µL, 3 µL, 4 µL e 5 µL, respectivamente, para cada nanoemulsão. Cada dose foi administrada em triplicata, utilizando-se 15 animais para cada grupo. A mortalidade e o comportamento dos animais foram registrados a cada uma hora, tendo o último registro 24 horas depois da administração oral. Posteriormente foram avaliados os parâmetros comportamentais e histopalógicos.

Rota de administração e tratamento por grupo

As nanoemulsões (NECHA, NECULT e NECOM) e o controle (Tween 20 a 5% e água) foram administradas oralmente, por gavagem (Borges et. al, 2018). O procedimento de gavagem foi realizado com cinco doses diferentes de cada nanoemulsão: 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg e 5 mg/kg. No zebrafish, com base no peso médio dos animais e concentração do OERO nas nanoemulsoes, estas doses corresponderam às concentrações brutas de 1000 µg/kg, 2000 µg/kg, 3000 µg/kg, 4000 µg/kg e 5000 µg/kg, respectivamente.

Os animais foram divididos nos grupos: A (2 mg/kg de NECHA), B (2 mg/kg de NECULT), C (2 mg/kg de NECOM); D (3 mg/kg de NECHA), E (3 mg/kg de NECULT), grupo F (3 mg/kg de NECOM), grupo G (4 mg/kg de NECHA), grupo H (4 mg/kg de NECULT), grupo I (4 mg/kg de NECOM), grupo J (Controle – 5% de Tween 20 e 95% de água), grupo K (1 mg/kg de NECHA), grupo L (1 mg/kg de NECULT), grupo M (1 mg/kg de NECOM), grupo N (5 mg/kg de NECHA), grupo O (5 mg/kg de NECULT), grupo P (5 mg/kg de NECOM). Apenas os animais pertencentes aos grupos A a J foram selecionados para a discussão dos resultados, tendo em vista que os animais tratados com a dose mais baixa (1 mg/kg) e a mais elevada (5 mg/kg) não apresentaram histopatologia significativa.

Avaliação dos parâmetros comportamentais

Os parâmetros comportamentais foram avaliados conforme descrito por Souza et al. (2016), com adaptações. Os animais foram mantidos em jejum por 24h antes do experimento. Após o procedimento de gavagem, os mesmos foram distribuídos individualmente em béqueres contendo água do sistema. O comportamento dos animais foi observado a cada uma hora, nas primeiras cinco horas que seguiram o tratamento. Posteriormente, a última observação ocorreu 24 horas depois da gavagem. As alterações de comportamento do zebrafish podem ser classificadas em três estágios: I (maior atividade natatória e presença de tremores de cauda), II (natação circular e perda de equilíbrio), e III (perda de motilidade, repouso no fundo e morte) (Souza et. al, 2016).

Analise histopatólogica

Para a preparação e analise microscópica tecidual dos órgãos analisados foram empregadas as técnicas descritas por Souza et al. (2016), Carvalho et al., (2017) e Borges et. al (2018) para zebrafish, assim como as alterações teciduais.

O Índice de Alterações Histológicas (IHA) foi calculado a partir dos níveis de alterações teciduais observadas em brânquias, fígado, rins e intestino. As alterações podem ser classificadas em níveis I, II e III, e o valor de IHA indica se o órgão se encontra normal (0 a 10), moderadamente alterado (11 a 20), com alterações moderadas a graves (21 e 50) ou contendo alterações severas irreversíveis (>100) (Poleksic and MitrovicTutundzic, 1994; Ringolin-Sá, 1999; Takashima and Hibiya, 1994).

Análise Estatística

A dose letal média (DL_{50}) foi determinada através da análise de probitos utilizando o programa GraphPad Prism 6.0. Para a analise estatística dos resultados dos parâmetros experimentais do estudo histopatológico foi utilizado ANOVA, seguido do teste post hoc não paramétrico de Kruskal Wallis, sendo considerado significativos os resultados com valores de $p < 0,05$.

Resultados

Os grupos de zebrafish tratados na menor dose (1 mg/kg) não apresentaram alterações comportamentais e histopatológicas significativas, enquanto o grupo tratado com a dose mais elevada (5 mg/kg) apresentou o nível máximo de alterações teciduais. Portanto, os animais tratados nas doses de 2 mg/kg, 3 mg/kg e 4 mg/kg apresentaram histopatologia relevante, sendo selecionados para a avaliação de seus resultados. De maneira geral, nos animais que receberam a dose de 2 mg/kg (100 µg de OERO), observou-se até 40% de morte. Os que receberam dose de 3 mg/kg (150 µg de OERO) apresentaram mortalidade de até 60%. A dose de 4 mg/kg (200 µg de OERO) induziu 80% de morte nos animais. Não ocorreu morte nos animais tratados com o grupo controle das nanoemulsões, indicando que os efeitos letais estão relacionados ao OERO nanoencapsulado, e não ao surfactante (Tabela 1).

Neste estudo foi observado que nenhuma das doses induziu 100% de morte nos animais, sendo a mortalidade aumentada em concentrações maiores da nanoemulsão. A Figura 1 mostra a mortalidade (%) a partir da admistracao das nanoemulsoes (DL_{50}-24h). Dentre as três nanoemulsões testadas, a NECHA apresentou a DL_{50} de 3.6 mg/kg, indicando menor toxicidade do que as nanoemulsões NECULT e NECOM, que apresentaram 2.8 mg/kg e 2.4 mg/kg, respectivamente.

Na análise dos parâmetros comportamentais, dentre os animais que receberam as doses de 2 mg/kg de nanoemulsões, os animais do grupo C apresentaram natação circular (estágio II) durante a primeira hora após a gavagem. Os animais do grupo A

apresentaram alterações de nível I duas horas após a gavagem. Na terceira hora, ocorreu morte de animais pertencentes aos grupos A e C, totalizando 3 animais mortos (20%) em cada grupo (Tabela 2).

Nos animais que receberam nanoemulsões na dose de 3 mg/kg via gavagem, pertencente aos grupos E (3 mg/kg de NECULT) e F (3 mg/kg de NECOM), foram registrados tremores na cauda (estágio I) perda de equilíbrio (estágio II) e morte (estágio III) ainda na primeira hora após o procedimento. Os animais do grupo D (3 mg/kg de NECHA) não apresentaram alterações neste intervalo de tempo. Duas horas após a gavagem, foram observadas alterações de nível II em animais dos grupos D e F. Na terceira hora, foi registrada morte de 20% dos animais dos grupos D e E. Quatro horas após a gavagem, morreram 40% animais do grupo F. No final do período de observação, o grupo D apresentou 20% de morte, enquanto os grupos E e F totalizaram morte de 60% dos animais (Tabelas 2 e 3).

Uma hora após a gavagem, os grupos de animais que receberam a dose de 4 mg/kg - G (4 mg/kg de NECHA), H (4 mg/kg de NECULT) e I (4 mg/kg de NECOM) – apresentaram aumento da atividade natatória, perda de equilíbrio e morte, alterações de níveis I, II e III, respectivamente. Na segunda hora, ocorreu morte de mais de 20% dos animais dos grupos G e I. A morte de animais dos três grupos também foi registrada durante a segunda e a quarta hora de observação. Na quinta hora, apenas o grupo H apresentou morte. No final do período de observação comportamental, o grupo G apresentou 60% de morte, enquanto 80% dos animais dos grupos H e I morreram (Tabelas 2 e 3).

No grupo J, formado por animais que receberam o controle das nanoemulsões (Tween 20 e água), ocorreu maior atividade natatória (nível I) na primeira hora após a gavagem. Os mesmos se recuperaram posteriormente e não foram observados outros tipos de alterações comportamentais até o final do período de observação (Tabelas 2 e 3).

Nas brânquias, os grupos A (2 mg/kg de NECHA) e B (2 mg/kg de NECULT) apresentaram somente alterações teciduais de nível I (hiperplasia das células de cloreto e/ou das células epiteliais, infiltração de leucócitos, dilatação dos capilares, deslocamento das células do epitélio, fusão parcial das lamelas secundárias), e no grupo C (2 mg/kg de NECOM) foi observada alteração de nível II (degeneração celular) (Figura 2). O IHA foi de 0.6, 0.6 e 3.0, e o percentual de alterações teciduais foi de 25%, 25% e 50%, respectivamente (Tabelas 3 e 4). Esses valores demonstram que a dose testada promoveu alterações de grau leve no tecido branquial. Os grupos D (3 mg/kg de NECHA), E (3 mg/kg de NECULT) e F (3 mg/kg de NECOM) apresentaram tecido branquial com

algumas alterações de nível II (ruptura epitelial, degeneração celular, aneurisma lamelar, fusão completa das lamelas secundárias) (Figura 2). Todos os grupos apresentaram valores similares de IHA (4.8, 4.8 e 5.0), mas no percentual de alterações histopatológicas do tecido branquial, o grupo F (58.3%) apresentou valor maior do que os grupos D e E (50.0%) (Tabelas 3 e 4).

Todos os animais dos grupos G (4 mg/kg de NECHA), H (4 mg/kg de NECULT) e I (4 mg/kg de NECOM) apresentaram necrose (nível III), com o IHA de 22.6, 26.6 e 26.6, respectivamente, e 58.3% de alterações teciduais em relação ao total de alterações encontradas nas brânquias. Não foram encontradas alterações histopatológicas no tecido branquial do grupo J (controle) (Figura 2; Tabelas 3 e 4).

No estudo histopatológico do fígado, os grupos A (2 mg/kg de NECHA), B (2 mg/kg de NECULT) e C (2 mg/kg de NECOM) apresentaram alterações histopalógicas de nível I (perda do contorno celular, diminuição de glicogênio, aumento do volume dos vasos) e nível II (hiperemia, atrofia nuclear, vacuolização nuclear) (Figura 3). A porcentagem de alterações teciduais nos grupos A e B foi de 33.3% em relação ao total de alterações encontradas no tecido, enquanto em C o valor foi de 25%. O índice de alterações histopatológicas (IHA) foi de 2.6, nos grupos A e B, e 4.2 no grupo C, demonstrando que o tecido hepático permaneceu funcionalmente normal após a exposição (Tabelas 3 e 4).

Nos grupos D (3 mg/kg de NECHA), E (3 mg/kg de NECULT) e F (3 mg/kg de NECOM) foram observadas as mesmas alterações de níveis I e II (Figura 3). Os grupos apresentaram IHA de 4.4, 6.4 e 4.6, respectivamente, indicando a presença de alterações leves no fígado, mas mantendo o funcionamento normal do órgão (Tabela 3). A porcentagem de alterações totais foi de 33.3% no grupo D, e 41.6% nos grupos E e F (Tabela 4). Foram identificadas alterações histopatológicas de nível III (necrose) nos grupos G (4 mg/kg de NECHA), H (4 mg/kg de NECULT) e I (4 mg/kg de NECOM) (Figura 3). Estes grupos apresentaram percentual de alterações teciduais de 58.3, 50 e 50%, respectivamente. O IHA (28.4, 28.2 e 26.4) em ambos grupos indicou alterações de grau moderado a grave (Tabelas 3 e 4). No grupo J (controle) não foram identificadas alterações histopatológicas, mantendo o IHA e o percentual de alterações teciduais iguais a zero (Figura 3; Tabelas 3 e 4).

Na avaliação histopalogicas dos rins, os grupos A (2 mg/kg de NECHA), B (2 mg/kg de NECULT) e C (2 mg/kg de NECOM) apresentaram desorganização tubular e hipertrofia das células tubulares (nível I), degeneração citoplasmática das células tubulares, hiperemia e degeneração tubular (nível II), com valores de índice de alterações histopatológicas de 4.2, 4.2, 6.2, respectivamente (Figura 4; Tabela 5). Os grupos A e B

apresentaram 25% das alterações totais encontradas no tecido, e no grupo C o valor foi de 33.3% (Tabela 3). Nos animais dos grupos D (3 mg/kg de NECHA), E (3 mg/kg de NECULT), F (3 mg/kg de NECOM), G (4 mg/kg de NECHA), H (4 mg/kg de NECULT) e I (4 mg/kg de NECOM) foi identificada necrose renal (alteração de nível III). Portanto, todos apresentaram IHA de 26.2 a 30.0, caracterizando danos moderados a graves no tecido, e percentual de alterações histopalógicas de 41.6% a 50%. Não foram identificadas alterações teciduais nos animais pertencentes ao grupo J (controle) (Figura 4; Tabelas 3 e 5).

Avaliando a histopalogia do tecido intestinal, foi possível identificar alterações de níveis I (atrofia dos vilos, infiltração de leucócitos e linfócitos, hiperplasia das células caliciformes, dilatação dos vasos presentes nos vilos, redução da espessura da lâmina própria, degeneração da camada muscular) e II (distanciamento da lâmina própria, hemorragia na lâmina própria) nos grupos A (2 mg/kg de NECHA), B (2 mg/kg de NECULT) e C (2 mg/kg de NECOM) (Figura 5). O IHA foi de 3.0, 2.8 e 2.8, respectivamente (Tabela 5). O grupo A apresentou 33.3% de alterações totais, enquanto os grupos B e C apresentaram 27.7% (Tabela 3). No intestino dos animais pertencentes aos grupos D (3 mg/kg de NECHA), E (3 mg/kg de NECULT) e F (3 mg/kg de NECOM), foi identificada a necrose tecidual (nível III) (Figura 5). Os grupos D e F apresentaram IHA no valor de 23.2, com 44.4% das alterações identificadas no tecido intestinal. No grupo E o IHA foi de 24.8, com 38.8% das alterações (Tabelas 3 e 5). Os três grupos apresentaram grau moderado a grave na histopatologia do intestino.

Da mesma maneira, os animais dos grupos G (4 mg/kg de NECHA), H (4 mg/kg de NECULT) e I (4 mg/kg de NECOM) também apresentaram necrose tecidual, mas com valor aumentado de IHA (27.0, 27.2, 31.2, respectivamente) e percentual expressivo de alterações no tecido intestinal (50%, 55.5%, 66.6%, respectivamente). No grupo J (controle) foram observadas alterações teciduais de nível I (dilatação dos vasos presentes nos vilos, infiltração de leucócitos), apresentando IHA no valor de 0.6 e 0.1% de alterações teciduais (Figura 5; Tabelas 3 e 5).

Discussão

Estudos anteriores demonstraram que a composição química encontrada nas amostras de óleo essencial de *Rosmarinus officinalis* L. apresentam componentes como 1,8-cineol, α-pineno, canfeno, β-pineno, cânfora, borneol, bornil acetato, p-cimeno, β-myrceno, limoneno, α-terpineno, verbenona, α-terpineol, linalol e terpinen-4-ol, geralmente destacando-se como majoritários o 1,8-cineol e a cânfora (Borges et. al, 2017; Borges et.

al, 2018; Takayama et al, 2016; Türkmen et al., 2014; Hcini et al., 2013; Salido et al., 2003).

Assim como descrito por Forgiarini et al., (2000), as nanoemulsões apresentaram aspecto translúcido, com reflexo levemente azulado e ausência de cremeação. O tamanho das gotículas foi menor que 200nm em todas as amostras, e os valores de índice de polidispersão foram baixos (Duarte et. al, 2015; Solans et al., 2005; Solè et al., 2012).

Na caracterização das nanoemulsões, a NECHA destacou-se por apresentar menor tamanho de gotículas quando comparada à NECULT e NECOM. Em geral, emulsões contendo gotículas de menores diâmetros tendem a ser fisicamente mais estáveis (Bruxel et al., 2012). De acordo com Borges et. al (2018), a nanoemulsão NECHA apresenta 75% de óleo encapsulado, demonstrando sua elevada eficácia em manter a maior parte do OERO no interior das micelas. Dentre as três nanoemulsões testadas, a NECHA apresentou maior DL_{50} (3.6 mg/kg), o que indica menor toxicidade em relação as outras nanoformulações. Desta maneira, ela poderia ser utilizada de forma mais confiável na avaliação de atividades biológicas. Este fato pode estar relacionado à elevada presença do composto 1,8-cineol na amostra de OECHA (50.82%), que possivelmente atuou como estabilizador das micelas. De acordo com Salido et al. (2003), nanoemulsões contendo o composto natural trans-cinamaldeído apresentam instabilidade por envelhecimento de Ostwald (OR). Essa desestabilização pode ser controlada a partir da adição de óleos, porém esse processo diminui a eficácia das atividades biológicas relacionadas ao composto. Ao adicionar o 1,8-cineol à nanoemulsão com trans-cinamaldeído, o mesmo foi capaz de inibir a instabilidade por OR sem interferir na atividade biológica. Desta maneira, o 1,8-cineol foi considerado adequado para a obtenção de nanoemulsões estáveis com processo de desestabilização controlado.

Após a administração das nanoemulsões NECHA, NECULT e NECOM por via oral, os animais apresentaram alterações comportamentais em todas as doses testadas. Esses resultados são similares aos de Souza et. al (2016), que afirma que a administração de nanoformulações em zebrafish pode induzir alterações no comportamento. De acordo com Everds et al. (2013), em ensaios de toxicidade é comum ocorrer estresse nos animais, podendo gerar alterações no peso corporal, no consumo de alimentos, no comportamento, na circulação sanguínea e nas funções reprodutivas. Entretanto, apenas alguns desses fatores são normalmente avaliados em determinado estudo.

As alterações comportamentais em zebrafish podem ser classificadas em níveis I, II e III, iniciando com o aumento da atividade natatória e perda de equilíbrio, seguida de

descanso no fundo e morte (Souza et. al, 2016). O aumento da atividade natatória, observado nos grupos E, F, G, H e I na primeira hora após o experimento, é um indicativo da reação do animal frente ao estresse, representando um possível mecanismo de defesa através da tentativa de fuga (Souza et. al, 2016). Este comportamento também foi observado no grupo J (controle), porém a recuperação dos animais e ausência de outros tipos de alterações pode indicar que o estresse tenha sido causado pelo procedimento de administração por via oral, ao contrário dos outros grupos citados, que apresentaram morte de animais ainda nas duas primeiras horas.

Em zebrafish, a toxicidade de nanopartículas pode ser avaliada através de diversos experimentos, incluindo a determinação da mortalidade (Martinez et al., 2017). De acordo com Bilberg et al. (2012), concentrações maiores de nanopartículas induzem níveis mais elevados de morte. Essa afirmação está de acordo com os valores de mortalidade observados no presente estudo, onde as nanoemulsões na menor dose (2 mg/kg) induziram até 40% de morte nos animais, enquanto as mais elevadas (3 mg/kg e 4 mg/kg) foram capazes de provocar mortalidade de até 80%.

Em estudo de toxicidade de nanoemulsão contendo álcool perílico, as concentrações mais elevadas (50 e 125 µg/L) induziram 100% de morte em zebrafish adultos (Souza et. al, 2016). Esses resultados são diferentes do presente estudo, pois nenhuma das nanoemulsões nas doses administradas foi capaz de induzir 100% de morte.

No zebrafish, as brânquias são consideradas órgãos fundamentais para a extração de oxigênio da água, a regulação do equilíbrio ácido-base e a excreção de resíduos. São órgãos sensíveis a substâncias tóxicas. (Souza et. al, 2016; Holden et al., 2012; Houlihan et al., 1982). Uma das primeiras alterações decorrentes de exposição a substâncias tóxicas nas brânquias é o deslocamento das células epiteliais. Essa alteração, observada em animais tratados com as três nanoemulsões (NECHA a 2 e 3 mg/kg, NECULT a 4 mg/kg e NECOM a 2, 3 e 4 mg/kg), indica tentativa de adaptação de animais aquáticos às novas condições fisiopatológicas. O espaço formado entre a lamela e o epitélio deslocado, pode ser preenchido por água, que leva à formação de edema. Essas alterações podem gerar disfunção das brânquias e sufocamento (Carvalho et. al, 2017; Rezende et al., 2014; Campagna, 2008).

