UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

BRUNA BENSO

AVALIAÇÃO DAS ATIVIDADES ANTIBACTERIANA, ANTI-INFLAMATÓRIA, ANTI-OSTEOCLASTOGÊNICA E ANTI-HIV DA *Malva sylvestris*

EVALUATION OF THE ANTIBACTERIAL, ANTI-INFLAMMATORY, ANTI-OSTEOCLASTOGENIC AND ANTI-HIV ACTIVITIES OF *Malva sylvestris*

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica.

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Orientador: Prof. Dr. Pedro Luiz Rosalen

Co-orientador: Prof. Dr. Gilson César Nobre Franco

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA BRUNA BENSO, ORIENTADA PELO PROF. DR. PEDRO LUIZ ROSALEN E CO-ORIENTADADA PELO PROF. DR GILSON CÉSAR NOBRE FRANCO.

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RESUMO

A natureza é fonte de descoberta de novos fármacos há séculos, originando inúmeras drogas de utilidade clínica. Plantas são reconhecidas por seu valor medicinal e nutracêutico, a exemplo, a *Malva sylvestris* possui literatura etnofarmacológica, que relata histórico de suas propriedades biológicas. Desta forma, o objetivo deste estudo foi realizar um *screening* das atividades farmacológicas da *Malva sylvestris*, portanto, investigou-se: (1) As atividades antibacteriana e anti-inflamatória do extrato de *M. sylvestris* (MSE) e frações utilizando método de cultura de células epiteliais e de tecido gengivais infectadas pelo micro-organismo *Aggregatibacter actinomycetemcomitans* e a quantificação da expressão de genes e citocinas relacionados ao processo inflamatório; (2) A atividade do MSE e frações quanto a atividade anti-inflamatória *in vivo* (migração de neutrófilos para cavidade peritoneal, edema de pata e quantificação de citocinas), capacidade de ação anti-osteoclastogênica (análise de expressão de gênica, contagem das células TRAP positivas e zimografia), atividade antioxidante (método DPPH e ABTS•+), e identificação química e confirmação da fração bioativa (MS/MS); (3) A ação anti-HIV da fração aquosa (AF) em células infectadas por HIV-BaL em modelo *dual chamber in vitro* por meio da quantificação antígeno p24, expressão gênica, citocinas e mecanismo de ação por transcriptase reversa. A análise estatística de variáveis quantitativas foram comparadas por análise de variância (ANOVA) e *post-hoc* de Dunnet. O nível de significância adotado foi de alfa= 0,05. Os resultados demonstraram que a fração clorofórmica (CLF) na concentração de 75 μg/mL foi eficaz na redução da colonização bacteriana e no controle dos mediadores inflamatórios promovendo a regulação dos genes IL-1beta, IL-6, IL-10, CD14, PTGS, MMP-1 e FOS, bem como, na redução da expressão das proteínas IL-1beta, IL-6, IL-8 e GM-CSF. A ação anti-inflamatória *in vivo* foi significativa (dose de 30 mg/kg, via oral) para todas as frações (CLF, EAF e AF) e extrato (MSE) estudados, com exceção da fração hexânica, na redução na migração de neutrófilos para cavidade peritoneal. AF (dose 30 mg/kg, via oral) reduziu o edema de pata nas 3 primeiras horas analisadas, apresentando uma ação mais rápida que o controle positivo, e ainda, reduziu os níveis de expressão de IL-1β. A análise da atividade de *M. sylvestris* sobre o processo de remodelação óssea demonstrou que AF na concentração de 10 μg/mL regulou a transcrição dos genes analisados (anidrase carbônica, catepsina K e fosfatase ácido-tártaro resistente), promoveu a redução no número de osteoclastos TRAP-positivos/área e controlou expressão de enzimas proteolíticas específicas MMP-9. Para a atividade antioxidante a AF e a

fração acetato de etila (EAF) apresentaram a melhor capacidade em capturar radicais livres. A identificação química revelou a presença do composto bioativo rutina na AF. Os resultados para atividade antiviral demonstraram uma redução na expressão de antígeno p24, ação sobre transcriptase reversa, controle da transcrição do genes CD4, Bcl-2 e TRIM5, e redução da expressão citocinas IL1-alpha, IL-beta, IL-6, IL-8 e GM-CSF após o tratamento com AF (50 μg/mL). Portanto, podemos concluir que a *M. sylvestris* e as frações bioativas encontradas apresentam compostos promissores como novos agentes terapêuticos.

Palavras-chave: Malvaceae. Infecções Bacterianas. Osteoclastos. Antioxidantes. Inflamação. Infecções por HIV

ABSTRACT

Nature has been a source of medicinal products for centuries, yielding many useful drugs. A wide variety of plants are well recognized for their medicinal and nutraceutical value, *Malva sylvestris* being one example; the ethnopharmacological literature has reported a long history of recognition of biological properties. The aim of this study was to conduct a pharmacological screening of *Malva sylvestris* and its interest to dentistry. Therefore, we investigated: (1) the antibacterial and anti-inflammatory activity of *M. sylvestris* extract (MSE) and fractions using a cell culture technique with epithelial and gingival cells infected with *Aggregatibacter actinomycetemcomitans* and a gene expression and cytokine quantification related to the inflammatory response; (2) The activity of MSE and fractions in the *in vivo* anti-inflammatory activity (neutrophil migration, paw edema and cytokine quantification, anti-osteoclastogenic action (gene expression, number of positive TRAP positive cells and zymography), antioxidant activity (DPPH and ABTS•+), and chemical identification of the bioactive fraction (MS/MS); (3) anti-HIV activity of aqueous fraction (AF) in cells infected with HIV-Bal using the *in vitro dual chamber* model, quantifying p24 antigen, gene expression and cytokines. Statistical analysis was performed by analysis of variance (ANOVA) and Dunnett's post-hoc test. The significance level adopted was alfa = 0.05. The results showed that chloroform fraction CLF (75 μg/mL) was efficient in reducing the bacteria colonization and inflammatory mediators, promoting the gene regulation of IL-1beta, IL-6, IL-10, CD14, PTGS, MMP-1 and FOS, as well as reducing protein expression IL-1beta, IL-6, IL-8 and GM-CSF. The *in vivo* reduction of anti-inflammatory effect (30 mg/kg, orally) was significant for the extract (MSE) and all fractions (CLF, EAF and AF) with the exception of the hexane fraction in the neutrophil migration assay. The AF (30 mg/kg, orally) reduced the paw edema in the first 3 hours analyzed, with a faster action than the positive control, reducing the levels of IL-1β expression. The activity of *M. sylvestris* in the bone remodeling assay demonstrated that the aqueous fraction (AF) in the concentration of 10 μg/mL regulated the gene transcription of the study genes (carbonic anhydrase, cathepsin K and tartrate-resistant acid phosphatase) and reduced the number of TRAP-positive osteoclasts and the specific proteolytic enzyme MMP-9. In terms of the antioxidant activity, the AF and the ethyl acetate fraction (EAF) had the best ability to capture free radicals. The chemical identification revealed rutin as the bioactive compound in the AF. Results for the antiviral activity showed a p24 antigen reduction, reverse transcriptase

mechanism of action, controlled transcription of the genes CD4, Bcl-2 and TRIM5, and a reduction in the cytokines IL-beta, IL-6, IL-8 and GM-CSF after treatment with AF (50 μg/mL). Therefore, we can conclude that *M. sylvestris* and its bioactive fractions are promising compounds as novel therapeutic agents.

Keywords: Malvaceae. Bacterial Infections. Osteoclasts. Antioxidants. Inflammation. HIV Infections.

SUMÁRIO

1 INTRODUÇÃO

Historicamente, os produtos naturais proveniente de plantas e animais são os responsáveis por cerca de 25 % dos medicamentos disponíveis no mercado (Cragg et al., 2014). As plantas, em particular, tem formado a base da medicina tradicional com registros de uso milenar que originam desde a Mesopotâmia, 2600 a.C, no entanto, apenas no século XIX iniciou-se a busca por princípios ativos, originando assim, os primeiros fármacos com características semelhantes aos atuais (Dias et al., 2012; Harvey, 2008).

As plantas constituem uma valiosa fonte de recursos para a síntese orgânica devido ao seu mecanismo de biossíntese de compostos chamado metabolismo secundário (Harvey, 2007). O metabolismo secundário, geralmente não é essencial para o crescimento, desenvolvimento ou reprodução dos organismos e são produzidos devido ao processo de adaptação ao meio ambiente, ou ainda, podem ser produzidos como mecanismo de defesa para sobrevivência (Dewick, 2001). A biossíntese pode ocorrer por fotossíntese, glicólise ou pelo ciclo de *Krebs* e podem produzir intermediários biossintéticos, que podem ser infinitos e reconhecidos como produtos naturais (Maplestone et al., 1992). Esse processo diferenciado de biossíntese proporciona características únicas na estrutura química e inúmeras atividades biológicas (Dewick, 2001).

O desenvolvimento de técnicas analíticas de separação e elucidação estrutural permitiram o isolamento de diversos metabólitos secundários com potencial farmacológico (Cragg et al., 2014). Há muitas áreas de conhecimento que se beneficiaram dos esforços de descobertas de novas drogas, entre elas, os antimicrobianos (Molinari, 2009). A medicina tradicional mostra interesse cada vez maior na utilização de drogas antimicrobianas derivadas de plantas, pois o antibióticos tradicionais, aqueles originados de produtos de micro-organismos ou derivados sinteticamente, tem se mostrado ineficazes no tratamento infeccioso em diversos momentos (Lai e Roy, 2004). A diversidade estrutural nos compostos derivados de plantas é imenso e o impacto produzido nos microrganismos é dependente da configuração química (Harvey, 2007). Para exemplificar, nas flavonas a presença do grupo (-OH) na posição 5´ da fórmula estrutural confere atividade contra cepas *Staphylococcus aureus* resistentes a meticilina. Esses achados mostram a relação direta entre a estrutura química e a atividade antimicrobiana (Lai e Roy, 2004).

Na lista das doenças crônicas que apresentam maior prevalência na população mundial estão presentes as doenças infecciosas de origem bucal (Petersen et al., 2005). A microbiota da cavidade oral tem estrutura complexa e é composta por mais de 600 espécies diferentes de bactérias (Dewhirst et al., 2010; Moore e Moore, 1994). As populações microbianas das estruturas dos dentes (biofilme dental) e o sistema de defesa do hospedeiro se mantem em equilíbrio dinâmico, no entanto, em algumas situações há colonização de novas espécies e um desequilíbrio pode ser iniciado, causando inflamação destrutiva dos tecidos circundantes, periodontais (Darveau, 2010; Alani e Seymour 2014).