A hiperplasia das células epiteliais e fusão das lamelas, observadas em todos os grupos tratados com as nanoemulsões, podem ser consideradas como mecanismo de defesa através da tentantiva de bloqueio da passagem de água e sangue para o interior

das lamelas. Entretanto, isso pode provocar a morte do animal por falta de oxigenação (Mazon et al., 2002, Souza et. al, 2016).

As células de cloreto localizam-se na base das lamelas secundárias e são responsáveis por absorver quantidades adequadas de sal (Holden et al. 2012). A hiperplasia dessas células, observada em grupos de animais tratados com as três nanoemulsões, pode ser um indicativo de desequilíbrio osmótico em peixes, que tentam adaptar-se através do aumento do transporte de cloreto de sódio e água para a circulação sanguínea, no intuito de estabelecer a homeostase (Rezende et. al, 2014).

Animais tratados com NECULT (nas doses de 3 e 4 mg/kg) e NECOM (na dose de 4 mg/kg) apresentaram aneurisma lamelar. Essa alteração pode ser indicativo morte de células pilares. As células pilares são fundamentais para a manutenção da circulação sanguínea na lamela, portanto, alterações em seu funcionamento normal provocam perda da integridade da lamela secundária e afetam o fluxo sanguíneo (Van Den Heuvel et al., 2000; Rezende et. al, 2014).

Todos os grupos tratados com as nanoemulsões apresentaram degeneração celular nas doses mais elevadas (3 e 4 mg/kg), sendo que apenas animais tratados com NECOM a apresentaram na menor dose (2 mg/kg). Essa alteração pode ser provocada por perda de função no tecido branquial (Takashima e Hibiya, 1984). A necrose foi observada nos animais que receberam a dose mais elevada das três nanoemulsões (4 mg/kg). Essa condição ocorre em organismos aquáticos expostos a condições de maior toxicidade (Abel, 1976).

O fígado do zebrafish apresenta semelhança ao de mamíferos nos principais processos fisiológicos realizados, apesar de sua estrutura ser diferente. Dentre eles, destacam-se as vias de metabolização de drogas, que incluem a ação do citocromo P450. Portanto, após exposição a substâncias tóxicas, sua histopatologia pode ser comparada às lesões hepáticas de mamíferos (Vliegenthart et al, 2014).

A vacuolização foi uma das alterações mais frequentes observadas no fígado de animais tratados com as nanoemulsões NECHA (na dose de 4 mg/kg), NECULT (nas doses de 2 e 3 mg/kg) e NECOM (nas doses de 3 e 4 mg/kg). Pode estar relacionada à redução das reservas de glicogênio nos hepatócitos e ao acúmulo de lipídios combinados com agentes tóxicos, podendo alterar o funcionamento normal do órgão (Borges et. al, 2018). Entretanto, a diminuição do glicogênio no tecido hepático também pode ser justificada pela agilidade do zebrafish, que diminui as reservas devido ao seu metabolismo acelerado (Carvalho et. al, 2017).

Outras alterações presentes no tecido hepático e consideradas como mecanismo de defesa a agentes tóxicos foram a degeneração celular, observada em animais tratados com as três nanoemulsões na dose mais elevada (4 mg/kg), e a hiperemia, observada em animais tratados com NECHA (na dose de 3 mg/kg), NECULT (nas doses de 3 e 4 mg/kg) e NECOM (nas doses de 3 e 4 mg/kg). A hiperemia ocorre como tentativa de aumentar o fluxo sanguíneo geral no fígado e ampliar a liberação de nutrientes e oxigênio para as áreas afetadas, evitando a hipóxia (Takashima e Hibiya, 1984; Carvalho et. al, 2017, Borges et. al, 2018). Na dose de 4 mg/kg, todos os grupos apresentaram necrose hepática. Essa alteração pode ser provocada por agentes tóxicos em doses elevadas.

O rim do zebrafish desempenha a função importante de excreção do volume de água que entra no peixe através da boca. Também realiza a filtração de resíduos e a absorção de sal e água (Borges et. al, 2018; Holden et al., 2012). É um dos órgãos mais afetados por substâncias tóxicas (Carvalho et. al, 2017).

Os grupos de animais tratados com NECULT e NECOM (na dose de 2 mg/kg) apresentaram hipertrofia das células tubulares no tecido renal. Essa condição ocorre devido ao ressecamento das células epiteliais dos túbulos renais, podendo preceder a degeneração hialina, que consiste no aumento da quantidade de grânulos eosinofílicos no citoplasma dessas células (Carvalho et. al, 2017). A degeneração hialina, condição observada em animais tratados com NECOM (nas doses de 3 e 4 mg/kg), pode estar relacionada à reabsorção de substâncias protéicas sintetizadas em excesso pelo glomérulo (Takashima e Hibiya, 1984).

A hiperemia, alteração observada em todos os grupos que receberam as nanomulsões em diferentes doses, consiste no aumento da quantidade de sangue circulante e pode estar associada a ruptura de vasos. Nos rins, pode ser provocada a partir da pressão exercida pela dilatação dos capilares glomerulares na presença de substâncias tóxicas (Carvalho et. al., 2017).

Outras alterações, como desorganização tubular, degeneração tubular e degeneração citoplasmática das células tubulares, foram frequentes em animais tratados com os três tipos de nanoemulsões nas diferentes doses. De acordo com Carvalho et. al (2017), alterações tubulares observadas em rins de zebrafish podem ser provocadas indiretamente pela disfunção metabólica induzida pela exposição a substâncias tóxicas. Essas alterações podem frequentemente culminar em necrose nos rins (Takashima e Hibiya, 1984). Este fato justifica a presença de necrose nos rins de zebrafish tratados com as três nanoemulsões, nas doses mais elevadas (3 e 4 mg/kg).

O intestino do zebrafish é formado por camada mucosa com células caliciformes, células inflamatórias e enterócitos, que auxiliam o epitélio na absorção de nutrientes, na resposta imune e no equilíbrio osmótico (Borges et. al, 2018; Carvalho et. al, 2017; Holden et al. 2012). A exposição a substâncias tóxicas causa danos à mucosa intestinal e ao desenvolvimento celular, podendo perturbar a fisiologia do órgão e provocar diversas alterações histológicas (Carvalho et. al, 2017; Takashima e Hibiya, 1984).

Em todos os grupos de animais tratados com as nanoemulsões, foram observadas infiltração de leucócitos e infiltração linfocítica na camada mucosa. O aumento de células de defesa no epitélio intestinal pode estar relacionado a presença de inflamação na lâmina própria, provocada pela exposição do zebrafish a substâncias tóxicas (Carvalho et. al, 2017; Roberts e Ellis 2012). O deslocamento da mucosa intestinal foi observado em animais tratados com as nanoemulsões na dose mais elevada (4 mg/kg). Essa alteração também é comum no intestino de peixes expostos a agentes tóxicos (Roberts and Ellis, 2012).

A vacuolização foi observada em animais tratados com NECHA (na dose de 4 mg/kg), NECULT (nas doses de 3 e 4 mg/kg) e NECOM (na dose de 3 mg/kg). Essa alteração é frequente após a exposição a substâncias com elevada toxicidade e, geralmente, precede a necrose (Takashima e Hibiya, 1984). Este fato justifica a presença de necrose intestinal em todos os grupos tratados com as nanoemulsões nas doses mais elevadas (3 e 4 mg/kg).

Conclusão

A exposição de *Danio rerio* a diferentes doses (2, 3 e 4 mg/kg) de nanoemulsões contendo OERO de procedências diferentes (NECHA, NECULT e NECOM) durante 24 horas, induziu alterações comportamentais em zebrafish e a DL_{50} foi estimada em 3.6, 2.8 e 2.4 mg/kg, respectivamente. Esses valores indicam que a NECHA apresentou menor toxicidade quando comparada às outras nanoemulsões, o que justifica os valores menores de taxa de mortalidade. Apesar de apresentarem as concentrações reais de apenas 100 µg, 150 µg e 200 µg de OERO, a nanoemulsões foram eficazes na avaliação da toxicidade, comprovando capacidade de ampliar a biodisponibilidade desse óleo. Os órgãos mais alterados no estudo histopatológico foram intestino e rins. Nos rins, alterações predominantes como hipertrofia das células tubulares e ruptura dos vasos, foram menos frequentes nos animais tratados com NECHA. Assim como no tecido intestinal de animais tratados com essa nanoemulsão, a vacuolização foi observada apenas na maior dose. Dentre as três nanoemulsões testadas, a NECHA apresenta

quantidade mais elevada de 1,8-cineol em sua composição. De acordo com estudos anteriores, também apresenta melhor atividade biológica entre as nanoemulsões e mantém 75% do óleo encapsulado de maneira eficaz, o que pode estar relacionado à capacidade do 1,8-cineol de atuar como estabilizador de nanoformulações.

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Tabela 1: Quantidade e porcentagem de animais mortos após tratamentos com diferentes concentrações de nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. em zebrafish (15 animais/grupo).

Concentration	Number of death animals	Percentage
A - NECHA 2 mg/kg	03	20%
B - NECULT 2 mg/kg	03	20%
C - NECOM 2 mg/kg	06	40%
D - NECHA 3 mg/kg	03	20%
E - NECULT 3 mg/kg	09	60%
F - NECOM 3 mg/kg	09	60%
G - NECHA 4 mg/kg	12	80%
H - NECULT 4 mg/kg	12	80%
I - NECOM 4 mg/kg	12	80%
J - Control	0	0%

Tabela 2: Alterações comportamentais após tratamento com diferentes doses das nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. em zebrafish em diferentes tempos de observação.

	Time	A	B	C	D	E	F	G	H	I	J
Stage I	1’					X	X	X	X	X	X
Stage II	1’			X		X	X	X	X	X	
Stage III	1’					X	X	X	X	X	
Stage I	2’	X									
Stage II	2’				X		X	X	X	X	
Stage III	2’							X	X	X	
Stage I	3’										
Stage II	3’	X	X	X	X	X					
Stage III	3’	X	X	X	X	X					
Stage I	4’										
Stage II	4’										
Stage III	4’						X	X	X	X	
Stage I	5’										
Stage II	5’										
Stage III	5’								X		
Stage I	24’										
Stage II	24’										
Stage III	24’										

Estágio I: aumento da atividade de natação, tremores na cauda; Estágio II: movimento de natação circular, perda de postura; Estágio III: morte. A: NECHA 2 mg / kg, B: NECULT 2 mg / kg, C: NECOM 2 mg / kg, D: NECHA 3 mg / kg, E: NECULT 3 mg / kg, F: NECOM 3 mg / kg, G: NECHA 4 mg / kg, H: NECULT 4 mg / kg, I: NECOM 4 mg / kg, J: NECONTROL (controle).

Tabela 3: Percentual de alterações histológicas apresentado em fígado, brânquias, rins e intestino de zebrafish após exposição as nanoemulsões a 2, 3 e 4 mg/kg.

Group/tissue	Stage I	Stage II	Stage III	Total	%
Liver					
A – 2 mg/kg NECHA	3/5	1/6	0/1	4/12	33,3
B - 2 mg/kg NECULT	3/5	1/6	0/1	4/12	33,3
C - 2 mg/kg NECOM	1/5	2/6	0/1	3/12	25,0
D - 3 mg/kg NECHA	2/5	2/6	0/1	4/12	33,3
E - 3 mg/kg NECULT	2/5	3/6	0/1	5/12	41,6
F - 3 mg/kg NECOM	3/5	2/6	0/1	5/12	41,6
G - 4 mg/kg NECHA	2/5	4/6	1/1	7/12	58,3
H - 4 mg/kg NECULT	1/5	4/1	1/1	6/12	50,0
I - 4 mg/kg NECOM	2/5	3/1	1/1	6/12	50,0
J – Control	0/5	0/6	0/1	0/12	0,0
Gills					
A – 2 mg/kg NECHA	3/6	0/5	0/1	3/12	25,0
B - 2 mg/kg NECULT	3/6	0/5	0/1	3/12	25,0
C - 2 mg/kg NECOM	5/6	1/5	0/1	6/12	50,0
D - 3 mg/kg NECHA	4/6	2/5	0/1	6/12	50,0
E - 3 mg/kg NECULT	4/6	2/5	0/1	6/12	50,0
F - 3 mg/kg NECOM	5/6	2/5	0/1	7/12	58,3
G - 4 mg/kg NECHA	**3/6**	**3/5**	**1/1**	**7/12**	**58,3**
H - 4 mg/kg NECULT	3/6	3/5	1/1	7/12	58,3
I - 4 mg/kg NECOM	3/6	3/5	1/1	7/12	58,3
J – Control	0/6	0/5	0/1	0/12	0,0
Intestine					
A - 2 mg/kg NECHA	4/11	1/6	0/1	5/18	27,7
B - 2 mg/kg NECULT	4/11	1/6	0/1	5/18	27,7
C - 2 mg/kg NECOM	4/11	1/6	0/1	5/18	27,7
D - 3 mg/kg NECHA	5/11	1/6	1/1	7/18	38,8
E - 3 mg/kg NECULT	4/11	2/6	1/1	7/18	38,8
F - 3 mg/kg NECOM	6/11	1/6	1/1	8/18	44,4
G - 4 mg/kg NECHA	5/11	3/6	1/1	9/18	50,0
H - 4 mg/kg NECULT	6/11	3/6	1/1	10/18	55,5
I - 4 mg/kg NECOM	6/11	5/6	0/1	12/18	66,6
J - Control	0/11	0/6	0/1	0/18	0,0
Kidney					
A - 2 mg/kg NECHA	1/4	2/7	0/1	3/12	25,0
B - 2 mg/kg NECULT	1/4	2/7	0/1	3/12	25,0
C - 2 mg/kg NECOM	1/4	3/7	0/1	4/12	33,3
D - 3 mg/kg NECHA	1/4	3/7	1/1	5/12	41,6
E - 3 mg/kg NECULT	1/4	3/7	1/1	5/12	41,6
F - 3 mg/kg NECOM	1/4	4/7	1/1	6/12	50,0
G - 4 mg/kg NECHA	1/4	4/7	1/1	6/12	50,0
H - 4 mg/kg NECULT	1/4	4/7	1/1	6/12	50,0
I - 4 mg/kg NECOM	0/4	5/7	1/1	6/12	50,0
J – Control	0/4	0/7	0/1	0/12	0,0

A porcentagem foi determinada sobre o número de peixes por grupo (n = 5). Segundo Poleksic and Mitrovic-Tutundzic (1994), Rigolin-Sá (1998) e Takashima and Hibiya (1995).

Tabela 4: Média do Índice de Alterações Histológicas (IHA) das brânquias e fígado de zebrafish após exposição a diferentes concentrações das nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. em triplicata (n = 15 animais/grupo).

Gills					
Replicate	**A**	**B**	**C**	**D**	**E**
1	0.7	0.5	3.5	4.6	5.5
2	0.6	0.7	3.0	5.8	4.5
3	0.5	0.7	2.5	4.0	4.4
Mean ± SD	0.6± 0.1	0.6±0.11	3.0±0.5	4.8±0.92	4.8±0.61
Replicate	**F**	**G**	**H**	**I**	**J**
1	4.6	20.6	25.7	28.0	0.0
2	5.6	24.8	27.2	25.8	0.0
3	4.8	22.4	26.8	26.1	0.0
Mean ± SD	5.0±0.53	22.6±2.11	26.6±0.78	26.6±1.12	0.0±0.0
Liver					
Replicate	**A**	**B**	**C**	**D**	**E**
1	2.6	2.6	4.6	4.2	6.6
2	3.0	2.5	3.7	4.6	5.9
3	2.2	2.7	4.4	4.4	7.0
Mean ± SD	2.6±0.40	2.6±0.10	4.2±0.47	4.4±0.20	6.4±0.56
Replicate	**F**	**G**	**H**	**I**	**J**
1	4.5	30.0	27.1	27.0	0.0
2	4.2	28.3	28.6	25.7	0.0
3	5.1	26.8	29.0	26.5	0.0
Mean ± SD	4.6±0.46	28.4±1,60	28.2±1.00	26.4±0.66	0.0±0.0

Tabela 5: Média do Índice de Alterações Histológicas (IHA) de rins e intestino de zebrafish após exposição a diferentes concentrações das nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. em triplicata (n = 15 animais/grupo).

Kidney					
Replicate	**A**	**B**	**C**	**D**	**E**
1	4.3	3.9	6.5	27.0	25.8
2	3.8	4.3	6.4	26.3	26.2
3	4.5	4.4	5.7	25.4	26.6
Mean ± SD	4.2±0.36	4.2±0.26	6.2±0.43	26.2±0.80	26.2±0.40
Replicate	**F**	**G**	**H**	**I**	**J**
1	27.5	29.0	28.3	30.4	0.1
2	28.2	27.2	28.1	30.0	0.0
3	29.0	28.4	28.1	29.7	0.0
Mean ± SD	28.2±0.75	28.2±0.91	28.2±0.11	30.0±0.35	0.0±0.6
Intestine					
Replicate	**A**	**B**	**C**	**D**	**E**
1	2.7	2.9	3.0	25.0	26.1
2	2.8	2.9	2.8	21.0	24.5
3	3.0	2.6	2.6	23..0	23.8
Mean ± SD	2.8±0.15	2.8±0.17	2.8±0.20	23.0±2.82	24.8±1.17
Replicate	**F**	**G**	**H**	**I**	**J**
1	23.3	27.0	27.3	32.2	0.0
2	22.8	26.8	28.1	30.8	0.0
3	23.5	27.1	26.2	30.6	0.0
Mean ± SD	23.2±0.36	27.0±0.15	27.2±0.95	31.2±0.87	0.0±0.0

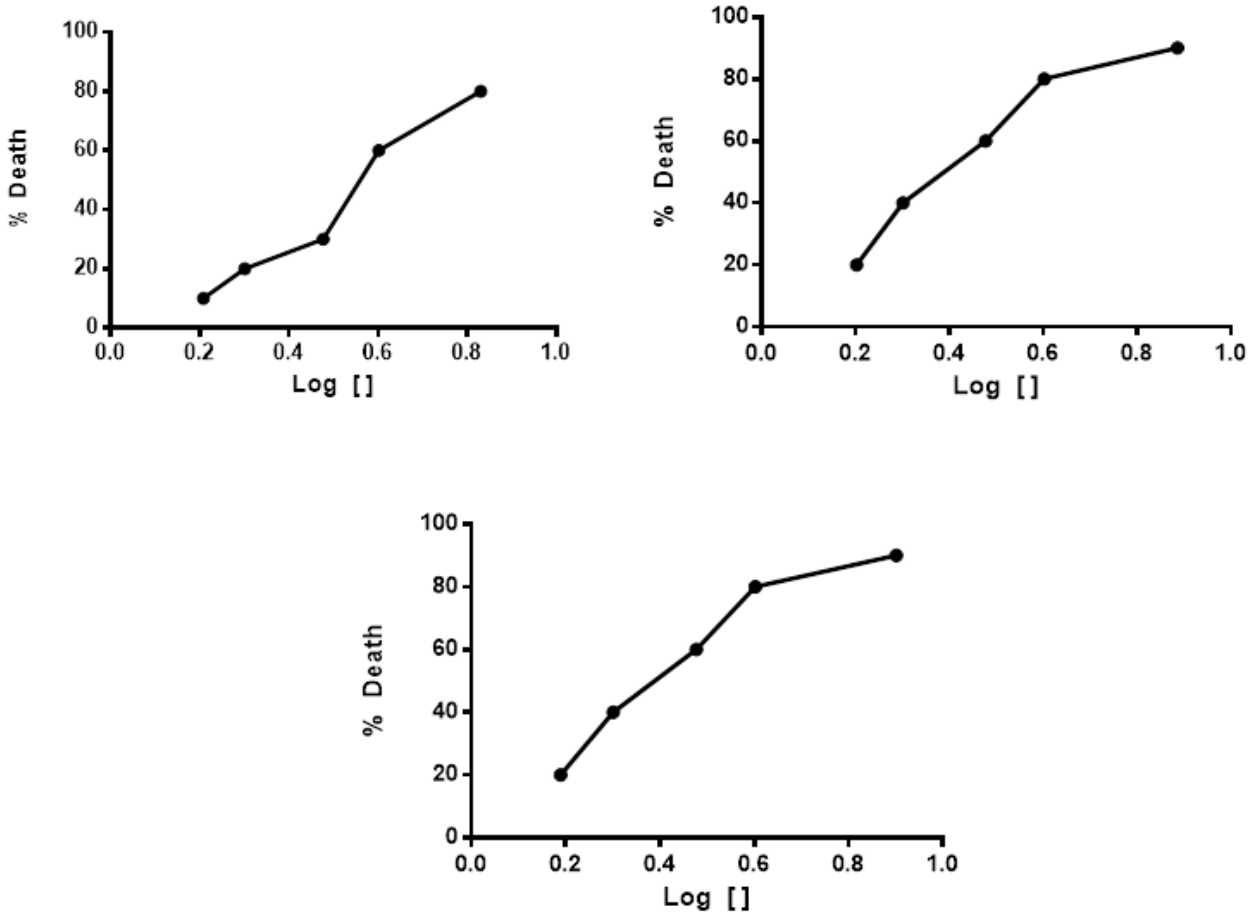

Figura 1: Efeito da administração via gavagem de diferentes doses de nanoemulsões contendo óleo essencial de *Rosmarinus officinalis* L. (1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg e 5 mg/kg) em zebrafish (n = 15 animais/grupo). DL_{50}: NECHA = 3.5 mg/kg, NECULT = 2.7 mg/kg, NECOM = 2.4 mg/kg).