O início e a manutenção da inflamação periodontal é determinado por bactérias que estão presentes no biofilme dentário e, em maior proporção, as gram-negativas (Johansson, 2011). *Aggregatibacter actinomycetemcomitans* é o micro-organismo relacionado de forma específica à periodontite agressiva, no entanto, também exerce papel na doença crônica (Slots, 1999; Kachlany, 2010). *A. actinomycetemcomitans* e outros micro-organismos incluindo *Porphyromonas gingivalis, Treponema denticola, Tannerela forsythia* estimulam a resposta imune promovendo inflamação dos tecidos moles e consequente destruição óssea (Darveau, 2010). Estas bactérias periodontopatogênicas produzem fatores de virulência como os lipopolissacarídeos e peptideoglicanos, que induzem a produção de citocinas pro-inflamatórias pelo hospedeiro (Salvi e Lang, 2005).

Os lipopolissacarídeos são macromoléculas que se associam a proteína CD14, formando o complexo LPS-CD14 que ativa o receptor de proteína tool-like (TLR-4), estimulando a sinalização intracelular e ativação de fosfolipase A, fosfolipase C e aumento dos níveis intracelulares de cálcio, p42/p44, e ainda, p38 (Han et al., 1993; Lima et al., 2010). Além disso, estimulam a liberação de diversos mediadores inflamatórios como: prostaglandinas (PGs), óxido nítrico (NO) e interleucinas (ILs) (Henderson et al., 1996; Johansson, 2011).

A ativação do sistema imune pode induzir ao estresse oxidativo e promover a produção e liberação de NO e espécies reativas de oxigênio (ROS) é um mecanismo utilizado para atrair mediadores para o local da inflamação (Conner e Grisham, 1996; Khansari et al., 2009). Uma ativação genética pode resultar na expressão de ROS pouco regulada e promover a apoptose de osteoblastos e a consequente reabsorção óssea e ativação do sinalizador NF-κB, responsável pelo mecanismo de osteoclasteogênse (Conner e Grisham, 1996). A progressão da doença periodontal permite a ativação dos osteoclastos e consequente

destruição óssea, fatores estimulatórios são reguladores do processo, como por exemplo, interleucina tipo 1 (IL-1), fator estimulador das colônias de macrófagos (MCSF), monócitos e células T (Henderson et al., 1996; Salvi and Lang, 2005).

O tratamento da doença periodontal é baseado nos fatores de virulência, nos micro-organismos que se estabelecem nos processos de saúde e doença, desta maneira, as terapias devem ser direcionadas para o controle desses micro-organismos (Seymour, 2006). Embora seja indiscutível o papel do biofilme bacteriano na etiologia das doenças periodontais, a severidade e a progressão destas doenças são determinadas por fatores relacionados a resposta do hospedeiro (Haffajee et al., 1997; Batchelor, 2015). Agentes moduladores são estudados como coadjuvantes no tratamento da doença periodontal não cirúrgica (Alani e Seymour, 2014). A partir da década de 90 foi incluído a terapia de modulação da resposta do hospedeiro como uma opção adjunta ao tratamento convencional da doença periodontal, são exemplos de moduladores: anti-inflamatórios sistêmicos e tópicos, sub-doses de doxicilina e o uso de bifosfonatos (Golub et al., 1992; Gokhale e Padhye, 2013). A terapia periodontal é realizada com sucesso, porém a recolonização da área subgengival pelos periodontopatógenos, resulta em uma terapia preventiva de manutenção falha e isto pode levar ao processo de doença recorrente (Teles et al., 2006).

O sistema imune também é desafiado por infecções de origem viral. Em condições fisiológicas a maior parte das células do sistema imune estão em repouso, no entanto, vários fatores podem participar como ativadores, e o vírus da imunodeficiência humana (HIV-1) é um exemplo (Younas et al., 2015). O processo infeccioso resulta na ativação de longa duração do sistema imunológico incluindo a perda progressiva de células de defesa T-$CD4^+$ e a produção elevada de citocinas pró-inflamatórias e quimiocinas que não são totalmente restabelecidos por terapias antirretrovirais (TARV) (Dagenais-Lussier et al., 2015). O efeito da TARV no tratamento de pacientes portadores de HIV trouxe inúmeros benefícios, em especial, diminuindo a mortalidade e risco de transmissão (Bahr, 2005). A terapia consiste na combinação de 3 classes de drogas: inibidores da transcriptase reversa, inibidores não-nucleosídeos da transcriptase reversa e inibidores de protease. A inserção desta terapia farmacológica permitiu que a doença infecciosa se transformasse em doença crônica (Maartens et al., 2014). No entanto, um significante número de novas terapias ainda não curativas e o alto custo ainda impede que algumas populações tenham acesso ao tratamento (Günthard et al., 2014).

A resposta imunológica mediante algumas patologias pode representar um desafio para a terapêutica, por exemplo, o tratamento de inflamações crônicas e infecções virais (Harvey, 2008). Há um interesse crescente no uso de plantas medicinais para a modulação do sistema imune e na prevenção de infecções relacionadas (Molinari, 2009). Compostos como flavonóides, polissacarídeos, lactonas, alcalóides, diterpenóides e glicosídeos presentes em muitas plantas, tem sido reportados pelas propriedades imunomoduladoras (Jantan et al., 2015).

A *Malva sylvestris*, popularmente conhecida como malva, é nativa da Europa, Norte da África e Ásia e tem o uso reportado desde 3000 a.C., devido a sua relevância terapêutica partes da planta tem sido empregadas na medicina tradicional e veterinária (Gasparetto et al., 2012). Etnofarmacologicamente é conhecida por suas propriedades anti-inflamatórias, antioxidantes, anticâncer, tratamento de bronquites e de lesões de pele (Gasparetto et al., 2012; Razavi et a., 2011). As folhas, flores e as parte aéreas da malva são conhecidas para tratamentos de doenças que afetam cavidade bucal como abcessos e dores dentárias (Guarrera, 2005). No Brasil a *M. sylvestris* é registrada na ANVISA como medicamento fitoterápico, na categoria para uso oral com expectorante, tratamento de inflamações e antisséptico da cavidade oral (Gasparetto et al., 2012; Kaileh et al., 2007). O uso da malva é disseminado e suas propriedades biológicas conhecidas ao redor do mundo (Romojaro et al., 2013). Desta forma, é necessária a investigação do potencial farmacológico e de interesse odontológico da *M. sylvestris*, constituindo uma base para o uso clínico desta planta, e ao mesmo tempo, um modelo para identificação de compostos bioativos.