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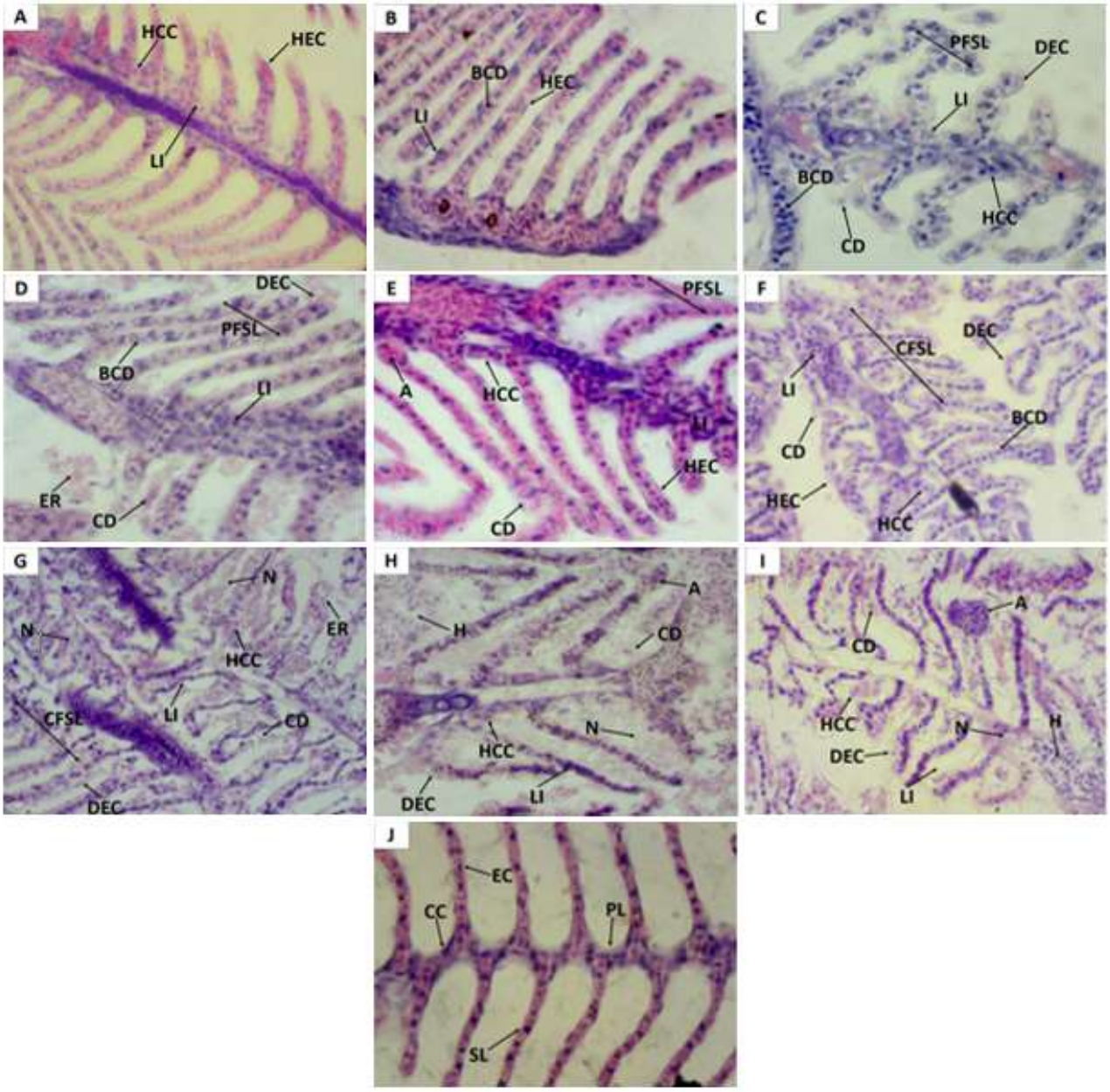

Figura 2: Secção transversal longitudinal das brânquias de zebrafish, todas as figuras foram ampliadas em 9400, H e E. A: NECHA 2 mg/kg mostrando HEC hiperplasia de células epiteliais, HCC hipertrofia de de células de cloreto, LI infiltração de leucócitos; B: NECULT 2 mg/kg mostrando LI infiltração de leucócitos, HEC hiperplasia de células epiteliais, BCD degeneração de capilares sanguíneos; C: NECOM 2 mg/kg mostrando LI infiltração de leucócitos, BCD degeneração de capilares sanguíneos, CD degeneração de células, HCC hipertrofia de de células de cloreto, DEC deslocamento de células epiteliais, PFSL fusão parcial de lamelas secundárias; D: NECHA 3 mg/kg mostrando a LI infiltração de leucócitos, BCD degeneração de capilares sangueos, CD degeneração de células, DEC deslocamento de células epiteliais, PFSL fusão parcial de lamelas secundárias, ER ruptura epitelial; E: NECULT 3 mg/kg mostrando LI infiltração de leucócitos, CD degeneração de células, PFSL fusão parcial de lamelas secundárias, HEC hiperplasia de células epiteliais, HCC hipertrofia de células de cloreto, A aneurisma; F: NECOM 3 mg/kg mostrando LI infiltração de leucócitos, CD degeneração de células, CFSL fusão completa de lamelas secundárias, HEC hiperplasia de células epiteliais, HCC hipertrofia de células de cloreto, A aneurisma, DEC deslocamento de células epiteliais, BCD degeneração de capilares sanguíneos; G: NECHA 4 mg/kg mostrando LI infiltração de leucócitos, CD degeneração de células, CFSL fusão completa de lamelas secundárias, HCC hipertrofia de de células de cloro, DEC deslocamento de células epiteliais, ER ruptura epitelial, N necrose; H: NECULT 4 mg/kg mostrando HCC hipertrofia de de clulas de cloreto, DEC deslocamento de células epiteliais, LI infiltração de leucócitos, CD degeneração de células, N necrose, A aneurisma, H hemorragia; I: NECOM 4 mg/kg mostrando HCC hipertrofia de células de cloreto, DEC deslocamento de células epiteliais, LI infiltração de leucócitos, CD degeneração de células, N necrose, A

aneurisma, H hemorragia; J: controle mostrando CC células de cloreto, EC células epiteliais, PL lamelas primárias, SL lamelas secundárias.

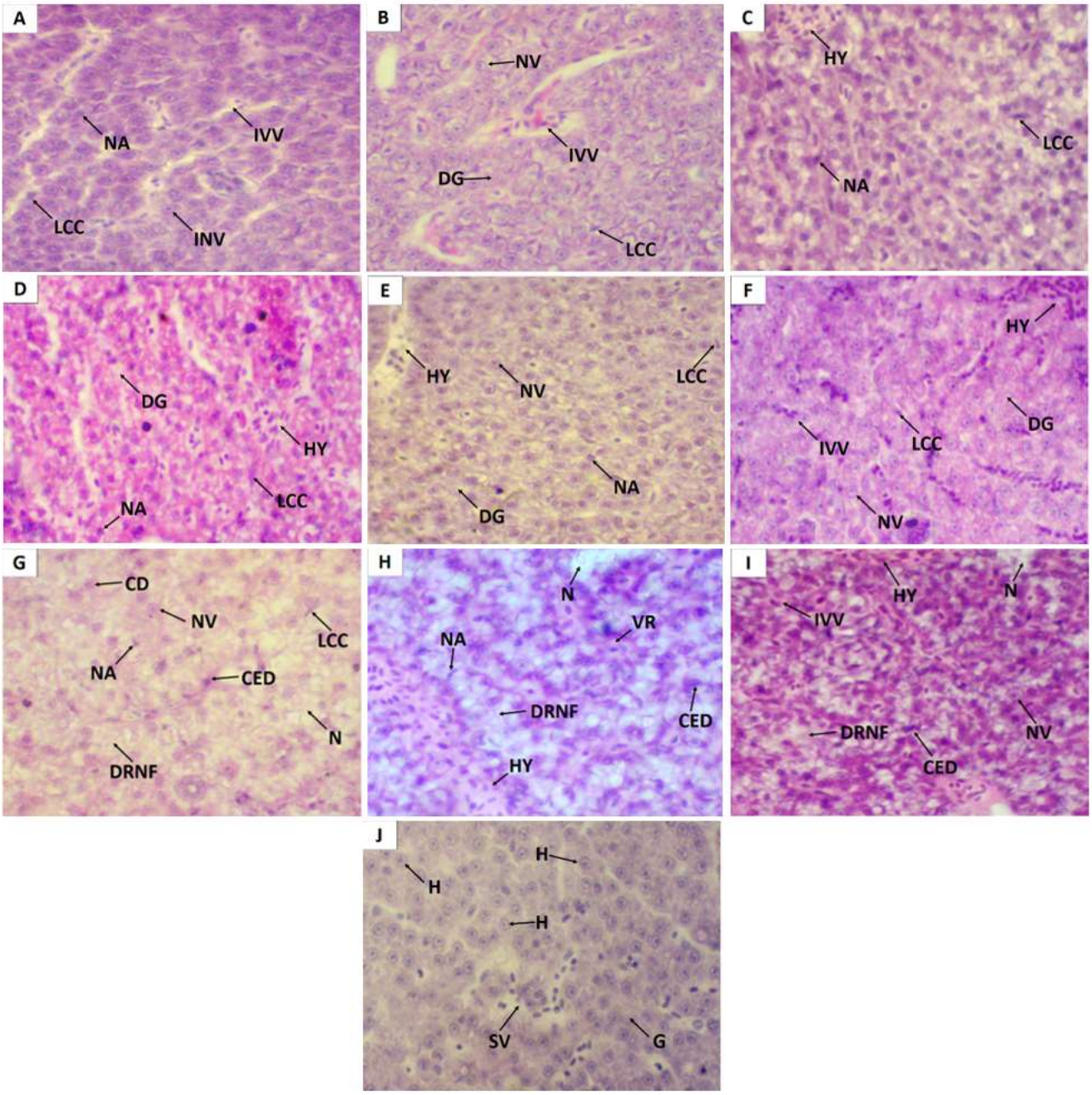

Figura 3: Secção transversal longitudinal do fígado de zebrafish, todas as figuras foram ampliadas em 9400, H e E. A: NECHA 2 mg/kg mostrando LCC perda do contorno celular, INV aumento do volume nuclear, IVV aumento do volume dos vasos, NA atrofia nuclear; B: NECULT 2 mg/kg mostrando NV vacuolização nuclear, LCC perda de contorno celular, IVV aumento do volume de vasos, DG diminuição de glicogênio; C: NECOM 2 mg/kg NA atrofia nuclear, LCC perda do contorno celular, HY hiperemia; D: NECHA 3 mg/kg mostrando DG diminuição de glicogênio, HY hiperemia, LCC perda do contorno celular, NA atrofia nuclear; E: NECULT 3 mg/kg mostrando HY hiperemia, LCC perda do contorno celular, NA atrofia nuclear, NV vacuolização nuclear, DG diminuição de glicogênio; F: NECOM 3 mg/kg mostrando LCC perda de contorno celular, HY hiperemia, DG diminuição de glicogênio, NV vacuolização nuclear, IVV aumento do volume de vasos; G: NECHA 4 mg/kg apresentando NA atrofia nuclear, NV vacuolização nuclear, CD degeneração citoplasmática, CED ruptura de células, LCC perda de contorno celular, DRNF diminuição da frequência relativa de núcleos, N necrose; H: NECULT 4 mg/kg apresentando NA atrofia nuclear de, DRNF diminuição da frequência relativa de núcleos, HY hiperemia, CED ruptura de células, RV ruptura de vasos de, N necrose; I: NECOM 4 mg/kg mostrando IVV volume aumentado de vasos, HY hiperemia, DRNF diminuiu frequência relativa de núcleos, CED ruptura de células, NV vacuolização nuclear, N necrose; J: controle mostrando H hepatócitos, SV vasos sinusóides, G glicogênio.

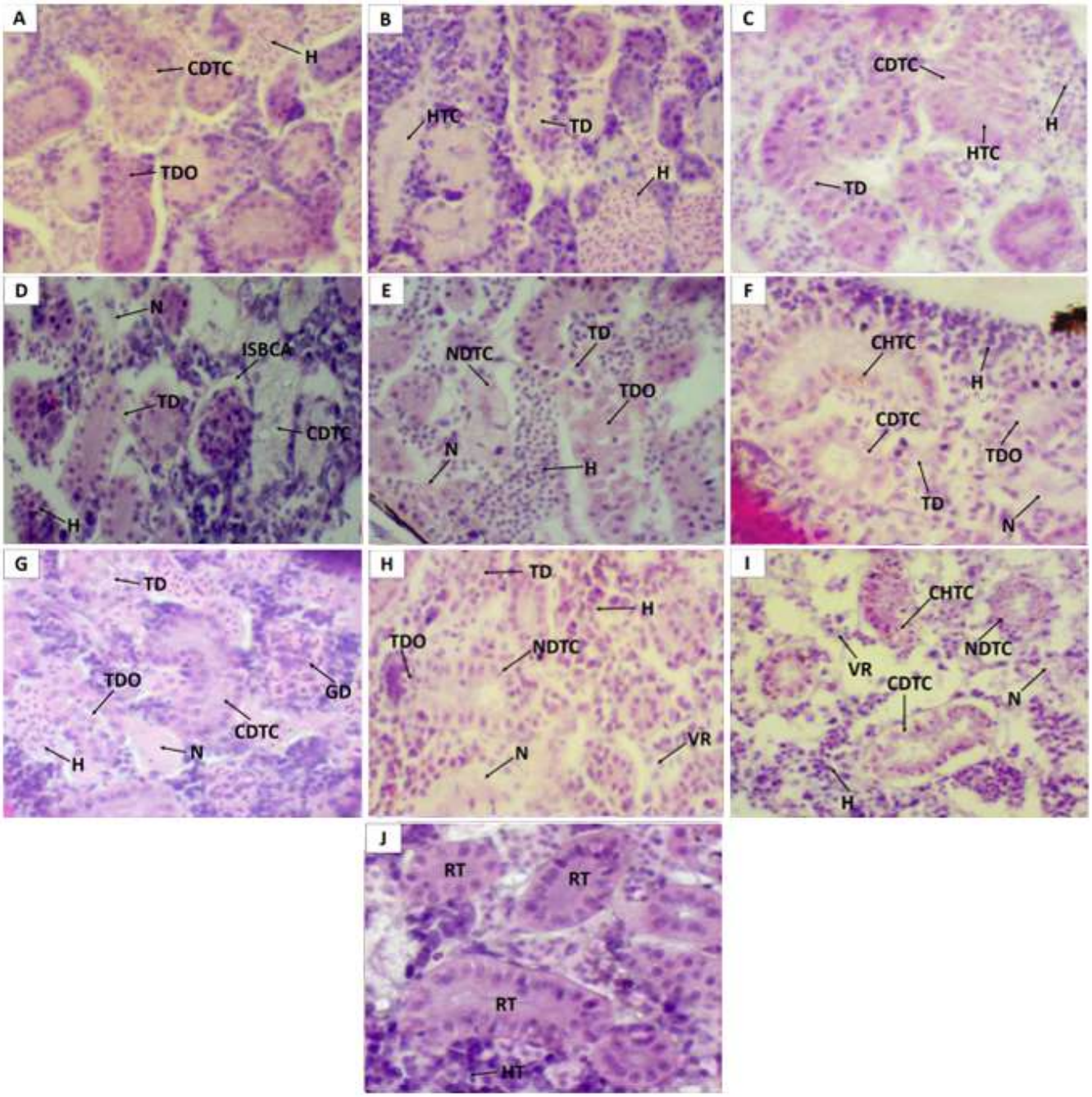

Figura 4: Secção transversal longitudinal do rim de zebrafish, todas as figuras foram ampliadas em 9400, H e E. A: NECHA 2 mg/kg mostrando CDTC degeneração citoplasmática de células tubulares, TDO desorganização tubular, H hiperemia; B: NECULT 2 mg/kg mostrando HTC hipertrofia de células tubulares, TD degeneração tubular, H hiperemia; C: NECOM 2 mg/kg mostrando CDTC degeneração citoplasmática de células tubulares, HTC hipertrofia de células tubulares, TD degeneração tubular, H hiperemia; D: NECHA 3 mg/kg mostrando TD degeneração tubular, H hiperemia, CDTC degeneração citoplasmática de células tubulares, ISBCA aumento do espaço da cápsula de Bowman, N necrose; E: NECULT 3 mg/kg mostrando H hiperemia, NDTC degeneração nuclear de culas tubulares, N necrose, TD degeneração tubular, TDO desorganização tubular; F: NECOM 3 mg/kg mostrando CDTC degeneração citoplasmática de células tubulares, CHTC degeneração hialina de células tubulares, TD degeneração tubular, TDO desorganização tubular, H hiperemia, N necrose; G: NECHA 4 mg/kg mostrando CDTC degeneração citoplasmática de células tubulares, TD degeneração tubular, TDO desorganização tubular, H hiperemia, N necrose, GD degeneração glomerular; H: NECULT 4 mg/kg mostrando TD degeneração tubular, TDO desorganização tubular, H hiperemia, N necrose, NDTC degeneração nuclear de células tubulares, VR ruptura de vasos; I: NECOM 4 mg/kg mostrando CDTC degeneração citoplasmática de células tubulares, H hiperemia, N necrose, CHTC degeneração hialina de

células tubulares, VR ruptura de vasos, NDTC degeneração nuclear de células tubulares; J: RT túbulos renais, HT tecido hematopoiético.