Assim, a proposta deste trabalho foi realizar um *screening* de diferentes atividades farmacológicas da planta *Malva sylvestris* e como objetivos específicos, investigar: (1) As atividades antibacteriana e anti-inflamatória do extrato de *M. sylvestris* (MSE) e frações em células infectadas por *Aggregatibacter actinomycetemcomitans*; (2) A atividade do MSE e frações quanto a atividade anti-inflamatória, anti-osteoclástica, antioxidante, e finalmente, identificar quimicamente a fração ativa; (3) A ação anti-HIV da fração aquosa de *Malva sylvestris* em células infectadas por HIV-BaL.

2 ARTIGOS

2.1 ARTIGO (artigo publicado – Anexo 1)[1]

Malva sylvestris inhibits inflammatory response in oral human cells. An *in vitro* infection model

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Abstract

The aim of this study was to investigate the *in vitro* anti-inflammatory activity of *Malva sylvestris* extract (MSE) and fractions in a co-culture model of cells infected by *Aggregatibacter actinomycetemcomitans*. In addition, we evaluated the phytochemical content in the extract and fractions of *M. sylvestris* and demonstrated that polyphenols were the most frequent group in all samples studied. An *in vitro* dual-chamber model to mimic the periodontal structure was developed using a monolayer of epithelial keratinocytes (OBA-9) and a subepithelial layer of fibroblasts (HGF-1). The invasive periodontopathogen *A. actinomycetemcomitans* (D7S-1) was applied to migrate through the cell layers and induce the synthesis of immune factors and cytokines in the host cells. In an attempt to analyze the antimicrobial properties of MSE and fractions, a susceptibility test was carried out. The extract (MIC 175 μg/mL, MBC 500μg/ mL) and chloroform fraction (MIC 150 μg/mL, MBC 250 μg/mL) were found to have inhibitory activity. The extract and all fractions were assessed using a cytotoxicity test and results showed that concentrations under 100 μg/mL did not significantly reduce cell viability compared to the control group ($p > 0.05$, viability > 90%). In order to analyze the inflammatory response, transcriptional factors and cytokines were quantified in the supernatant released from the cells. The chloroform fraction was the most effective in reducing the bacterial colonization ($p< 0.05$) and controlling inflammatory mediators, and promoted the down-regulation of genes including IL-1beta, IL-6, IL-10, CD14, PTGS, MMP-1 and FOS as well as the reduction of the IL-1beta, IL-6, IL-8 and GM-CSF protein levels ($p< 0.05$). *Malva sylvestris* and its chloroform fraction minimized the *A. actinomycetemcomitans* infection and inflammation processes in oral human cells by a putative pathway that involves important cytokines and receptors. Therefore, this natural product may be considered as a successful dual anti-inflammatory–antimicrobial candidate.

Introduction

Periodontal disease is characterized by bacterial infection associated with the presence of biofilm, resulting in chronic inflammation of the tooth-supporting tissues and leading to progressive destruction of periodontal tissue. This disease affects up to 90% of the world's population [1], [2]. Dental biofilm with a large quantity of gram-negative bacteria is responsible for the initiation and maintenance of periodontal inflammation [3]. *Aggregatibacter actinomycetemcomitans* has been described as an important agent of localized aggressive periodontic lesions, but is also related to chronic periodontitis [4], [5]. In addition, *A. actinomycetemcomitans* and other pathogenic microbiota including *Porphyromonas gingivalis, Treponema denticola, Tannerela forsythia* trigger both innate and acquired immune responses, resulting in the progression of periodontal disease, and promote soft tissue inflammation and destruction with consequent bone resorption [6].

The development of new therapeutic agents that can inhibit biofilm formation and modulate the inflammatory response will have a major impact on the prevention and treatment of periodontal disease [7].

Nature has been a source of medicinal products for centuries, yielding many useful drugs [8]. A wide variety of plants are well recognized for their medicinal and nutraceutical value, and the exploration of biodiversity from rich environments has led to the discovery of many pharmacologically active chemicals [9], [10]. *Malva sylvestris* is one example. Commonly known as mallow, it is a plant native to Europe, North Africa and Asia. The ethnopharmacological literature has reported a long history of recognition for its potent anti-inflammatory, antioxidant, anticancer and antiulcerogenic properties [11], [12]. Some reports have indicated that *M. sylvestris* contains phytochemicals including several classes of terpenoids, including monoterpenes, diterpenes, sesquiterpenes and norterpenes [11], [13], [14]. Since natural products do not have a standard composition, there is increasing interest in

identifying biological therapeutic potential in new plant extracts [15]. Thus, the aim of this study was to investigate *in vitro* the antimicrobial and anti-inflammatory activity of *Malva sylvestris* extract and fractions in a dual chamber model of epithelial and subepithelial cells infected by *A. actinomycetemcomitans*.