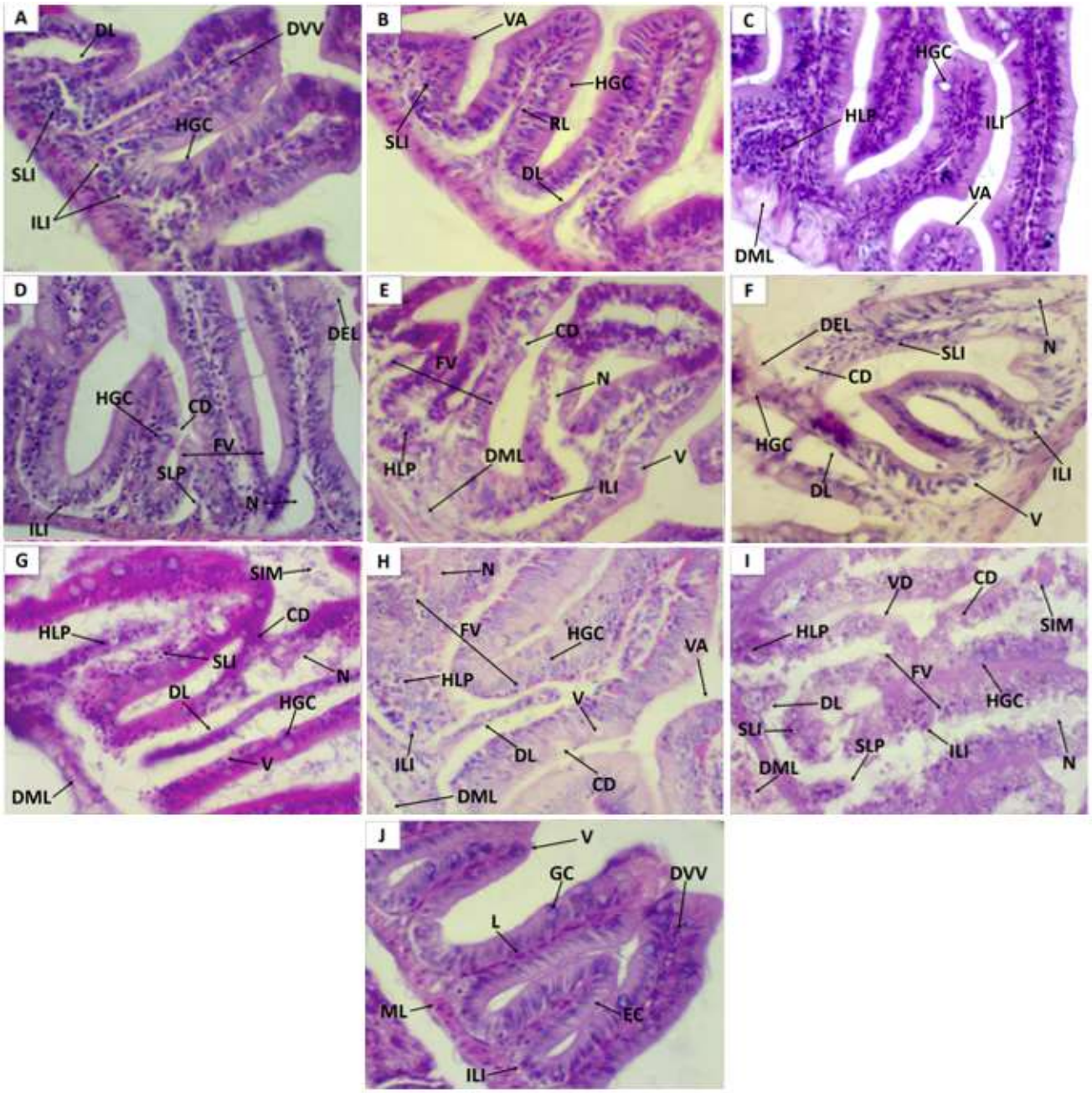

Figura 5: Secção transversal longitudinal de intestino de zebrafish, todas as figuras foram ampliadas em 9400, H e E. A: NECHA 2 mg/kg mostrando DL deslocamento da lâmina própria, SLI infiltrado linfocítico estromal, ILI infiltração de leucócitos, DVV dilatação de vasos, HGC hiperplasia de células caliciformes; B: NECULT 2 mg/kg mostrando DL deslocamento da lâmina própria, SLI infiltração linfocítica estromal, HGC hiperplasia de células caliciformes, VA atrofia dos vilos, RL redução na espessura da lâmina própria; C: NECOM 2 mg/kg mostrando HLP hemorragia na lâmina própria, VA atrofia nos vilos, ILI infiltração de leucócitos, HGC hiperplasia de células caliciformes, DML degeneração da camada muscular; D: NECHA 3 mg/kg mostrando ILI infiltração de leucócitos, HGC hiperplasia de células caliciformes, CD degeneração de células, DEL deslocamento do revestimento epitelial do ápice dos vilos, FV fusão total ou parcial dos vilos, SLP encurtamento da lâmina própria, N necrose; E: NECULT 3 mg/kg mostrando FV fusão total ou parcial dos vilos, HLP hemorragia na lâmina própria, DML degeneração da camada muscular, ILI infiltração de leucócitos, CD degeneração de células, V vacuolização de enterócitos, N necrose; F: NECOM 3 mg/kg mostrando HGC hiperplasia de células caliciformes, CD degeneração de células, DL deslocamento da lâmina própria, DEL deslocamento do revestimento epitelial do ápice dos vilos, SLI infiltração linfocítica estromal, V vacuolização de enterócitos, N necrose, ILI infiltração de leucócitos; G: NECHA 4 mg/kg mostrando HLP hemorragia na lâmina própria, DML degeneração da camada muscular, DL

deslocamento da lâmina própria, SLI infiltração linfocítica estromal, CD degeneração de células, SIM descamação da mucosa intestinal, V vacuolização de enterócitos, N necrose, HGC hiperplasia de células caliciformes; H: NECULT 4 mg/kg mostrando HGC hiperplasia de células caliciformes, CD degeneração de células, DL deslocamento da lâmina própria, V vacuolização de enterócitos, ILI infiltração de leucócitos, DML degeneração da camada muscular, VA atrofia dos vilos, FV fusão parcial ou completa dos vilos, HLP hemorragia na própria lâmina, N necrose; I: NECOM 4 mg/kg mostrando HGC hiperplasia das células caliciformes, CD degeneração das células, DL deslocamento da lâmina própria, ILI infiltração de leucócitos, DML degeneração da camada muscular, FV fusão total ou parcial dos vilos, HLP hemorragia na lâmina própria, N necrose, SLI infiltração linfocítica estromal, SLP encurtamento da lâmina própria, VD degeneração dos vilos, SIM descamação da mucosa intestinal; J: L lâmina própria, ML camada muscular, GC células caliciformes, CE enterócitos, V vilos, ILI infiltração leucocitária, DVV dilatação dos vasos.

7 CAPÍTULO 5

EFEITO RELAXANTE DE NANOEMULSÕES CONTENDO ÓLEO ESSENCIAL DE *Rosmarinus officinalis* L. SOBRE O MÚSCULO LISO TRAQUEAL ISOLADO DE COBAIA

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Manuscrito a ser encaminhado para publicação.

Abstract

A espécie vegetal *Rosmarinus officinalis* L. tem sido utilizada na medicina tradicional em forma de chá, infusões e óleo essencial, para o tratamento de diversas manifestações, inclusive as de ordem inflamatória das vias aéreas. Neste estudo foi investigado o efeito relaxante de nanoemulsões obtidas a partir de quatro amostras de óleo essencial de *Rosmarinus officinalis* L. (OERO), denominadas NECHA, NECULT, NECOM e NEMEX, em músculo liso de tráqueia isolada de cobaia. Posteriormente os possíveis mecanismos de ação foram avaliados na presença de propranolol, glibenclamida e em solução livre de Ca^{2+} com contrações induzidas por cloreto de cálcio. Todas as nanomulsões diminuíram o percentual de contração do músculo liso traqueal em relação aos seus respectivos óleos a 300 µg/mL. Os óleos apresentaram faixa de 60.0 - 55.8%, enquanto as nanoemulsões reduziram as contrações para a faixa de 43.1 - 17.9%, sendo que a NECHA apresentou melhor desempenho em relação as outras nanoemulsões. Isto pode ser devido ao elevado teor de 1,8-cineol na composição do óleo essencial. Este composto tem sido relacionado ao efeito relaxante em músculo liso. A NECHA não diminuiu ou modificou o bloqueio dos canais de K^{+}_{ATP} e nem dos receptores β2-adrenérgicos. Também não apresentou efeito relaxante na presença de $CaCl_2$, sugerindo que não atua através dessas vias. Entretanto, isto não exclui a possibilidade de o seu mecanismo de ação estar relacionado ao controle da concentração de Ca^{2+} intracelular ou à regulação de calmodulina. No entanto, mais estudos são necessários para confirmar esta hipótese.

Keywords: Rosemary, *Rosmarinus officinalis*, smooth muscle, nanoemulsions.

Introdução

A espécie vegetal *Rosmarinus officinalis* L. tem sido utilizada no preparo de chás e infusões para tratar enfermidades desde a antiguidade (Ribeiro-Santos et al., 2015). Estudos relatam o uso de seu óleo essencial (OERO) para o tratamento de distúrbios digestivos e doenças respiratórias, devido aos efeitos anti-inflamatório e analgésico (Raut e Karuppayil 2014; Ribeiro-Santos et al., 2015).

Investigações anteriores sobre a composição química do óleo essencial desta espécie relataram a presença de terpenos, como 1,8-cineol, α-pineno, cânfora, borneol, canfeno, α-terpineol, limoneno, β-pineno, β-cariofileno e mirceno (Raskovic et. al, 2014; Napoli et. al, 2015; Chávez- González 2016). A quantidade dos componentes varia de

acordo com a área geográfica, clima, partes da planta e método de extração (Mouahid et al., 2017; Tawfeeq et al., 2016; Yosr et al., 2013).

Uma metodologia de encapsulamento do OERO em nanomulsão foi desenvolvida usando sistema de baixa energia (Duarte et. al, 2015). Essas nanoformulações demonstraram atividades anti-inflamatória e antiálgica em modelos farmacológicos em nivel pré-clinico (Borges et. al, 2017; Borges et. al, 2018).

Há evidencias de que o OERO diminui os efeitos provocados pela reação inflamatória das vias aéreas, devido a sua capacidade de provocar o relaxamento do músculo liso. Em estudo realizado por Aqel (1991), o OERO inibiu as contrações estimuladas por acetilcolina, histamina e solução contendo elevada concentração de K^+ na musculatura lisa de traqueia de coelhos e cobaias, sendo sugerida a possível ação antagonista ao cálcio. Entretanto, há poucos estudos adicionais sobre o mecanismo da ação miorrelaxante do OERO nas vias aéreas. Neste estudo foi avaliada a ação do OERO na forma de nanoemulsão sobre sistema isolado de musculo liso traqueal de cobaia.

Material e Métodos

Produtos químicos

O cloreto de acetilcolina (ACh), cloreto de carbacol (CCh), propranolol e glibenclamida foram adquiridos da Sigma Chemical Co. (St. Louis, Millstone, EUA). O pentobarbital sódico foi adquirido da Pfizer S.A. de C.V. (México).

Material vegetal e óleo essencial de *Rosmarinus officinalis*

O material vegetal (partes aéreas de *Rosmarinus officinalis* L.) e as amostras do OERO foram obtidos, identificados e extraídos de acordo com a técnica descrita por Borges et al., 2018. Ressaltando que essas mesmas amostras foram utilizadas em estudos anteriores (Borges et. al, 2017; Borges et. al, 2018).

Analise do OERO por Cromatografia Gasosa com Epectrometria de Massa

Foram realizadas em Borges et al. (2017) e Borges et al. (2018), que consistiu no uso de sistema Shimadzu / GC 2010 acoplado a self-gun Shimadzu / AOC-5000 e detector de massa (Shimadzu MS2010 Plus) com um impacto de elétrons de 70 eV e equipado com uma coluna de sílica fundida de DB-5MS (Agilent Advanced J & W 30 m × 0.25 mm × 0.25 µm). O tempo total de análise foi de 35 min e a identificação dos compostos foi realizada utilizando a biblioteca de equipamentos NIST 5.0.

Preparo das nanoemulsões e caracterização

As nanoemulsões foram obtidas através de método de baixa energia, descrito por Duarte et. al (2015). Para uma massa final de 50 g, utilizou-se 90% de água, 5% de OERO e 5% de Tween 20. Inicialmente preparou-se uma fase orgânica, adicionando OERO e o surfactante em béquer. Agitou-se a mistura utilizando um agitador magnético (750 rpm) durante 30 minutos. Em seguida, a fase aquosa foi adicionada, com fluxo de 0,5 mL / min com agitação contínua durante 60 min (Borges et al., 2018).

O tamanho das gotículas e o índice de polidispersão das amostras NECHA, NECULT e NECOM foram determinados em Borges et. al (2018), através de espectroscopia de correlação de fótons utilizando o Zetasizer 5000 (Malvern Instruments, Malvern, UK). O tamanho médio das gotículas foi expresso como diâmetro médio (Orafidiya & Oladimeji, 2002).

Animais

Cobaias machos (300-450 g) foram adquiridos do Biotério Central do Departamento de Farmácia da Facultat de Quimica da Universidad Autonoma de Mexico. Estes foram mantidos em caixas de polietileno, sob condições convencionais e alimentados com dieta padrão (purina pellets) com livre acesso à água.

Esta pesquisa foi aprovada pelo Comitê de Ética em Uso de Animais da Universidade Federal do Amapá, através da autorização nº. 0021/2015. Os procedimentos envolvendo animais e seus cuidados foram conduzidos de acordo com a Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999), e em conformidade com as normas internacionais sobre cuidados e uso de animais de laboratório.

Preparo dos anéis de traquéia

Os animais foram submetidos a eutanasia por injeção intraperitoneal de pentobarbital sódico (75 mg/kg). A traquéia foi retirada e mantida em solução de Krebs (KHS). Em seguida, o excesso de tecidos conjuntivo e adiposo foi retirado e a traquéia foi dividida em oito pequenos anéis (2 mm) contendo dois a três segmentos cartilaginosos. Cada anel foi pendurado entre dois ganchos inseridos nas câmaras e mantidos em banho contendo solução de Krebs (NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 25, glicose 11.1. mM) a 37°C aerada com 95% de O_2 e 5% de CO_2. A tensão isométrica foi obtida através de polígrafo MP100 de oito canais (Biopack

System) com um transdutor de força Grass FT 03E. Os dados foram digitalizados e analisados por software. Os tecidos foram estabilizados na tensão de 1.5 × g durante 60 min e foram lavados com solução de Krebs em intervalos de 15 min antes de iniciar os experimentos.

Avaliação do efeito relaxante

Após o período de estabilização, os tecidos foram contraídos com cloreto de acetilcolina (3 µM) duas vezes em intervalos de 30 min, após estimulação com solução de Krebs. Trinta minutos depois, os tecidos foram contraídos com cloreto de carbacol (3 µM) e, em seguida, foram inseridas as concentrações crescentes dos OERO ou nanoemulsões (10, 50, 100 e 300 µg/mL). Os efeitos relaxantes das amostras foram expressos em percentual de contração (%).

Para avaliar os possíveis mecanismos de ação no relaxamento do músculo liso traqueal, a nanoemulsão NECHA foi selecionada por apresentar maior percentual de relaxamento. Foi avaliado o seu efeito sobre os canais de potássio (K^+) sensíveis a ATP e os receptores β2-adrenérgicos utilizando glibenclamida (10 µM) e propranolol (3 µM), respectivamente. Os mesmos foram adicionados nas câmaras contendo os anéis de traquéia previamente contraídos. As concentrações da nanoemulsão foram adicionadas (10, 50, 100, 300 e 500 µg/mL). Para a avaliação da inibição de contrações induzidas por íon cálcio (Ca^{2+}), os anéis de traqueia foram preparados da mesma maneira, porém foram mantidos em solução KHS 80µM livre de cloreto de cálcio. A ACh e o CCh também foram inseridos, seguidos de lavagens. Em seguida, a NECONTROL foi adicionada para avaliar o efeito da nanoemulsão controle e, após 15 minutos, foram inseridas as concentrações crescentes de cloreto de cálcio (0.01, 0.03, 0.1, 0.8, 1, 3, 10, 30, 100 µg/mL) para induzir a contração do musculo liso. Após lavagem, diferentes concentrações de NECHA foram adicionadas (10, 50, 100, 300 e 500 µg/mL).

Análise Estatística

Para a análise estatística dos resultados foi utilizado o programa GraphPad Prism 6.0. Os dados foram apresentados como média ± SEM (n = 10/grupos) expressos em percentual, considerando significativos os valores de $p < 0,05$. Utilizou-se ANOVA seguido do teste post hoc de Dunnett´s.

Resultados

No processo de extração, o óleo essencial OEMEX apresentou rendimento médio de 1.5%. Os óleos OECHA e OECULT apresentaram 1.0% e 2.5%, respectivamente (Borges et. al, 2018). Na caracterização das amostras OECHA, OECULT, OEMEX e OECOM os componentes majoritários identificados foram: 1,8-cineol (50.82, 16.84, 14.10, 33.70%) cânfora (19.16, 30.93, 33.18 e 27.68%) e α-pineno (10.12, 14.24, 8.04 e 8.13%), respectivamente (Tabela 1 e Figura 1) (Borges et. al, 2018). O óleo essencial OEMEX também apresentou 12.28% de Limoneno.

Na avaliação do tamanho das gotículas e índice de polidispersão (pdI) todas as nanoemulsões apresentaram gotículas de tamanho médio variável entre 77.32 – 152.30 nm e pdI abaixo de 0.3 nm. Avaliadas em Borges et. al (2018), a nanoemulsão NECHA apresentou gotículas de 77.32 ± 1.192000 nm e polidispersividade de 0.239 ± 0.006 nm, a NECOM apresentou gotículas de 89.87 ± 0.083 nm e pdI de 0.193 ± 0.008 nm, enquanto em NECULT o valor médio das gotículas foi de 98.01 ± 0.302 nm e a polidispersividade de 0.182 ± 0.001 nm. No presente estudo, a NEMEX demonstrou gotículas de 152.30 ± 60.550 nm e pdI de 0.158 ± 0.006 nm (Figura 2). Esses resultados demonstram que ao comparar as nanoemulsões obtidas, a NECHA destacou-se por apresentar gotículas de menores tamanhos.

O percentual de contração do músculo liso de traqueia provocado pelos OEROs e nanoemulsões foi estimado para avaliar a atividade miorrelaxante. Os OEROs produziram efeito relaxante do músculo liso, sendo capazes de diminuir os valores do percentual de contração. Na concentração máxima testada (300 µg/mL), reduziu as contrações para a faixa de 60.0 - 55.8% (Figura 3a).

A nanoemulsão controle NECONTROL, contendo apenas Tween 20 e água, apresentou 116.2 % de contração muscular, na concentração de 300 µg/mL. Esse resultado indica que o surfactante utilizado para preparar as nanoemulsões, Tween 20, não influenciou na atividade avaliada. Todas as nanoemulsões foram capazes de provocar relaxamento do músculo liso, assim como os seus respectivos óleos essenciais a 300 µg/mL.

As nanoemulsões, obtidas a partir dos OEROs, diminuíram o percentual de contração para uma faixa de 43.1 – 17.9 %, sendo OECOM 56.4 % e NECOM 43.1 %, OEMEX 55.8 % e NEMEX 20.5 %, OECULT 60.0 % e NECULT 18.8 %, OECHA 56.5 % e NECHA 17.9 % (Figura 3b e Figura 4). Observa-se que o óleo na forma de nanoemulsão produziu efeito relaxante mais eficaz do que o óleo essencial bruto. A nanoemulsão

NECHA foi a que apresentou menor percentual de contração do tecido muscular, sugerindo melhor efeito miorrelaxante dentre as nanoemulsões.

Quando empregada no ensaio em que o músculo liso foi tratado previamente com a glibenclamida (10 µM), a nanoemulsão NECHA (a 10, 50, 100, 300 e 500 µg/mL) não reduziu o efeito do pré-tratamento, apresentando percentual de contração na faixa de 107 – 8.8 % em concentrações crescentes. Na ausência de pré-tratamento com a glibenclamida, apresentou perfil similar de percentual de contração, na faixa de 103.9 – 22.6 %. Esses resultados sugerem que a nanoemulsão não atue através dos canais de K^+ dependentes de ATP. Ao ser avaliada no tecido tratado com propranolol, a NECONTROL (Tween 20 e água) manteve o percentual de contração próximo a 100% (102 – 97 %) nas concentrações testadas, demonstrando que não interferiu no mecanismo avaliado (Figura 5).

No ensaio onde o tecido foi tratado com propranolol (3 µM), observou-se que a NECHA apresentou percentual de contração entre 105 a -8.6 %, em concentrações crescentes (10, 50, 100, 300 e 500 µg/mL), indicando que a nanoemulsão não foi capaz de modificar o efeito do propranolol. Na ausência de propranolol, apresentou valores entre 103.9 a 22.6% de contração do tecido. Os resultados sugerem que a NECHA não atue através dos canais β-adrenérgicos. Nas concentrações testadas, a NECONTROL demonstrou não interferir no mecanismo avaliado, tendo em vista que o tecido apresentou percentual de contração na faixa de 101.6 – 97.1% (Figura 6).

Em solução livre de Ca^{2+} com KCl (80 mM), a adição cumulativa de $CaCl_2$ (0.01, 0.03, 0.1, 0.8, 1, 3, 10, 30, 100 µg/mL) induziu aumento gradual da tensão no músculo liso traqueal (22.9 – 100%). A nanoemulsão NECHA não inibiu essas contrações nas concentrações testadas, 50 µg/mL (10.6 – 100%), 100 µg/mL (8.5 – 100%) e 300 µg/mL µg/mL (7.3 – 100%) (Figura 7).

Discussão

Em estudo realizado por Napoli (2015), foram identificados 82 compostos químicos no óleo essencial de *Rosmarinus officinalis* L., destacando-se 1,8-cineol, α-pineno, limoneno, β-pineno, terpinoleno, β-mirceno, borneol, linalool, verbenona, cânfora, e β-cariofileno. As amostras de OECHA, OECULT e OECOM apresentaram como compostos majoritários 1,8-cineol e cânfora (Borges et al., 2018). A amostra de OERO, proveniente de *R. officinalis* cultivada na cidade do México (OEMEX), avaliada neste estudo também apresentou estes compostos dentre os majoritários.

Nanoemulsões viáveis tendem a apresentar reflexo levemente azulado, aspecto translúcido e ausência de cremeação (Forgiarini et. al, 2000). A nanoformulação anteriormente desenvolvida com o OERO deve apresentar valor médio de tamanho de gotículas menor que 200 nm (Duarte et. al, 2015). Em estudo realizado por Borges et. al (2018) as nanoemulsões NECHA, NECULT e NECOM apresentaram características de viabilidade, tendo destaque para a NECHA por apresentar menor valor médio de gotículas e capacidade de manter 75% do óleo efetivamente encapsulado. A NEMEX, avaliada no presente estudo, também demonstrou valor médio de gotículas e características de viabilidade de acordo com o estudo anterior.

A contração do músculo liso das vias aéreas ocorre através da constrição da parede brônquica, que reduz a passagem de ar. Essa contração ocorre frequentemente na asma, uma síndrome complexa relacionada ao aparelho respiratório caracterizada pela presença de inflamação crônica (Gerthoffer et. al, 2013).

Durante a contração das células musculares lisas das vias aéreas diversos mecanismos podem ser ativados. De acordo com Delmotte et. al (2010), a contração muscular lisa na asma ocorre quando a fosforilação da miosina promove a interação actina-miosina. Esse processo pode ocorrer a partir da cadeia leve da miosina (MLC) que é ativada pela miosina quinase (MLCK), cuja atividade é estimulada pelo aumento da concentração de Ca^{2+} intracelular. Ou também pode estar relacionado à redução da atividade da fosfatase da cadeia leve da miosina (MLCP), que desfosforila a MLC, resultando em diminuição da contração. A atividade da MLCP pode ser diminuída pela ativação da Rho-quinase (RHOK) ou da proteína quinase C (PKC) (Figura 8).

Nas fibras musculares lisas da traqueia, são encontrados receptores muscarínicos M_3, acoplados à proteína G. Estes, ao serem estimulados por agonistas, provocam broncoconstrição e secreção de muco a partir de glândulas submucosas. A ativação dos receptores muscarínicos pode promover a liberação de Ca^{2+} do retículo sarcoplasmático e a abertura dos canais de K^+ ativados pelo Ca^{2+}. Pode também provocar a despolarização da membrana e ampliar o potencial de ação em todo o músculo (Bolton & Lim, 1991). Os receptores muscarínicos podem ser ativados pela acetilcolina (Figura 8).

No presente estudo, os OEROs e as nanoemulsões inibiram as contrações induzidas por acetilcolina, estando de acordo com estudos anteriores que relatam a ação miorrelaxante do OERO no músculo liso (Aqel, 1991; Aqel, 1992). Além disso, observou-se que as nanoemulsões promoveram ação relaxante mais potente do que os óleos brutos. Isso provavelmente ocorreu devido a capacidade das nanoemulsões de aumentarem a possibilidade dos princípios ativos presentes no óleo essencial, que são

apolares, interagirem com os receptores membranares envolvidos no processo de contração dessa musculatura, uma vez que requerem menor concentração do OERO para apresentar efeito similar ao do óleo não encapsulado (Borges et al., 2018).

Dentre os compostos majoritários identificados nas amostras de OERO, o 1,8-cineol tem sido associado à atividade relaxante do músculo liso. Em estudo realizado por Nascimento et. al (2009), o cineol relaxou o músculo liso traqueal obtido de ratos e cobaias através de um mecanismo inespecífico. Quando os anéis de traqueia foram sensibilizados com ovoalbumina, essa atividade foi aumentada. Este efeito pode estar relacionado à sua ação anti-inflamatória através da inibição da produção de leucotrienos.

Principalmente ao 1,8-cineol o efeito anti-inflamatório do OERO tem sido atribuído. Isso ocorre devido a sua capacidade de diminuir a ação da Lipoxigenase e inibir a síntese de citocinas pró-inflamatórias e a geração de leucotrienos (Juerges et al., 1998; Santos e Rao; 2000; Borges et al., 2018). A diminuição dos níveis de leucotrienos pode interferir na liberação de histamina, uma das substâncias mediadoras do processo de contração do músculo liso nas vias aéreas de mamíferos (Nascimento et al., 2009). Este fato sugere uma possível via de ação indireta do 1,8-cineol na diminuição da contração do músculo liso traqueal.

Outro estudo atribui a ação miorrelaxante do 1,8-cineol a um possível efeito neurogênico, devido a sua capacidade de bloquear a amplitude do potencial de ação neuronal em nervo ciático isolado de rato. Isso possivelmente resultaria na diminuição da velocidade de condução de impulso nervoso que chega às fibras musculares e, consequentemente, na redução da contração (Soares et. al, 2005).

Além dos diversos possíveis mecanismos de ação estudados para o cineol na inibição da contração muscular, também foi relatada a baixa toxicidade desse monoterpeno para o músculo liso de traquéia, em análise *in vitro* e, a partir da ausência de sinais de toxicidade aguda em ratos após sua administração por via oral (Nascimento et al., 2009).

A glibenclamida é um inibidor seletivo dos canais de potássio regulados por ATP (K_{ATP}) encontrados em muitas células (Sobey, 2001). Quando estes canais estão abertos ocorre efluxo de K^+, promovendo a hiperpolarização da membrana que resulta no relaxamento das células musculares lisas (Nichols et. al, 1995).

O óleo essencial proveniente das folhas de *Rosmarinus officinalis* L. foi capaz de inibir as contrações do músculo liso aórtico de coelho induzidas por norepinefrina, em solução isenta de cálcio e em meio elevado em K^+. Estes dados sugerem que o OERO apresenta efeito relaxante direto do músculo liso vascular (Aqel, 1992). Já no músculo liso

traqueal, o 1,8-cineol não produziu relaxamento do tecido muscular em meio elevado em K^+, sugerindo que o seu mecanismo de ação não ocorre através da abertura dos canais de K^+ (Nascimento et al., 2009). Estes resultados estão de acordo com os apresentados neste estudo, onde a NECHA, nanoemulsão contendo óleo essencial com alto teor de 1,8-cineol, não atuou sobre os canais de K^+ sensíveis a ATP nas concentrações testadas (Figura 8).

A ativação dos receptores β2-adrenérgicos por agonistas pode resultar na diminuição das oscilações causadas pelo Ca^{2+} e também reduzir a sensibilidade das células musculares lisas a esse íon, sendo esse efeito variável entre as espécies (Delmotte et. al, 2010). É um mecanismo importante para o relaxamento do músculo liso das vias aéreas (Mangprayool et al., 2013).

O óleo essencial de *Zingiber officinale*, contendo 1,8-cineol entre os componentes majoritários, apresentou atividade espasmolítica em traqueia isolada de ratos pré-contraída com carbacol. Na investigação do possível mecanismo de ação, foi mostrado que este óleo pode apresentar ação agonista aos receptores β2–adrenérgicos devido à redução significativa do efeito miorrelaxante na presença de propranolol. Entretanto, como o teor de 1,8-cineol na amostra era relativamente baixo em relação a outros compostos majoritários, os autores afirmam que a contribuição desse composto para o efeito avaliado não foi representativa (Mangprayool et al., 2013). Isso sugere que o efeito agonista aos receptores β2-adrenérgicos não estava relacionado ao cineol. Esta hipótese corrobora com os resultados obtidos no presente estudo, onde a NECHA, contendo elevado teor de 1,8-cineol, não atuou sobre os receptores β2–adrenérgicos nas concentrações testadas (Figura 8).

O carbacol pode provocar oscilações nas concentrações de Ca^{2+} na membrana e no sarcoplasma em músculo liso de cobaias (Kohda et al., 1996). A acetilcolina pode provocar oscilação nas concentrações de Ca^{2+} dependentes, em parte, da presença de Ca^{2+} extracelular (Liu & Farley, 1996).

Em células musculares de cobaias estimuladas por carbacol, foram observadas oscilações, respostas induzidas por agonistas, na concentração de Ca^{2+} tanto no sarcolema quanto no sarcoplasma (Kohda et al., 1996). Essas oscilações podem surgir a partir do influxo desse íon através do sarcolema ou das reservas intracelulares (Roux et. al, 1997).

Como os óleos e as nanoemulsões foram capazes de relaxar o músculo liso, o seu mecanismo de ação poderia ocorrer através da interação com o receptor de agonistas ou através da regulação do influxo de Ca^{2+}. Entretanto, de acordo com Aqel (1991), é

improvável que o OERO atue através do receptor de agonistas devido a sua ação antagonica com os efeitos da histamina e da acetilcolina, sendo mais provável que atue através da interação com os canais de íon cálcio.

Ao avaliar a ação da NECHA na presença de cloreto de cálcio, foi observado que a mesma não apresentou atividade miorrelaxante através da abertura de canais de íon cálcio da membrana plasmática. Entretanto, isso não exclui a possibilidade de seu mecanismo de ação estar relacionado à regulação do controle da concentração de Ca^{2+} das reservas intracelulares. O Ca^{2+} proveniente das reservas intracelulares pode ser liberado pelo retículo sarcoplasmático através de canal dependente de voltagem ligado ao receptor de rianodina (RyR). Os RyRs são uma família de canais de liberação de Ca^{2+} encontrados em diversos tipos de células e envolvidos em mecanismos importantes (Fill e Copello, 2002).

Neste caso é importante considerar que o OERO pode interferir na liberação de cálcio a partir de fontes intracelulares (Aqel, 1991). Em músculo liso traqueal de ratos o 1,8-cineol (1 – 30 mg/kg) inibiu as contrações fásicas induzidas por solução elevada em K^+. Essa atividade foi relacionada à possível ação intracelular como segundo mensageiro (Nascimento et. al, 2009). Ou também é possível que atue através da regulação de calmodulina.

Desta maneira, pode-se afirmar que mais estudos são necessários para elucidar o mecanismo de ação pelo qual o OERO promove o relaxamento das fibras musculares lisas e também para confirmar a suposta relação deste efeito ao componente majoritário 1,8-cineol. Embora seja necessária uma análise mais aprofundada para correlacionar esta atividade, aqui fornecemos pela primeira vez informações sobre uma nova formulação com atividade miorrelaxante capaz de aumentar o efeito do óleo essencial, provavelmente, por favorecer a interação dos princípios ativos com os sítios alvo envolvidos no processo de contração da musculatura lisa de traqueia empregada.

Conclusão

As nanoemulsões NECHA, NECULT, NECOM e NEMEX foram capazes de potencializar o efeito relaxante dos óleos essenciais de *Rosmarinus officinalis* L. sobre o músculo liso de traqueia isolada de cobaia. Dentre as nanoemulsões, a NECHA destacou-se por aumentar significativamente o efeito relaxante do seu respectivo óleo essencial, apresentando melhor resultado quando comparada às outras nanoemulsões. Isso provavelmente ocorreu devido a sua maior estabilidade por apresentar gotículas de tamanho reduzido. Além de estudos anteriores demonstrarem a capacidade dessa

nanoformulação de manter elevada quantidade do óleo essencial efetivamente encapsulado, e possivelmente ampliar a disponibilidade do óleo em sistemas biológicos.

O efeito relaxante da nanoemulsão NECHA em músculo liso traqueal provavelmente não ocorre através dos canais de K^+(ATP) ou por receptores β2-adrenérgicos. A nanoemulsão também não apresentou efeito miorrelaxante na presença de $CaCl_2$, o que não exclui a possibilidade de o seu mecanismo de ação estar relacionado ao controle da concentração de Ca^{2+} intracelular ou à regulação de calmodulina. No entanto, mais estudos são necessários para confirmar esta hipótese.

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Tabela 1. Compostos químicos identificados no óleo essencial de *Rosmarinus officinalis* L. (OERO) através de CG-MS (IR calculado, ** IRlit: tabela IR para composto, * não identificado)

Pico	RT (min)	Composto	(%) GC-MS OECHA	(%) GC-MS OECOM	(%) GC-MS OECULT	(%) GC-MS OEMEX	IR exp.	IR lit.*
1.	4.872	α-thujene	-	0.11	-	-	928	926
2.	**5.054**	**α-pinene**	**10.12**	**8.13**	**14.24**	**10.12**	**935**	**939**
3.	5.546	β-Thujene	-	**-**	0.35	0.32	955	971
4.	5.424	Camphene	3.26	1.68	4.48	4.45	950	954
5.	6.045	Sabine	-	0.21	-	-	955	976
6.	6.152	β-pinene	1.10	0.58	1.27	4.90	979	979
7.	6.347	3- octanone	-	-	0.24	0.28	987	983
8.	6.482	β-myrcene	0.65	0.90	10.20	1.91	993	990
9.	6.911	α-phellandrene	0.13	0.77	0.37	5.48	1007	1002
10	7.282	α-Terpinene	0.33	0.45	0.87	0.96	1018	1017
11	7.532	o-cymene	1.92	1.65	1.67	2.03	1026	1026
12	**7.674**	**Limonene**	2.14	**21.99**	4.49	**12.28**	**1030**	**1031**
13	**7.773**	**1,8-cineole**	**50.82**	**33.70**	**16.84**	**14.10**	**1033**	**1033**
14	8.666	γ -Terpinene	0.16	0.39	1.18	1.41	1059	1059
15	9.724	Terpinolene	0.22	0.20	0.93	0.56	1091	1088
16	10.128	β-linalool	1.01	0.16	1.67	1.03	1102	1098
17	10.350	***	-	0.44	0.36	-	1108	***
18	10.662	***	0.10	-	-	0.10	1216	
19	**11.897**	**Camphor**	**19.16**	**27.68**	**30.93**	**33.18**	**1147**	**1146**
20	12.620	Isopinocamphone	-	**-**	0.20	0.14	1165	1160
21	12.736	Borneol	4.32	0.32	1.61	2.52	1168	1169
22	13.194	Isopinocamphone	0.83	-	1.06	0.83	1179	1173
23	13.739	α-terpineol	2.98	0.12	2.23	0.94	1193	1188
24	13.899	α-campholenal	-	0.20	-	-	1197	1125
25	14.005	Myrtenol	-	-	0.19	-	1199	1194
26	14.532	Verbenone	-	0.18	3.71	0.57	1213	1205
27	17.723	Bornyl acetate	0.26	-	0.38	1.17	1288	1288
28	23.220	β-caryophyllene	0.29	0.12	0.54	1.40	1421	1427
29	29.850	***	0.19	-	-	0.19	1960	

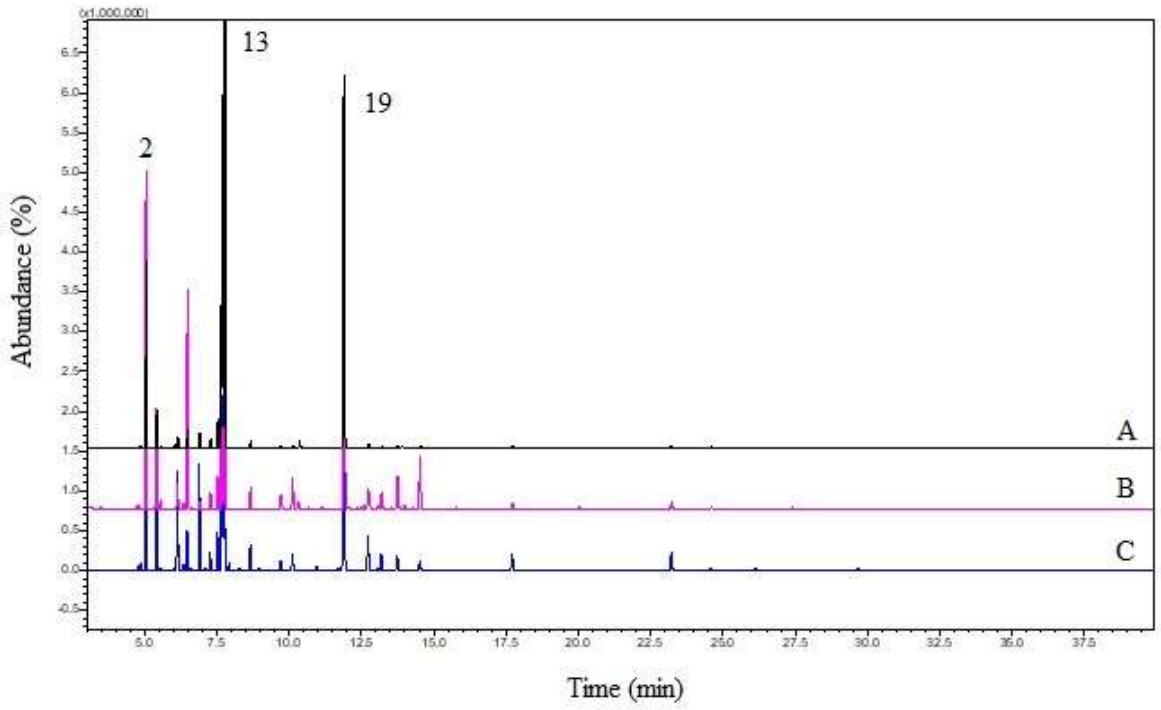


Figura 1. Cromatrogramas dos OEROs obtidos através de chromatography-mass spectrometry (GC-MS) com: A = OECOM corresponding to **2** – α-pinene (8.13%), **13** – 1,8-cineole (33.70%), **19** – camphor (27.68%); B = OECULT: corresponding to **2** – α-pinene (14.24%), **13** – 1,8-cineole (16.84%), **19** – camphor (30.93%) and C = OEMEX: corresponding to **2** – α-pinene (10.12%), **13** – 1,8-cineole (14.10%), **19** – camphor (33.18%).