Material and Methods

Preparation of the extract and fractions

Malva sylvestris leaves were purchased from a local farmer in the municipality of Princesa Isabel, Paraiba (northeast Brazil) in March and April 2013. This plant is not an endangered or protected species and was registered in the herbarium of the University of Sao Paulo (USP), receiving an identification number (ESA voucher # 121403). Absolute ethanol (800 mL) at room temperature was used to create extracts of *M. sylvestris* leaves (100 g) using exhaustive maceration (for 7 days). Filtration was used to obtain the ethanolic extract of *M. sylvestris* (MSE). The material was lyophilized, homogenized, weighed and stored at -*20°C*. The MSE was successively partitioned using liquid-liquid extraction with hexane, chloroform, and ethyl acetate solvents. The final residue obtained after ethyl acetate fractionation was totally soluble in water and thus was called the aqueous fraction (AF)[16]. The extract (MSE), chloroform fraction (CLF) and aqueous fraction were re-suspended in 1% ethanol and used in the biological assays.

Determination of total flavonoid, phenol and condensed tannin content

For the flavonoid determination, the aluminum chloride method was used. Total flavonoid contents were calculated using quercetin for the calibration curve. The absorbance was measured at 425 nm with a microplate reader (SpectraMax M5, Molecular Devices

Sunnyvale, CA, USA). The polyphenol content was measured by the Folin-Ciocalteu method and gallic acid was used as a standard equivalent [17]. For the content of condensed tannins, a vanillin solution was added to the extract, followed by 37% hydrochloric acid. The calibration curve was determined based on catechin as a reference.

Bacterial Strains

The *A. actinomycetemcomitans* (D7S-1) was cultivated from the subgingival plaque of an African American female patient diagnosed with generalized aggressive periodontitis. The strain was kindly donated by Dr. Casey Chen (University of Southern California) [18]. In addition, the following reference strains were used: *Fusobacterium nucleatum* ATCC 25586*, Prevotella intermedia* 25611 and *Porphyromonas gingivalis* ATCC BAA-308.

Cell culture

Keratinocytes were processed and isolated, and the cell line established was named OBA-9 [19]. The cell line was kindly donated by Dr. Kusumoto. OBA-9 cells used in this experiment were cultured in a specific medium for keratinocytes (Defined Keratinocyte-SFM, Life Technologies, Carlsbad, CA, USA). The human gingival fibroblasts HGF-1 (*ATCC CRL-2014)* were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen Life Technologies, CA, USA). Cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

Susceptibility testing

The susceptibility of four potential periodontopathogenic bacteria (*A. actinomycetemcomitans* DS7-1, *Fusobacterium nucleatum* ATCC 25586*, Prevotella*

intermedia 25611 and *Porphyromonas gingivalis* ATCC BAA-308) to MSE extract and fractions were tested. Tests were performed according to Clinical and Laboratory Standards Institute guidelines [20]. The minimum inhibitory concentration (MIC) was determined as follows. Bacteria were inoculated at a concentration of 5×10^5 CFU/mL in 96-well microplates, using a trypticase soy broth and yeast extract medium (TSB, YE, Difco, Franklin Lakes, NJ, USA) for *A. actinomycetemcomitans* and enriched with 5 μg/mL hemin and 1 μg/mL of menadione for the other microorganisms. The concentrations of MSE and fractions ranged from 3.125 to 1000 μg/mL. The vehicle control was ethanol (final ethanol concentration: 1%, v/v), and the positive control was gentamicin (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). The plates for the evaluation of antimicrobial activity against facultative aerobes were incubated at 37°C, 5% CO_2 and the plates for evaluation of activity against strict anaerobes were placed in an anaerobic chamber at 37°C, 10% H_2, 10% CO_2 and 80% N_2. The MIC was defined as the lowest concentration of MSE or fraction that allowed no visible growth, confirmed by 0.01% resazurin dye (Promega, Madison, WI, USA). The minimum bactericidal concentration (MBC) was determined by subculturing in trypticase soy agar (TSA, Difco, Franklin Lakes, NJ, USA) or TSA containing 2 μg/mL hemin, 1 μg/mL menadione and sheep blood (5.0%) and 20 μL aliquots from each incubated well with a concentration equal to or greater than the MIC. The experiments were conducted in triplicate in three independent assays.

Cell viability test

HGF-1 cells were seeded (~ $1x10^5$ cells/mL) in a 96-well plate and incubated for 24 h at 37ºC with 5% CO_2. *M. sylvestris* extract and fractions (0.1-1000 μg/mL) were added to the cell culture and incubated for 24 h. After the incubation time, the supernatant was discarded and the cells were washed with PBS (Lonza, Walkersville, MD, USA). Fresh

medium and 20 μL of CellTiter-Blue (Promega Corp, Madson, WI, USA) were added and incubated at 37°C and 5% CO_2. The CellTiter-Blue test is a fluorescent assay that measures cell viability via non-specific redox enzyme activity. After incubation, the well contents were transferred to a new microplate and the fluorescence was read in a microplate reader (SpectraMax M5 Molecular Devices Sunnyvale, CA, USA) with 550 nm excitation, 585 nm emission [21].

Invasion dual chamber assay

The activity of MSE and its fractions in cells infected by *A. actinomycetemcomitans* were investigated using an adapted dual chamber model to mimic the periodontum [22]. Keratinocytes (OBA-9) were seeded in a transwell insert with an 8 μm pore and 0.3 cm^2 culture surface (Grenier Bio-One, Monroe, NC, USA) and positioned in a 24 well plate. The basal chamber was seeded with HGF-1 fibroblasts. After 24 h the transepithelial resistance (TEER) was measured for each cell layer using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA). The cell layer confluence in the transwell insert was measured to reach the optimal TEER (>150 Ohm/cm^2). On day 2, an overnight *A. actinomycetemcomitans* culture was harvested by centrifugation at 900 X *g* for 10 min at room temperature and incubated in the dual chamber with KSFM culture medium (~1x10^6 CFU/mL) passing through the upper layer of cells (OBA-9) and reaching the bottom layer (HGF-1) for 2h. Extracellular, unattached bacteria were removed by washing with saline buffer (PBS) two times. After this initial incubation, epithelial and subepithelial cell layers were incubated with gentamicin 100 μg/mL (Sigma, St Louis, MO, USA) to kill the extracellular bacteria. The medium was removed and washed with saline buffer. Fresh new culture medium was added and the culture was treated with MSE or fractions at a concentration of 75 μg/mL. In light of the dose-dependent effects of MSE and CLF

treatments, this concentration was determined to be the highest concentration that possessed antimicrobial activity but was still non-cytotoxic after an exposure time of 24 h.

Sample analysis

Antimicrobial activity

The antimicrobial activity of MSE and fractions in the co-culture model was accessed after 24 h of treatment. Aliquots of 20 µL were cultured from each sample in TSB-YE plates to determine the CFU/mL and quantify the numbers of viable bacterial cells.

Analysis using the RT^2 Profiler PCR Array

One microgram of RNA was converted in cDNA using RT^2 First Strand Kit (Qiagen, Valencia, CA, USA) according to the manufacture's instructions. 84 genes were analyzed using inflammatory response & Autoimmunity Array RT^2 profiler (Qiagen Sabiosciences, Valencia, CA, USA) with buffers supplied by the manufacturer. The full list of genes detected by the SYBR Green-optimized primer assays is shown in (Table 1). A reaction mixture was prepared using 102 µL cDNA, 1248 µL water and 1350 µL SYBR Green/ROX. Analysis was performed using the Sabioscences web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), according to the $2^{\Delta\Delta}CT$ method. DataSet is assigned a GEO accession number GSE72443.

Functional Gene Grouping	Subgroup	Gene symbol
Cytokine	**Chemokines**	CCL11 (eotaxin), CCL13 (MCP-4), CCL16 (HCC-4), CCL17 (TARC), CCL19, CCL2 (MCP-1), CCL21 (MIP-2), CCL22 (MDC), CCL23 (MPIF-1), CCL24 (MPIF-2), (Eotaxin-2), CCL3 (MIP-1A), CCL4 (MIP-1B), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CXCL1 (GRO1, GROa, SCYB1), CXCL10 (INP10), CXCL2 (GRO2, GROb, SCYB2), CXCL3, CXCL5 (ENA-78, LIX), CXCL6 (GCP-2), CXCL9 (MIG).
	Interleukins	IL10, IL15, IL17A, IL18, IL1A, IL1B, IL1RN, IL22, IL23A, IL5, IL6, CXCL8
	Other Cytokines	IL10, IL15, IL17A, IL18, IL1A, IL1B, IL1RN, IL22, IL23A, IL5, IL6, CXCL8, CSF1(MCSF), FASLG (TNFSF6), LTB, TNFSF14
Cytokines Receptors	**Cytokine Receptor**	IL10RB, IL1R1, IL1RAP, IL23R,IL6R.
	Chemokine Receptors	CCR1, CCR2, CCR3, CCR4, CCR7, CXCR1 (IL8RA), CXCR2 (IL8RB), CXCR4.
Cytokine Metabolism	-	IL10, IL18, TLR1, TLR3, TLR4, TLR6
Cytokine-Mediated Signaling	-	CCL2 (MCP-1), CCL5 (RANTES), CCR1, CCR2, IFNG, IL1A, IL1B, IL1R1, IL1RN, IL5, IL6, IL6R, MYD88, RIPK2, TNF
Acute-phase response		CEBP, CRP, PTGS2
Chronic Inflammatory Response	-	CCL11 (eotaxin), CCL5 (RANTES), IL1B, LTA (TNFB), TNF
Humoral Immune Response	-	C3, CCL16 (HCC-4), CCL2 (MCP-1), CCL22 (MDC), CCL3 (MIP-1A), CCL7 (MCP-3), CCR2, CCR7, CD40 (TNFRSF5), IL10, IL18, IL1B, IL6, ITGB2, LY96 (MD-2), NFKB1.
Regulation of the inflammatory response	-	BCL6, C3AR1, CD14, CD40LG, FOS, IL9, KNG91, NOS2, NR3C1, SELE, TIRAP, TLR3, TLR5, TLR7, TOLLIP

Table 1. The Human Inflammatory Response & Autoimmunity RT² Profiler PCR Array. This assay profiles 84 key genes involved in autoimmune and inflammatory immune responses. It profiles genes related to inflammatory cytokines and chemokines as well as their receptors and also genes related to the metabolism of cytokines and those involved in cytokine-cytokine receptor interactions.

Quantitative Real-Time PCR

Quantitative PCR (qPCR) was performed to evaluate the possible effects of the *A. actinomycetemcomitans* invasion in the lower chamber compartment upon reaching the subepithelial cells (HGF-1). In addition, we aimed to analyze genes related to the inflammation process to verify whether MSE and fractions could promote some biological activity in the infection process. RNA was isolated from cell culture after 24h of treatment

using the RNeasy Mini Kit (Qiagen; Valencia, CA, USA). Purity and quantity of RNA were measured in the NanoPhotometer P360 (Implen; Westlake Village, CA, USA). RNA sample has been treated with DNase. Reverse transcription of RNA to cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Based on PCR array analysis, genes were selected that presented significant levels of down-regulation (Quantitech Primers, Qiagen). The threshold was manually adjusted within the logarithmic curve above the background level and below the plateau phase. A comparative Ct method was used to calculate the relative gene number. The relative gene copy number was calculated using the $2^{\Delta\Delta}$CT method.