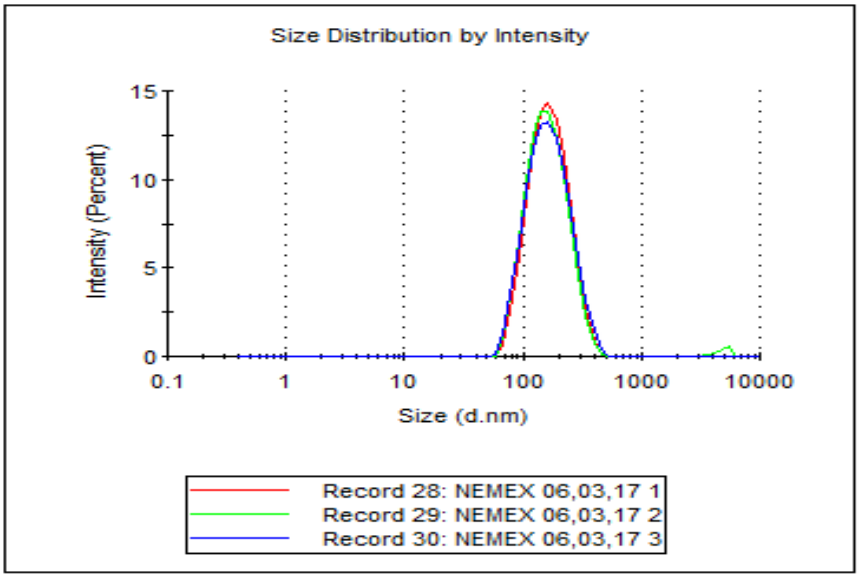


Figura 2. Análise de tamanho das gotículas e índice de polidispersão de NEMEX (152.30 ± 60.550 nm; polidispersividade 0.158 ± 0.006 nm).

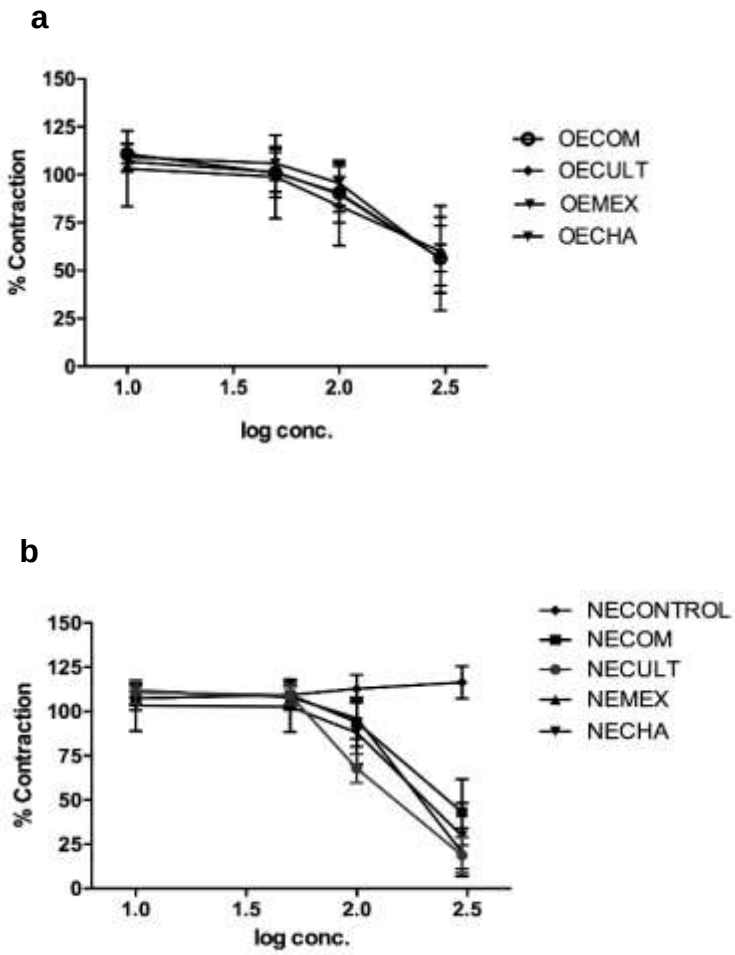


Figura 3. Efeito relaxante dos óleos essenciais (a) e nanoemulsões (b) de *Rosmarinus officinalis* L. através do percentual de contração (%) nas concentrações de 10, 50, 100 e 300 µg/mL, respectivamente: a: OECOM (110.9±2.1, 101.0±5.2, 90.5±6.4 e 56.4±11.1), OEMEX (109.3±2.4, 106.0±2.9, 95.7±3.9 e 55.8±5.9), OECULT (103.2±7.0, 99.0±7.7, 83.7±7.3 e 56.9±6.7), OECHA (106.9±1.8, 101.4±3.4, 89.8±3.0 e 56.5±2.4); b: NECONTROL (107.4±1.9, 109.3±2.6, 112.9±3.5 e 116.4±4.1), NECOM (107.4±1.9, 109.5±2.4, 93.9±3.8 e 43.1±5.2), NECULT (110.9±2.5, 109.2±2.0, 67.8±3.1 e 18.8±3.9), NEMEX (111.8±1.5, 108.0±2.3, 96.2±4.4 e 20.5±5.2), NECHA (103.3±3.8, 102.7±3.8, 88.1±4.8 e 17.9±8.2). Os dados são apresentados como média ± SEM (n = 10/grupos) expressos em percentual, * p 0,05 *vs*. control (NECONTROL), a análise estatística foi feita pelo teste ANOVA one-way seguida de post-hoc Dunnett´s.

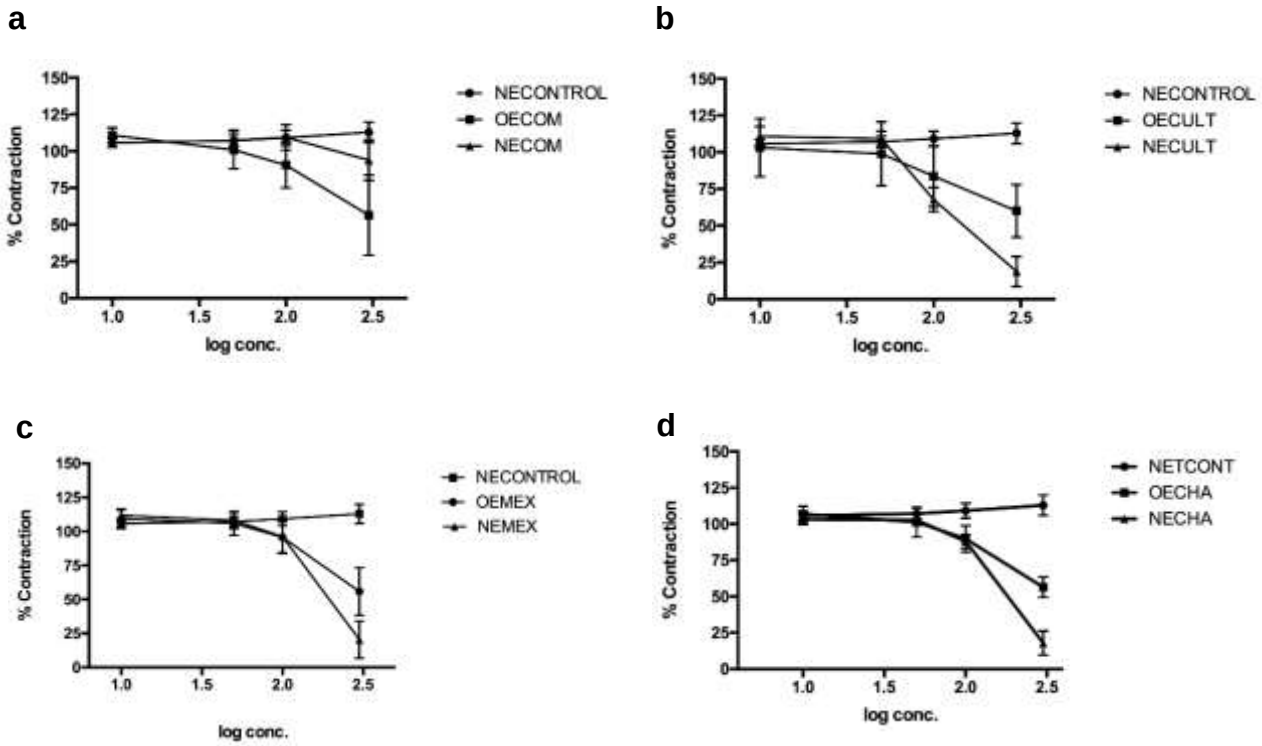
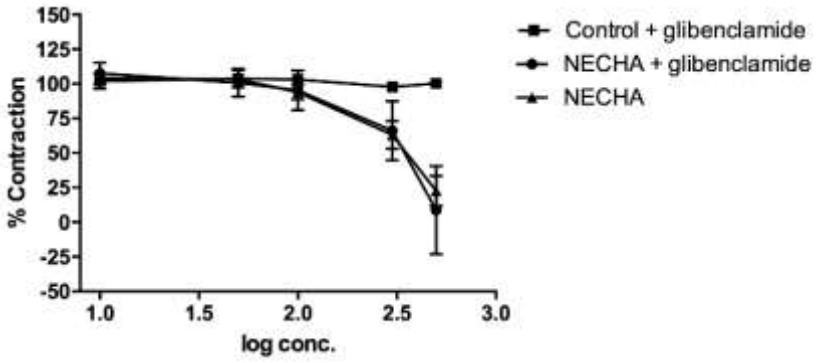


Figura 4. Comparação do efeito relaxante dos óleos essenciais de *Rosmarinus officinalis* L. com suas respectivas nanoemulsões, através do percentual de contração (%), em relação ao controle das nanoemulsões NECONTROL (116.4%), na maior concentração (300 µg/mL). a: OECOM (56.4±11.1) *vs* NECOM (43.1±5.2), b: OECULT (56.9±6.7) *vs* NECULT (18.8±3.9), c: OEMEX (55.8±5.9) *vs* NEMEX (20.5±5.2), d: OECHA (56.5±2.4) *vs* NECHA (17.9±8.2). Os dados são apresentados como média ± SEM (n = 10/grupos) expressos em percentual, * p 0,05 *vs*. control (NECONTROL), $^{\&}$ p 0,05 *vs*. OEs, a análise estatística foi feita pelo teste ANOVA one-way seguida de post-hoc Dunnett´s.

a



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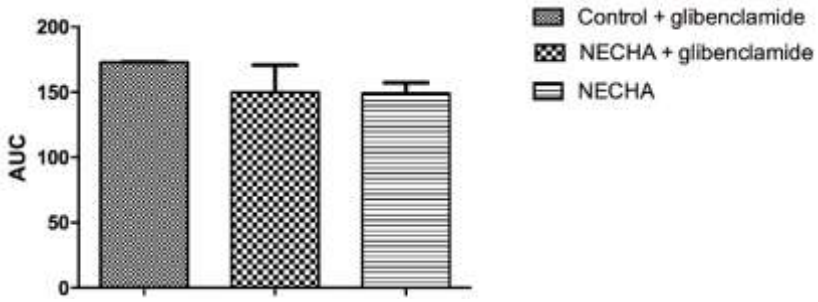

Figura 5. Efeito da glibenclamida sobre a ação relaxante da NECHA a 10, 50, 100, 300 e 500 µg/mL. a: percentual de contração (%) do controle + glibenclamida (102.0±3.9, 103.8±4.5, 103.2±1.1, 97.8±1.2 e 100.2±0.9), NECHA + glibenclamida (107.5±3.5, 100.7±4.5, 95.2±6.5, 66.1±9.5 e 8.8±14.3) e NECHA (103.9±1.2, 103.4±1.5, 94.0±2.2, 63.1±4.1 e 22.6±4.4); b: área sob a curva do controle + glibenclamida, NECHA + glibenclamida e NECHA. Os dados são apresentados como média ± SEM (n = 10/grupos) expressos em percentual, * p 0,05 *vs*. control + glibenclamide, a análise estatística foi feita por teste ANOVA one-way seguida de post-hoc Dunnett´s.

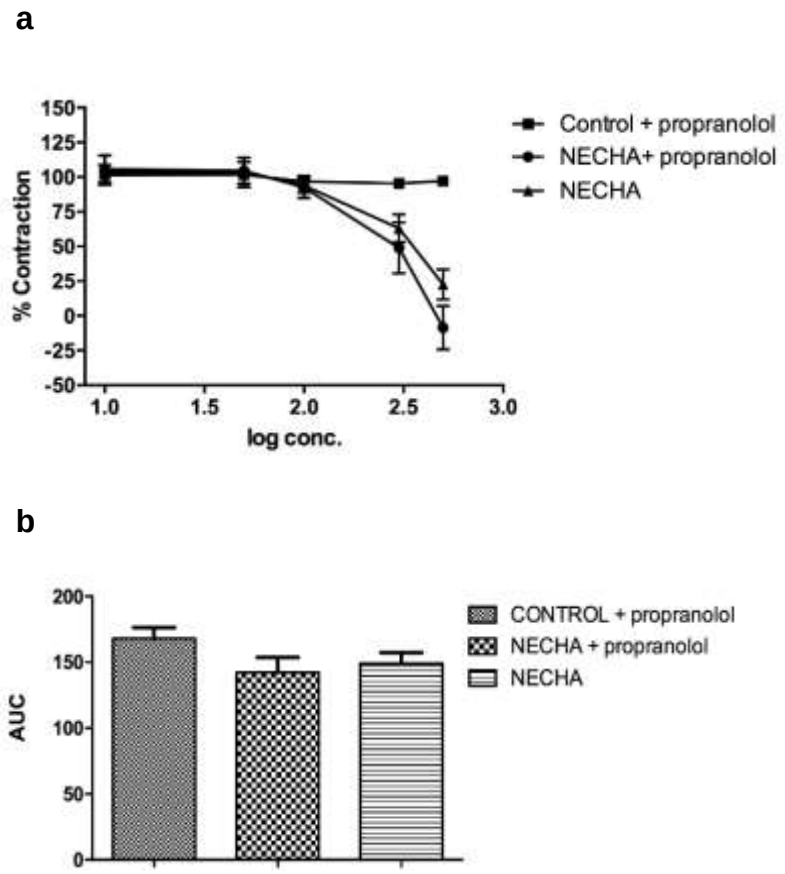

Figura 6. Efeito do propanolol sobre a ação relaxante da NECHA a 10, 50, 100, 300 e 500 µg/mL. a: percentual de contração (%) do controle + propanolol (101.6±5.1, 101.8±6.4, 96.7±2.9, 95.4±0.4 e 97.1±0.4), NECHA + propanolol (105.7±4.4, 104.6±4.2, 92.6±3.4, 49.0±8.2 e -8.6±7.0) e NECHA(103.9±1.2, 103.4±1.5, 94.0±2.2, 63.1±4.1 e 22.6±4.4); b: área sob a curva do controle + propranolol, NECHA + propanolol e NECHA. Os dados são apresentados como média ± SEM (n = 10/grupos) expressos em percentual, * p 0,05 *vs*. control + propanolol, a análise estatística foi feita por ANOVA one-way seguida de post-hoc Dunnett´s.

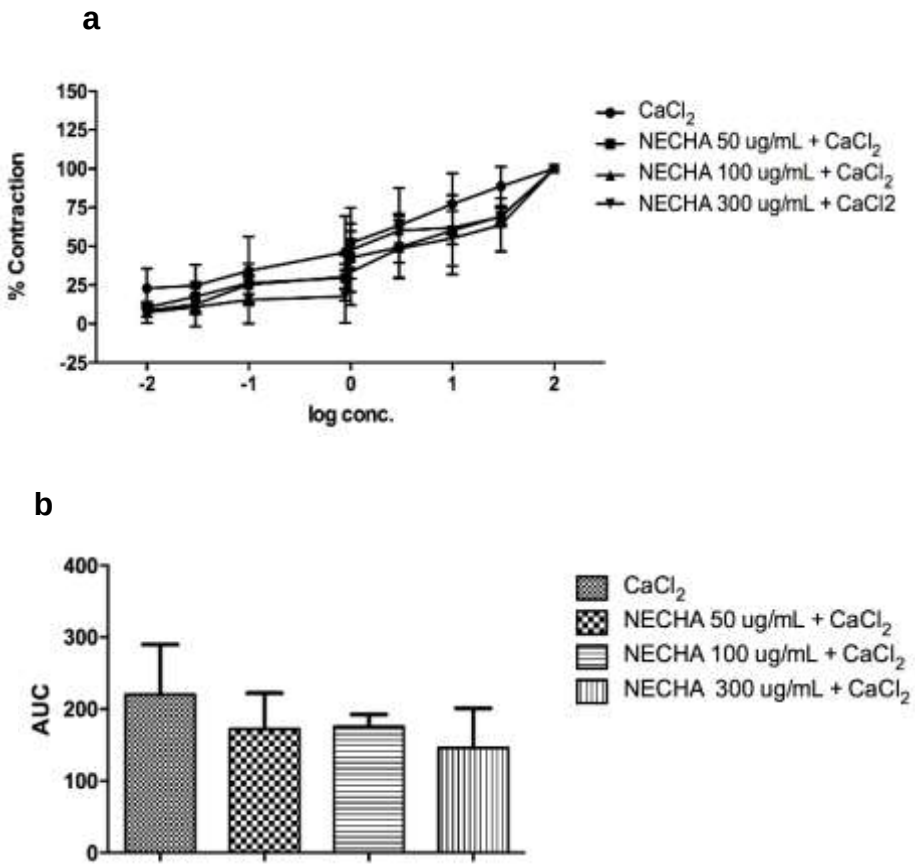

Figura 7. Efeito do $CaCl_2$ sobre a ação relaxante da NECHA (50, 100, 300 µg/mL) a: percentual de contração (%) a 0.01, 0.03, 0.1, 0.8, 1, 3, 10, 30, 100 µg/mL de $CaCl_2$, que também foi usado como controle. a: $CaCl_2$ (22.9±3.7, 24.7±3.9, 34.1±6.4, 46.1±6.7, 52.1±6.6, 63.4±6.9, 77.1±5.8, 88.6±3.7 e 100±0.0), NECHA 50 µg/mL + $CaCl_2$ (10.6± 1.9, 17.5±5.5, 26.2±7.3, 29.6±8.4, 42.6±12.7, 49.3±11.7, 60.1±13.1, 69.3±2.9 e 100±0.0), NECHA 100 µg/mL + $CaCl_2$ (8.5±2.0, 12.5±3.2, 25.3±3.1, 30.4±4.1, 47.5±6.1, 60.0±5.4, 62.0±5.3, 68.8±3.1 e 100±0.0) e NECHA 300 µg/mL + $CaCl_2$ (7.3±3.1, 10.9±5.7, 15.4±6.9, 17.5±7.6, 33.4±9.5, 48.4±8.3, 55.1±10.5, 63.8±7.6 e 100±0.0); b: área sob a curva (b) do $CaCl_2$, NECHA 50 µg/mL+ $CaCl_2$, NECHA 100 µg/mL+ $CaCl_2$ e NECHA 300 µg/mL+ $CaCl_2$. Os dados são apresentados como média ± SEM (n = 10/grupos) expressos em percentual, * p 0,05 *vs*. control ($CaCl_2$), a análise estatística foi feita com ANOVA one-way seguida de post-hoc Dunnett´s.