Cytokine assay

Cytokine assays were performed on all samples using specific enzyme-linked immunosorbent assay (ELISA) kits (Qiagen, Valencia, CA, USA). The cytokines were selected in order to confirm the encoded genes that exhibited down-regulation in the gene expression analysis. The concentration of IL-1alpha, IL-1beta, IL6, IL8, IL10 and GM-CSF were measured according to the manufacturer's instructions.

Results

Chemical analysis

Determination of total flavonoid, phenol and condensed tannin content

The phytochemical characterization revealed that the total polyphenol contents of MSE, CLF and AF were 38%, 26% and 22% gallic acid equivalents, respectively. Tannin represented 0.02%, 0.54% and 0.8% catechin equivalent in MSE, CLF and AF respectively; flavonoid content was 2.7%, 7% and 5.6% quercetin equivalent in MSE, CLF and AF respectively.

Susceptibility testing

Table 2 shows the MIC and the MBC values of MSE and fractions screened for different periodontopathogenic bacteria. The results demonstrated that MSE and CLF had inhibitory activity for the all microorganisms tested: *A. actinomycetemcomitans*, *Fusobacterium nucleatum, Prevotella intermedia* and *Porphyromonas gingivalis*. CLF was the most potent, with an MIC against *A. actinomycetemcomitans* of 150 μg/mL, an MIC against *F. nucleatum* of 500 μg/mL and an MIC against *P. intermedia* of 125 μg/mL. The MSE had the lowest MIC against *P. gingivalis* (15.6 μg/mL). AF had no inhibitory activity against any of the bacteria tested. Gentamicin was used as the positive control (10 μg/mL).

Extract/fraction	***A. actinomycetemcomitans***		***F. nucleatum***		***P. intermedia***		***P. gingivalis***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MSE	175	500	1000	-	250	-	15.6	125
CLF	150	250	500	-	125	500	62.5	1000
AF	-	-	-	-	-	-	-	-

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MIC and MBC for the ethanolic extract of *Malva sylvestris* and its chloroform and aqueous fraction against four different periodontopathogens: *Aggregatibacter actinomycetemcomitans* D7S1, *Fusobacterium nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611 and *Porphyromonas gingivalis* ATCC BAA-308. The highest concentration evaluated was 1000 μg/mL and the minus symbol (-) means no inhibitory activity.

Cell viability test

The cytotoxicity of the extract and all fractions was assessed at concentrations of 0.1, 1, 10, 100 and 1000 μg/mL. AF did not affect the cell viability ($p>0.05$) at any of the concentrations tested when compared to the control group (non-treated). MSE and CLF

reduced the number of viable cells at concentrations of 100 μg/mL and 1000 μg/mL ($p<0.05$). MSE and CLF were non-toxic at concentrations of 0.1, 1 and 10 μg/mL (Fig. 1).

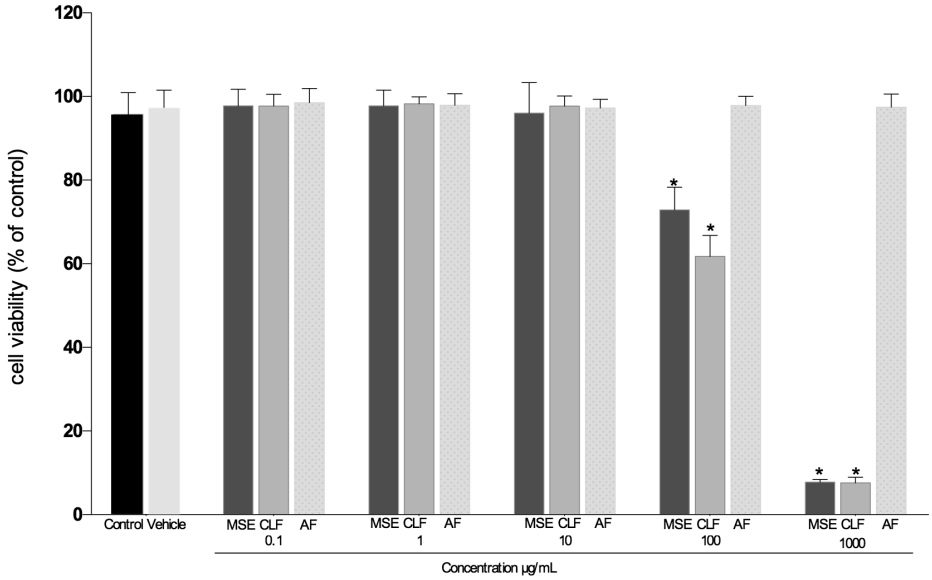

Fig 1. Cytotoxicity test. Cytotoxic effect of MSE, CLF and AF on fibroblasts HGF-1 cells**.** Data are expressed as mean ± SEM using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests as compared to non-treated. The level of statistical significance was set at 0.05.

Invasion dual chamber assay

MSE, CLF and AF activity in the co-culture model: susceptibility test, ELISA and gene expression

A viability test was used to determine the effects of the treatments and select the best culture conditions after *A. actinomycetemcomitans* infection. In addition, cells were analyzed for the ability to form an impervious epithelial layer by measuring the TEER. Dose-dependent antimicrobial effects were found for MSE and CLF treatment but high concentrations (≥ 100 μg/mL) were toxic for the cells; thus we tested the highest non-toxic

concentration for all the samples. A sub-MIC concentration of 75 μg/mL was established. Results confirmed that treatment with 75 μg/mL MSE or CLF did not significantly affect the percentage of viable cells or the integrity of the tight epithelial conjunction (Fig. 2).