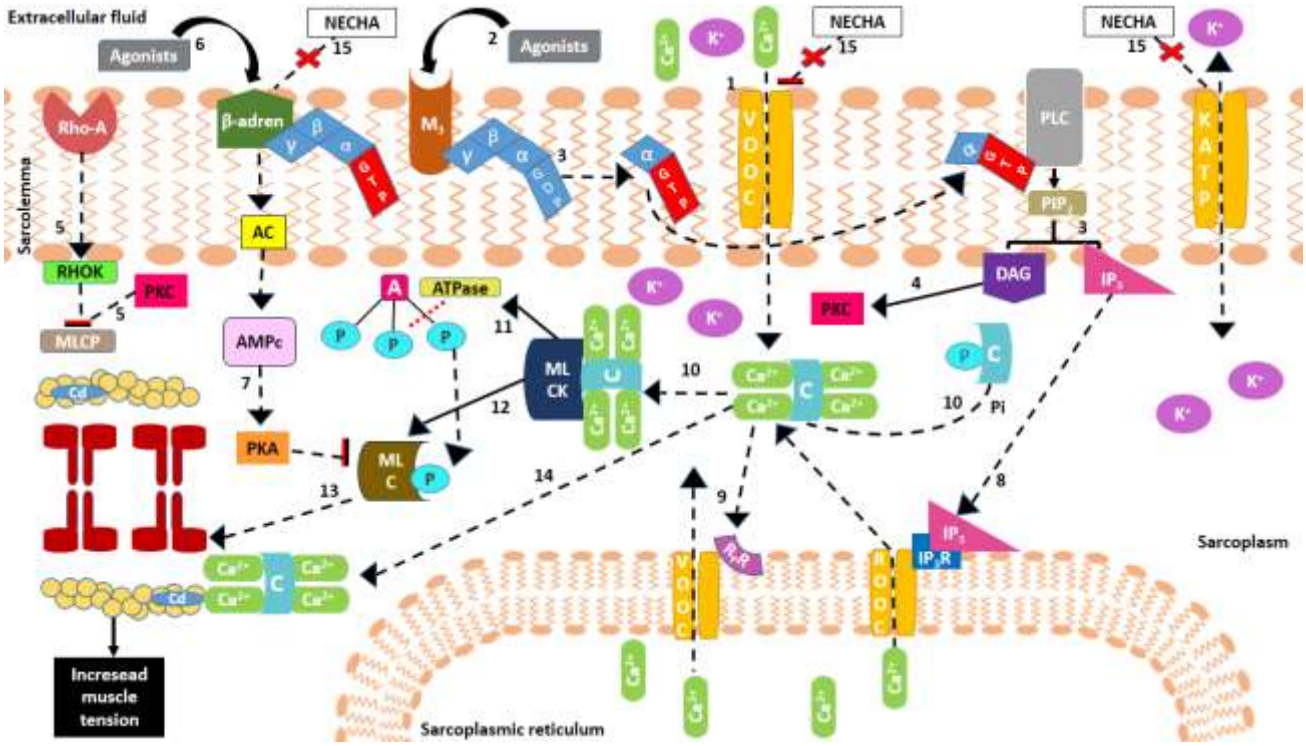

Figura 8. Mecanismos de contração e inibição da contração do músculo liso. 1. A variação da concentração de íons cálcio (Ca^{+}) e potássio (K^{+}) entre os meios intra e extracelular provoca a despolarização da membrana, que promove a abertura dos canais de cálcio operados por voltagem (VOCC) e entrada de íons Ca^{2+} para o sarcoplasma; 2. Receptores acoplados à proteína G (GPCR), como os receptores M_3 ativados por acetilcolina, ao serem estimulados por agonistas promovem despolarização da membrana e ocorre a ativação do difosfato de guanosina (GDP). 3 O GDP é ativado em trifosfato de guanosina (GTP), que resulta na liberação da porção α-GTP para ativar a Fosfolipase C (PLC), que cliva o Fosfatidilinositol bifosfato (PIP_2) em Diacilglicerol (DAG) e Inositol trifosfato (IP_3); 4. O DAG formado a partir do PIP_2 ativa a proteína quinase C (PKC); 5. A PKC pode atuar diminuindo a atividade da MLCP, a Rho quinase (RHOK) também pode apresentar essa ação ao ser ativada; 6. Ao serem estimulados por agonistas, os receptores β-adrenérgicos sofrem ativação da proteína G e ativam a adenililcilase (AC), que regula a formação do nucleotídeo cíclico AMPc; 7. Níveis elevados de AMPc ativam a proteína quinase A (PKA), que reduz a fosforilação da cadeia leve da miosina (MLC); 8. O IP_3, formado a partir do PIP_2, se liga ao receptor de IP_3 (IP_3R) da membrana do retículo sarcoplasmático induzindo a abertura do canal de cálcio operado por receptor (ROOC), que promove a liberação de Ca^{2+} para o sarcoplasma; 9. O aumento da concentração de Ca^{2+} no sarcoplasma induz a ativação do receptor de rianodina (R_YR) ligado ao canal de cálcio operado por voltagem (VOOC) e libera mais Ca^{2+} para o sarcoplasma; 10. A Calmodulina fosforilada se liga a quatro íons cálcio livres formando o complexo Ca^{2+}-Calmodulina (CaCM), que ativa a quinase da cadeia leve da miosina (MLCK); 11. A MLCK ativa a enzima ATPase a hidrolisar a molécula de adenosina trifosfato (ATP) em adenosina difosfato (ADP) e fosfato inorgânico (P); 12. A MLCK liga o P à cadeia leve da miosina (MLC); 13. A MLC fosforilada promove a fosforilação da região globosa da Miosina (M); 14. O complexo *CaCM* se liga à Caldesmona (Cd), que libera o sítio de ligação da actina, possibilitando a contração muscular; 15. Ensaios realizados no presente estudo sugerem que a nanoemulsão NECHA não

atue através dos canais de K^+(ATP) ou os β2-adrenérgicos, assim como também não bloqueia os canais de Ca^{2+} operados por voltagem (VOOC) presentes na membrana plasmática.

8 DISCUSSÃO GERAL

Rosmarinus officinalis L. é uma espécie vegetal da família Lamiaceae (LORENZI; MATOS, 2002) comumente usada para fins medicinais, em forma de infusão e óleo essencial (MARCHIORI, 2004). O óleo essencial de *Rosmarinus officinalis* L. (OERO) é um líquido de coloração amarelada e aroma intenso, cuja importância comercial está relacionada à sua aplicação na indústria farmacêutica e alimentar (KFOURY et al., 2015).

Com base nos estudos fitoquímicos disponíveis na literatura, e apresentados no **capítulo 1**, foram identificados 150 compostos químicos em amostras de OERO derivadas de regiões diversas. A variação de componentes e suas quantidades ocorre devido a influência do clima, área geográfica, partes da planta utilizadas e o método de extração empregado (YOSR et al., 2013; MOUAHID et al., 2017).

Os componentes químicos característicos deste óleo são 1,8-cineol, α-pineno, cânfora, acetato de bornilo, borneol, canfeno, α-terpineol, limoneno, β-pineno, β-cariofileno e mirceno (CHÁVEZ-GONZÁLEZ, 2016). Neste caso, as amostras de OERO podem ser classificadas de acordo com o componente químico majoritário, sendo denominado cineolífero (quando há a prevalência de 1,8-cineol), canforífero (com elevada concentração de cânfora) ou α-pineno (quando ocorre predominância de α-pineno) (NAPOLI et al., 2015; TOMI et al., 2016).

Estes estudos estão de acordo com os resultados apresentados nos **capítulos 2 e 3**, onde foram avaliadas quatro amostras de OERO, de procedência diversificada (OECULT, OECHA, OECOM e OEMEX) através de Cromatografia gasosa acoplada a espectrômetro de massa (GC-EM). Os principais componentes identificados foram 1,8-cineol (16.84, 50.82, 33.70, 14.10%), cânfora (30.93, 19.16, 27.68, 33.18%) e α-pineno (14.24, 10.12, 8.13, 10.12%), respectivamente.

De acordo com a revisão apresentada no **capítulo 1**, estudos relacionam ao 1,8-cineol as atividades antidepressiva, antiálgica, antioxidante e anti-inflamatória (JUHÁS et al., 2009; FARIA et al., 2011; MACHADO et al., 2013; VILELA et al., 2016; TAKAYAMA et al., 2016; BAJALAN et al., 2017; SELMI et al., 2017). À cânfora são atribuídas as atividades anti-inflamatória, antiálgica, antimutagência e antioxidante (FAHIN et al., 1999; FARIA et al., 2011; MELO et al., 2011; TAKAYAMA et al., 2016; BAJALAN et al., 2017). O α-pineno tem sido relacionado às atividades antifúngica, antioxidante e antibacteriana (LIN

et al., 2016; MEKONNEN et al., 2016; TAKAYAMA et al., 2016).

Entretanto, os óleos essenciais apresentam baixa disponibilidade em sistemas biológicos devido às suas características lipofílicas. Esta questão tem sido considerada um desafio tecnológico. Indústrias farmacêuticas têm usado sistemas de transporte coloidal para encapsular compostos lipofílicos, permitindo a sua dispersão em meio aquoso (MCCLEMENTS; RAO, 2011). As nanoemulsões são sistemas promissores para a veiculação de fármacos com pouca solubilidade em água e já foram propostas para serem associadas ao OERO (DUARTE et al., 2015). São caracterizadas por sua estabilidade termodinâmica e apresentam tamanhos de gotas entre 20 e 200 nm (OSTERTAG et al., 2012).

Os resultados obtidos nos **capítulos 2, 3 e 5** demonstram que as nanoemulsões obtidas a partir do OERO apresentaram características de estabilidade como ausência de cremeação, aspecto translúcido e reflexo levemente azulado. Na avaliação do tamanho das gotículas, todas as nanoemulsões apresentaram gotículas de tamanho médio variável entre 77.32 – 152.30 nm e pdI abaixo de 0.3 nm. A nanoemulsão NECHA se destacou por apresentar tamanhos menores de gotículas (77.32 ± 1.192000 nm), além de sua capacidade de manter grande parte do óleo encapsulado no interior das micelas, apresentando eficácia de encapsulação de 75% (**capítulo 2**).

Este fato pode estar relacionado ao elevado teor do composto 1,8-cineol na amostra de OECHA (50.82%), que possivelmente atuou como estabilizador das micelas devido a sua capacidade de inibir a instabilidade por Ostwald Ripening (OR) sem interferir na atividade biológica (SALIDO et al., 2003).

No **capítulo 2**, a nanoemulsão obtida a partir do óleo essencial de *R. officinalis* (NOERO) demonstrou atividade anti-inflamatória no teste de edema de pata de rato induzido por carragenina. A NOERO, a 498 µg/kg, inibiu a formação do edema em 46%, enquanto o OERO, a 300 mg/kg, inibiu em 50%. A nanoemulsão também apresentou atividade antiálgica, no teste de contorção induzida por ácido acético em ratos, inibindo de forma mais eficaz o processo algésico, em 84%, do que o OERO, que inibiu 55%. Em estudo anterior o OERO demonstrou ação anti-inflamatória nos processos inflamatórios agudos e crônicos e atividade analgésica periférica, além de não provocar danos na mucosa gástrica. Os efeitos anti-inflamatório e antinociceptivo foram relacionados à presença de 1,8-cineol e cânfora (FARIA et al., 2011).

É importante considerar que os doadores de H_2S apresentam capacidade de reduzir a formação de edema e a adesão de leucócitos ao endotélio vascular, além de inibir a síntese de citocinas pró-inflamatórias e aumentar a resistência da mucosa gástrica

a lesões (WALLACE, 2007). Considerando que a administração de OERO e NOERO foi realizada por via oral e o H_2S é um composto gasoso bastante relacionado à ação gastroprotetora, neste capítulo foi avaliada a produção de H_2S na mucosa estomacal de ratos. A NOERO inibiu a produção de H_2S em todas as fases de medida e o OERO reduziu 60% dessa produção. Os resultados apresentados neste estudo podem estar relacionados ao efeito anti-inflamatório do OERO na mucosa gástrica de não provocar lesões e aumentar a produção de muco (FARIA et al., 2011). Neste caso, a nanoemulsão pode ter potencializado este efeito e induzido a formação de uma camada de muco consistente, que poderia interferir na detecção desse mediador pelo sistema de eletrodos.

Os efeitos antiálgico e anti-inflamatório avaliados no **capítulo 2** foram associados à molécula de cânfora, que apresentou o maior número de interações com alvos terapêuticos relacionados ao processo inflamatório. A ancoragem molecular é um método computacional amplamente utilizado no processo de descoberta de possíveis mecanismo de ação de fármacos (CHANDAK et al., 2014), através da identificação dos modos de interação das moléculas no sítio de enzimas ou receptores.

Nos ensaios *in vitro*, a NECHA apresentou resultados de destaque em relação as outras nanoemulsões. No ensaio de citotoxicidade em macrófagos, nenhuma das amostras reduziu significativamente a quantidade de células, apresentando, em algumas concentrações, efeito de proliferação celular. Dentre as nanoemulsões, a NECHA apresentou aumento no número de células em todas as concentrações avaliadas. Por este motivo, foi realizado o teste de viabilidade celular com fibroblastos, onde a NECHA apresentou proliferação de células viáveis, com valores similares aos demonstrados pelo controle positivo, o ácido ascórbico. A nanoemulsão NECHA também demonstrou maior efeito inibitório da produção de NO· do que o óleo essencial OECHA em todas as concentrações avaliadas (**capítulo 3**). É importante considerar que essas células participam da resposta inflamatória e/ou produzem fatores de crescimento que controlam a proliferação e diferenciação celular, atuando também nos processos de cicatrização (TRAN et al., 2011).

Para ampliar o estudo da atividade anti-inflamatória *in vivo*, foi realizado o teste de edema abdominal induzido por carragenina em zebrafish. Esta é uma proposta nova de adaptação do ensaio realizado frequentemente em ratos, utilizando o modelo animal do zebrafish, que tem sido aplicado de forma exponencial em pesquisas farmacêuticas nos últimos anos (HUANG et al., 2014). O zebrafish apresenta vantagens em relação a outros animais por apresentar curto ciclo de vida, tamanho diminuto, maior custo-benefício e cerca de 70% de similaridade genética com os seres humanos (HOWE et al., 2013).

Em zebrafish, a NECHA foi capaz de inibir 77.99% da formação de edema abdominal induzido por carragenina, atuando de maneira mais eficaz do que os fármacos avaliados, Diclofenaco (49.5%) e Dexametasona (10.35%) (**capítulo 3**). Estes resultados demonstram que os compostos terpênicos majoritários (α-pineno, 1,8-cineol e cânfora) se tornaram mais disponíveis nos locais-alvo, inibindo o processo inflamatório. De acordo com Huang et al. (2014), este fato pode estar relacionado à capacidade dos compostos que apresentam propriedades anti-inflamatórias de modular as respostas induzidas pela carragenina em zebrafish adultos.

Estudos toxicológicos tem sido realizados com o zebrafish, principalmente para a avaliação da toxicidade de nanoformulações e/ou de extratos de origem vegetal (SOUZA et al., 2016). Tendo em vista que os OEROs e as NOEROs apresentaram potencial de inibição da formação de edema inflamatório em zebrafish, este também foi selecionado como modelo ideal para a avaliação da toxicidade das nanoemulsões obtidas no presente estudo.

No **capítulo 4**, as nanoemulsões NECHA, NECULT e NECOM, administradas por via oral em doses de 2 mg/kg, 3 mg/kg e 4 mg/kg em zebrafish, provocaram alterações comportamentais em todas as doses empregadas. Isto ocorreu poque a administração de nanoformulações em zebrafish pode induzir estresse nos animais (SOUZA et al., 2016). Entretanto, nenhuma das amostras provocou 100% de mortalidade. As nanoemulsões na menor dose (2 mg/kg) induziram até 40% de morte nos animais, enquanto as mais elevadas (3 mg/kg e 4 mg/kg) foram capazes de provocar mortalidade de até 80%.

Apesar de apresentarem as concentrações reais de apenas 100 µg, 150 µg e 200 µg de OERO, as nanoemulsões foram eficazes na avaliação da toxicidade, comprovando capacidade de ampliar a biodisponibilidade desse óleo. Por apresentarem diferença de tamanho e área superficial, ao serem aplicadas em organismos vivos, as substâncias em escala nano podem provocar toxicidade diferencial de acordo com sua estrutura e composição química (MARTINEZ et al., 2017). O efeito de nanopartículas em organismos aquáticos depende da solubilidade, composição química, estrutura da superficie, entre outros (BRUNDO et al., 2016).

A NECHA apresentou menor toxicidade quando comparada às outras nanoemulsões, sendo esta hipótese comprovada no estudo histopatológico onde os órgãos mais alterados, rins e intestino, apresentaram alterações predominantes como hipertrofia das células tubulares, vacuolização e ruptura dos vasos, que foram menos frequentes nos animais tratados com essa nanoemulsão. O zebrafish tem sido considerado um modelo confiável em ensaios de toxicidade por apresentar

metabolização de substâncias exógenas e histopatologia equivalente à de mamíferos (MUELLER; WULLIMANN, 2002; VLIEGENTHART et al., 2014; RYU et al., 2018).

Considerando que a atividade relaxante do músculo liso tem sido associada por estudos anteriores como um efeito importante na redução dos sintomas provocados pela inflamação nas vias aéreas, e que, em estudo com humanos, um dos componentes do OERO, o 1,8-cineol, mostrou-se eficaz na redução da inflamação em pacientes com asma aguda (JUERGENS et al., 2003), foi realizado o ensaio em traquéia isolada de cobaia para avaliar a atividade relaxante de OEROs e NOEROs no músculo liso traqueal **(capítulo 5)**. As nanoemulsões NECHA, NECULT, NECOM e NEMEX foram capazes de potencializar o efeito miorrelaxante dos OEROS, reduzindo o percentual de contração de 60.0-55.8% para 43.1-17.9%. Dentre elas, a NECHA destacou-se por aumentar significativamente o efeito relaxante de OECHA. Estes resultados corroboram com estudos anteriores que demonstram a capacidade do OERO em promover o relaxamento do músculo liso (AQEL, 1991; AQEL, 1992).

O efeito miorrelaxante da nanoemulsão NECHA não foi modificado ou bloqueado pelos canais de K^{+}_{ATP} ou por receptores β2-adrenérgicos nas concentrações avaliadas. A nanoemulsão também não apresentou efeito relaxante na presença de $CaCl_2$, o que sugere que a mesma não atue por essas vias. Estudos demonstram a atividade miorrelaxante do composto 1,8-cineol e a associam a outros mecanismos possíveis para a sua ação no músculo liso. O mesmo poderia atuar através da inibição da produção de leucotrienos (JUERGES et al., 1998; SANTOS; RAO, 2000; NASCIMENTO et al., 2009), que interfere na liberação de histamina, uma das substâncias mediadoras do processo de contração do músculo liso nas vias aéreas de mamíferos (NASCIMENTO et al., 2009). Poderia também bloquear a amplitude do potencial de ação neuronal em nervo ciático isolado de rato (SOARES et al., 2005), interferir na liberação de Ca^{2+} intracelular ou regular a ação da enzima Calmodulina.

Está bem estabelecido que o OERO apresenta atividade anti-inflamatória, relacionada aos compostos terpênicos. Entretanto, a lipofilicidade intrínseca dos óleos essenciais reduz a sua disponibilidade em sistemas biológicos. Neste estudo, as nanoemulsões obtidas a partir do OERO demonstraram eficácia no aumento da biodisponibilidade dos componentes majoritários e inibiram de maneira eficaz o processo inflamatório nos modelos avaliados. Apresentaram também capacidade de ampliar a ação antioxidante dos seus respectivos óleos essenciais em fibroblastos e potencializaram o efeito relaxante do OERO no músculo liso traqueal isolado de cobaia.

9 CONCLUSÃO

A utilização de produtos naturais para a obtenção de novos fámacos tem se tornado crescente no cenário mundial. Tradicionalmente, diversas plantas medicinais são empregadas no tratamento de enfermidades, e, por este motivo, têm sido estudadas amplamente para a investigação de suas atividades biológicas. O alecrim, de nome científico *Rosmarinus officinalis* L*.,* é usado popularmente para tratar inflamações, sendo ingerido em forma de infusões obtidas a partir do caule e das folhas. Diversos estudos ratificam a presença de propriedade anti-inflamatória, principalmente relacionada aos componentes terpênicos presentes no óleo essencial.

Entretanto, a baixa solubilização dos óleos essenciais dificulta a interação de seus componentes com os receptores ou canais presentes nas membranas biológicas. Por serem sistemas com estabilidade termodinâmica e ampla área superficial, as nanoelmulsões representam uma alternativa para a veiculação de substâncias com características lipofílicas. A obtenção de nanoformulações pode ser um processo oneroso quando se aplicam métodos de alto aporte energético. No entanto, a técnica de baixo aporte de energia empregada neste estudo pode tornar o processo mais acessível economicamente, facilitando sua aplicação em âmbito industrial.

É importante considerar que em todos os ensaios realizados, as nanoemulsões, contendo apenas 5% do OERO em sua formulação, demonstraram eficácia superior ou equivalente à do óleo essencial bruto. Dentre as nanoemulsões obtidas neste estudo, a NECHA destacou-se por apresentar menores tamanhos de gotículas, elevada estabilidade e capacidade de manter a maior parte do óleo efetivamente encapsulado. Também apresentou maior capacidade de melhorar a atividade anti-inflamatória e relaxante muscular atribuída ao seu respectivo óleo essencial (OECHA). Este efeito pode estar relacionado ao elevado teor de 1,8-cineol na composição do OECHA, tendo em vista que este composto tem sido relacionado à atividade anti-inflamatória e à capacidade de atuar como estabilizador de nanoemulsões.

Espera-se que os resultados apresentados neste trabalho possam servir de base para estudos posteriores e, consequentemente, impulsionem pesquisas clínicas com perspectivas de avaliação farmacológica em pacientes com enfermidades de origem inflamatória.

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ANEXOS

Anexo 1 – Parecer do Comitê de Ética em Pequisa no Uso de Animais.

Anexo 2 – Declaração de Estágio na Universidade Federal do Amazonas.

Anexo 3 – Declaração de Estágio na Universidad Nacional Autonoma de Mexico.

Anexo 4 – Certificado de apresentação de pôster na Semana Nacional de Ciência e Tecnologia – Macapá, AP / 2015.

Anexo 5 – Certificado de apresentação oral no I Encontro do PPGIF e PPGCF - Macapá, AP / 2016.

Anexo 6 – Certificado de premiação de 1º lugar na categoria comunicações orais do I Encontro do PPGIF e PPGCF – Macapá, AP/ 2016.

Anexo 7 – Cerificado de participação no 3º IPLeiria International Health Congress – Leiria, Portugal / 2016.

Anexo 8 – Certificado de participação no II Workshop da Rede PPGIF – Manaus, AM / 2016.

Anexo 9 – Artigo publicado na Revista Inflammopharmacology – Julho de 2017.

Anexo 10 - Artigo publicado na Revista Inflammopharmacology – Fevereiro de 2018.

Anexo 11 - Comprovante de submissão ao Journal of Ethnopharmacology.

Anexo 1 – Parecer do Comitê de Ética em Pequisa no Uso de Animais

UNIVERSIDADE FEDERAL DO AMAPÁ
PRÓ-REITORIA DE PESQUISA E PÓS- GRADUAÇÃO
COMITE DE ÉTICA NO USO DE ANIMAIS – CEUA – UNIFAP

CERTIFICADO

A Comissão de Ética no Uso de Animais da Universidade Federal do Amapá **APROVOU,** na reunião de 05 de janeiro de 2016, o parecer referente ao protocolo no. **0021/2015** e certifica que o Projeto de Pesquisa intitulado "**ESTUDO DA AÇÃO ANTI-INFLAMATÓRIA DE NANOEMULSÃO A BASE DO ÓLEO ESSENCIAL DE ROSMARINUS OFFICINALIS L.**" coordenado por **Raphaelle Sousa Borges**, está de acordo com os princípios de ética e bem-estar animal.

CERTIFICATE

The Ethics Committee on Animal Use of the Amapá Federal University **APPROVED** at the meeting of 05 January 2016, the final decision about the Protocol **0021/2015** and certify that the research project entitled " **ESTUDO DA AÇÃO ANTI-INFLAMATÓRIA DE NANOEMULSÃO A BASE DO ÓLEO ESSENCIAL DE ROSMARINUS OFFICINALIS L.**" coordinated by **Raphaelle Sousa Borges**, is in accordance with the principles of ethics and animal welfare.

Macapá, 05 de janeiro de 2016

Prof. Tit. José Carlos Tavares Carvalho
Presidente CEUA-UNIFAP
Port. No. 1733/2014

Universidade Federal do Amapá
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Anexo 2 – Declaração de Estágio na Universidade Federal do Amazonas

UFAM

UNIVERSIDADE FEDERAL DO AMAZONAS
Faculdade de Ciências Farmacêuticas

DECLARAÇÃO

Declaro para os devidos fins que a acadêmica Raphaelle Sousa Borges do Programa de Pós-Graduação em Inovação Farmacêutica, regularmente matriculada sob o nº 201512150001, realizou estágio no Laboratório de Atividade Biológica da Faculdade de Ciências Farmacêuticas da Universidade Federal do Amazonas – UFAM, durante o período de 01 a 30 de Março de 2016, com o propósito de realizar parte dos experimentos previstos em seu projeto de Doutorado, sob minha coordenação.

Atenciosamente,

Manaus, 31 de outubro de 2017.

UNIVERSIDADE FEDERAL DO AMAZONAS
Faculdade de Ciências Farmacêuticas
Prof. Dr. Emerson Silva Lima
Professor Associado

Anexo 3 – Declaração de Estágio na Universidad Nacional Autonoma de Mexico

FACULTAD DE QUÍMICA, UNAM • Dirección

OFICIO FQUI/DIR/214/17

M. en C. Raphaelle Sousa Borges
Estudiante de Doctorado en Innovación Farmacéutica
Facultad de Farmacia
Universidad Federal de Amapá, Brasil

Sirva el presente para informar que esta Facultad no tiene ningún inconveniente en recibirlo para realizar una estancia del 20 de febrero al 31 de marzo del año en curso, con el propósito de evaluar nanoformulaciones de aceites esenciales en los modelos de íleo y tráquea aislada de cobayo, en el laboratorio 126, del Dr. Andrés Navarrete Castro, Profesor Titular "C", adscrito al Departamento de Farmacia.

Sin otro particular, reciba un cordial saludo.

Atentamente.
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., febrero de 2017

EL DIRECTOR

Dr. Jorge Vázquez Ramos

JVR/FCG/ina

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Ciudad Universitaria, México, D.F. 04510
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jormaz@unam.mx

Anexo 4 – Certificado de apresentação de pôster na Semana Nacional de Ciência e Tecnologia – Macapá, AP / 2015

UNIFAP
UNIVERSIDADE FEDERAL DO AMAPÁ

Certificada

Certificamos que **Raphaelle Sousa Borges** apresentou o Pôster Intitulado "**Estudo da ação anti-inflamatória de nanoemulsão a base do óleo essencial de *Rosmarinus Officinalis* L**" *no II Encontro da Pós-Graduação da Semana Nacional de Ciência e Tecnologia 2015 – SNCT*, que ocorreu no período de 19 a 23 de outubro de 2015 na Universidade Federal do Amapá.

Profa Dra. Helena Cristina G. Queiroz Simões
Pró-Reitora de Pesquisa e Pós-Graduação
Portaria nº 1324/2014 - UNIFAP

Anexo 5 – Certificado de apresentação oral no I Encontro do PPGIF e PPGCF - Macapá, AP / 2016

UNIFAP

I ENCONTRO DO PPGCF E PPGIF DA UNIFAP

Certificado

Certificamos que o trabalho intitulado "**Estudo da ação anti-inflamatória de nanoemulsões a base de óleo essencial de *rosmarinus officinalis l. in vitro***" com autoria de Borges, R.S., Cruz, R.A.S., Fernandes, C.P., Ferreira, I.M., Santos, C.B.R., Duarte, J.L., Rosales, L.D., Silva, M.J.A., Lima, E.S., Conceição, E.C., Rodrigues, A.B.L., Carvalho, J.C.T., foi apresentado sob a forma de comunicação oral por **Raphaelle Sousa Borges** no **I Encontro do PPGCF e PPGIF da UNIFAP**, em 18 de Novembro de 2016.

Prof. Dr. Francisco Fábio Oliveira de Sousa
Coordenador do Programa de Pós-Graduação em Ciências Farmacêuticas
Coordenador do Programa de Pós-Graduação em Inovação Farmacêutica

Profª. Drª. Clárissa Silva Lima
Presidente da Comissão Organizadora do I Encontro do PPGCF e PPGIF da UNIFAP

Anexo 6 – Certificado de premiação de 1º lugar na categoria comunicações orais do I Encontro do PPGIF e PPGCF – Macapá, AP/ 2016

UNIFAP

I ENCONTRO DO PPGCF E PPGIF DA UNIFAP

Certificado

Certificamos que o trabalho intitulado **"Estudo da ação anti-inflamatória de nanoemulsões a base de óleo essencial de *Rosmarinus officinalis L. in vitro*"**, de autoria de **Raphaelle Sousa Borges**, Cruz, R.A.S., Fernandes, C.P., Ferreira, I.M., Santos, C.B.R., Duarte, J.L., Rosales, L.D., Silva, M.J.A., Lima, E.S., Conceição, E.C., Rodrigues, A.B.L., Amaral, F. A. S., e Carvalho, J.C.T., recebeu o prêmio de **PRIMEIRO LUGAR** na categoria de comunicações orais no **I Encontro do PPGCF e PPGIF da UNIFAP**, realizado em 17 e 18 de Novembro de 2016.

Prof. Dr. Francisco Fábio Oliveira de Sousa
Coordenador do Programa de Pós-Graduação em Ciências Farmacêuticas
Coordenador do Programa de Pós-Graduação em Inovação Farmacêutica

Prof. Drª. Clarissa Silva Lima
Presidente da Comissão Organizadora do I Encontro do PPGCF e PPGIF da UNIFAP

Anexo 7 – Cerificado de participação no 3º IPLeiria International Health Congress – Leiria, Portugal / 2016

Anexo 8 – Certificado de participação no II Workshop da Rede PPGIF – Manaus, AM / 2016

Universidade Federal do Amazonas

Programa de Pós-Graduação em Inovação Farmacêutica

CERTIFICA que

RAPHAELLE SOUZA BORGES, discente da Universidade Federal do Amapá, participou do *II Workshop da Rede de Inovação Farmacêutica*, nos dias 01 e 02 de dezembro de 2016. Manaus-AM, 02 de dezembro de 2016.

Prof. Dr. Emerson Silva Lima
Coordenador

Anexo 9 – Artigo publicado na Revista Inflammopharmacology – Julho de 2017

Inflammopharmacol
DOI 10.1007/s10787-017-0374-8

Inflammopharmacology

ORIGINAL ARTICLE

Anti-inflammatory and antialgic actions of a nanoemulsion of *Rosmarinus officinalis* L. essential oil and a molecular docking study of its major chemical constituents

Raphaelle Sousa Borges[1,7] · Emerson Silva Lima[2] · Hady Keita[1,3] · Irlon Maciel Ferreira[1] · Caio Pinho Fernandes[4,7] · Rodrigo Alves Soares Cruz[4] · Jonatas Lobato Duarte[1] · Josué Velázquez-Moyado[5] · Brenda Lorena Sánchez Ortiz[1,7] · Andrés Navarrete Castro[5] · Jaderson Vieira Ferreira[6,7] · Lorane Izabel da Silva Hage-Melim[6,7] · José Carlos Tavares Carvalho[1,7]

Received: 13 June 2017 / Accepted: 6 July 2017

Abstract We evaluate the anti-inflammatory and antialgic potency of a nanoemulsion (NEORO) containing the essential oil of *Rosmarinus officinalis* L. (EORO), which is composed primarily of limonene, camphor and 1,8-cineole. The EORO and NEORO were administered orally 30 min prior to starting the experiments. In a test of rat paw oedema induced by carrageenan, NEORO was effective in doses of 498 μg/kg, and it inhibited 46% of the maximum peak of the oedema; in a dose of 300 mg/kg, EORO inhibited 50% of the maximum peak of the oedema. In an acetic acid-induced writhing test, NEORO yielded a dose-dependent effect, and a dose of 830 μg/kg inhibited 84% of the algesic process; a dose of 100 mg/kg of EORO inhibited 55%. In an assay for H_2S production in rat stomachs, a dose of 498 μg/kg of NEORO inhibited H_2S production in all of the measurement phases, and a dose of 100 mg/kg EORO inhibited 60% and influenced the effect of the ethanol significantly, reducing the production of H_2S. We suggest that NEORO potentiated the effect of EORO, demonstrating effectiveness in doses 600 times lower than those applied with EORO. Among the major compounds of EORO, the camphor molecule exhibited the largest number of interactions with the therapeutic targets related to the inflammatory process, suggesting that it is responsible for EORO's anti-inflammatory and antialgic effects. This work paves the way for future investigations related to the therapeutic role of NEORO in the inflammation process.

Keywords *Rosmarinus officinalis* · Nanoemulsions · H_2S · Anti-inflammatory · Antialgic · Molecular docking

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Anexo 10 - Artigo publicado na Revista Inflammopharmacology – Fevereiro de 2018

Inflammopharmacology
https://doi.org/10.1007/s10787-017-0438-9

Inflammopharmacology

ORIGINAL ARTICLE

Anti-inflammatory activity of nanoemulsions of essential oil from *Rosmarinus officinalis* L.: in vitro and in zebrafish studies

Raphaelle Sousa Borges[1,2] · Hady Keita[1,3] · Brenda Lorena Sánchez Ortiz[1] · Tafnis Ingret dos Santos Sampaio[1] · Irlon Maciel Ferreira[1] · Emerson Silva Lima[3] · Márcia de Jesus Amazonas da Silva[4] · Caio Pinho Fernandes[5] · Anna Eliza Maciel de Faria Mota Oliveira[5] · Edemilson Cardoso da Conceição[6] · Alex Bruno Lobato Rodrigues[1] · Arlindo César Matias Pereira Filho[1] · Andrés Navarrete Castro[7] · José Carlos Tavares Carvalho[1,2,8]

Received: 5 November 2017 / Accepted: 26 December 2017

Abstract

The essential oil from *Rosmarinus officinalis* L. (OERO) has bioactive compounds with anti-inflammatory activity. The objective of this study was to evaluate the anti-inflammatory potency of nanoemulsions containing essential oil of *Rosmarinus officinalis* L. (NOERO, NECHA, NECULT, and NECOM) in vitro and in vivo. This study was accomplished in a quantitative format through tests with diphenyl picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), cellular antioxidant activity (CCA), determination of nitric oxide production, cellular viability and anti-inflammatory activity in zebrafish. OERO's were submitted to the analysis-coupled gas chromatography–mass spectrometry (GC–MS), which highlighted 1,8-cineol and camphor as major compounds. NOEROs were obtained by a low-energy method and presenting the medium size smaller than 200 nm. The efficiency of encapsulation by spectrometry and gas chromatographic analysis was 67.61 and 75.38%, respectively. In the CCA assay, all of the samples presented percentage values of inhibition similar to the quercetin pattern, indicating antioxidant activity. In the test for determination of NO·, all of the samples inhibited the production of NO· when compared to LPS, and NOEROS were more effective than OEROS to 5 µg/mL. In the cell viability assay, the cells remained viable after contact with the samples, demonstrating an absence of cytotoxicity. This study showed that all nanoemulsions (NECHA, NECULT, and NECOM) showed no toxicity to macrophages, besides demonstrating antioxidant activity and potentiation of the essential oil effect in the proliferation of viable fibroblasts. Nanoemulsions has also shown the ability to potentiate the anti-inflammatory action of essential oils by exerting immunomodulatory activity by inhibiting the production of the pro-inflammatory mediator nitric oxide. The results obtained with NECHA in zebrafish confirm the hypothesis that prominent terpenic compounds, alpha-pinene, 1,8-cineole, and camphor, became more available at the target sites, inhibiting the inflammatory process in this animal species.

Keywords *Rosmarinus officinalis* · Essential oil · Nanoemulsions · Anti-inflammatory · In vitro · In vivo · Zebrafish

Introduction

The Rosemary, scientific name *Rosmarinus officinalis* L., is a medicinal plant of the Lamiaceae family, that presents erect subshrub feature with little branch and height of up 1.5 m (Lorenzi and Matos 2002). Their leaves, quite aromatic, and stem are used popularly for medicinal purposes, ingested as tea (Marchiori 2004). It has been widely used in traditional medicine and as a flavouring in food (Ohno et al. 2003).

The essential oil of *Rosmarinus officinalis* L. (EORO), from the leaves, has been related to the presence of chemical compounds with specific properties (Celiktas et al. 2007). The chemical profile of EORO may vary according to environmental conditions; however, the most known chemotypes in the literature are cineoliferous (higher concentration of 1,8-cineole), camphorliferous (high concentration of camphor), and verbenoniferous (predominance of verbenona) (Napoli et al. 2015). Studies report bactericidal and fungicidal properties (Mekonnen et al. 2016),

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Anexo 11 - Comprovante de submissão ao Journal of Ethnopharmacology

Manuscript Details

Manuscript number	JEP_2018_1374
Title	Phytochemical and pharmacological studies of Rosmarinus officinalis L. essential oil related to anti-inflammatory activity
Article type	Review Article

Abstract

Ethnopharmacological relevance The plant species Rosmarinus officinalis L. is widely used all over the world for many purposes ranging from use in cooking, spiritual healing and as a popular medicine for inflammatory processes in general. Several authors have demonstrated in several studies that the essential oil of Rosmarinus officinalis L. (EORO) presents biological activities, as its bioactive compounds. The major components, such as 1,8-cineol, camphor, and α-pinene, have been associated with the anti-inflammatory activity. Aim of the study This review aimed to describe the chemical composition of EORO and the main studies related to anti-inflammatory activity and its possible mechanisms of action. Materials and methods We researched Medline, Embase, BVS Regional Portal, Science Direct, CAPES Journals, Scopus, using the keywords Rosmarinus officinalis L., anti-inflammatory and essential oil, complementary information was obtained from related textbooks. Results 150 chemical compounds were identified in EORO samples. Studies report that the anti-inflammatory effect of EORO may possibly occurs through inhibition of arachidonic acid metabolites formation and possibly inhibition of NF-κB transcription, which is synergistic with the antioxidant and relaxing activities of smooth muscle. Conclusions Although it has been widely evaluated in recent years, EORO will most likely continue to be subject of research, since more trials are needed to elucidate the various biological activities reported and consolidate it as an anti-inflammatory agent.

Taxonomy	Ethnopharmacology, Ethnopharmacological Field Study
Corresponding Author	José Carlos Tavares Carvalho
Corresponding Author's Institution	Universidade Federal do Amapa
Order of Authors	José Carlos Tavares Carvalho, Raphaelle Sousa Borges, Brenda Lorena Sánchez Ortiz, hady keita, Arlindo César Matias Pereira Filho

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