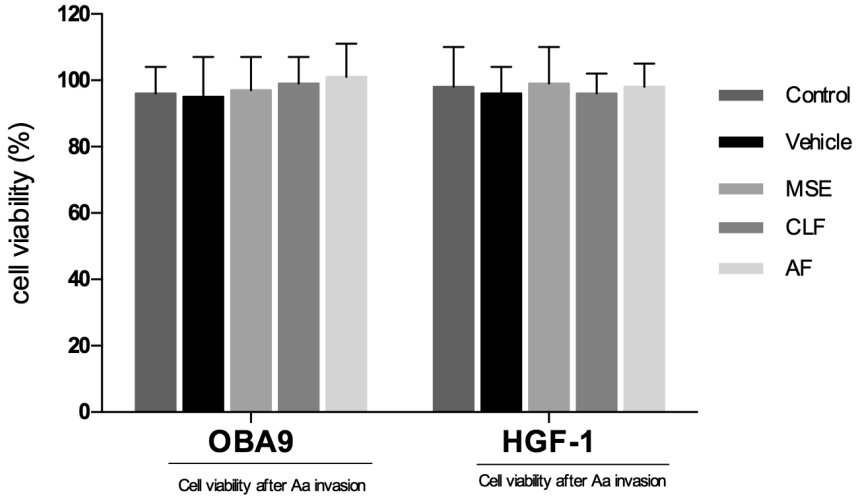

Fig 2. Cytotoxicity effect in the dual chamber model. Cytotoxic effects of MSE, chloroform and aqueous fraction on fibroblast HGF-1 and keratinocyte OBA-9 cell lines were found after 24 h hours of invasion by *A. actinomycetemcomitans***.** Data are expressed as mean ± SEM using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests as compared to the control group (non-treated). The level of statistical significance was set at 0.05.

In order to confirm the invasion assay of *A. actinomycetemcomitans* we first recovered the cultures from the dual compartment chambers. The time 0 h represents this time point at which the CFU quantification was performed after 2h infection period followed by 1h gentamicin treatment. There were no differences among the groups in the amount of internalized bacteria at time 0h ($p>0.05$). Twenty-four hours after the infection was initiated, the groups treated with MSE and AF had no ability to reduce or eliminate the invasion in the host cells; however, the number of microorganisms in the CLF group was reduced significantly compared to the control vehicle group ($p<0.05$)(Fig. 3).

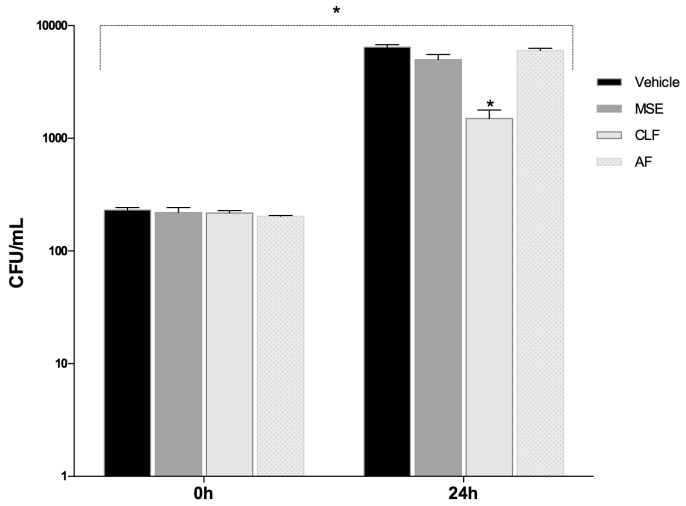

Fig 3. Comparison of colony-forming units. Comparison of colony-forming units (CFU/mL) among groups treated with MSE, CLF and AF after *A. actinomycetemcomitans* infection. Data are expressed as mean ± SEM using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests as compared to vehicle control. The level of statistical significance was set at 0.05.

Analysis Using the RT^2 Profiler PCR Array

Alterations in the transcript levels for all treatment groups were initially analyzed using the RT^2 Profiler PCR Array and 84 genes were screened and analyzed using the SABiosciences web portal software. The transcriptional profile in the lower chamber cell lines after 24 hours invasion assay is showed on Table 3. It was found a down-regulation of 6 different genes among 84 target genes. For the fold changes, values less than 1 are considered down regulated.

Symbol	Gene	Gen Bank	Fold changes		
			MSE	CLF	AF
BCL6	B-cell CLL/lymphoma 6	NM_001130845	0.37	0.45	0.68
CD14	CD14 molecule	NM_000591	0.64	0.63	0.57
FOS	FBJ murine osteosarcoma viral oncogene homolog	NM_005252	0.23	0.05	0.81
IL-1beta	Interleukin 1, beta	NM_000576	0.57	0.64	0.57
IL-6R	interleukin 6 receptor	NM_000565	0.07	0.08	0.29
IL-8	Interleukin 8	NM_000584	0.36	0.48	0.89

Table 3. The Human Inflammatory Response & Autoimmunity RT² Profiler PCR Array. Genes in the inflammatory pathway down-regulated by the treatments with MSE, CLF and AF.

Quantitative Real-Time PCR

The following genes were analyzed using qRT-PCR: IL-1alpha, IL-1beta, IL-6, IL-8, IL-10, CD14, PTGS, FOS, BCL6 and MMP-1. The reference control gene was GAPDH and the calibrator for the $2^{\Delta\Delta}$CT method was the non-treated control group. A minus-reverse transcriptase control was included in all qPCR experiments.

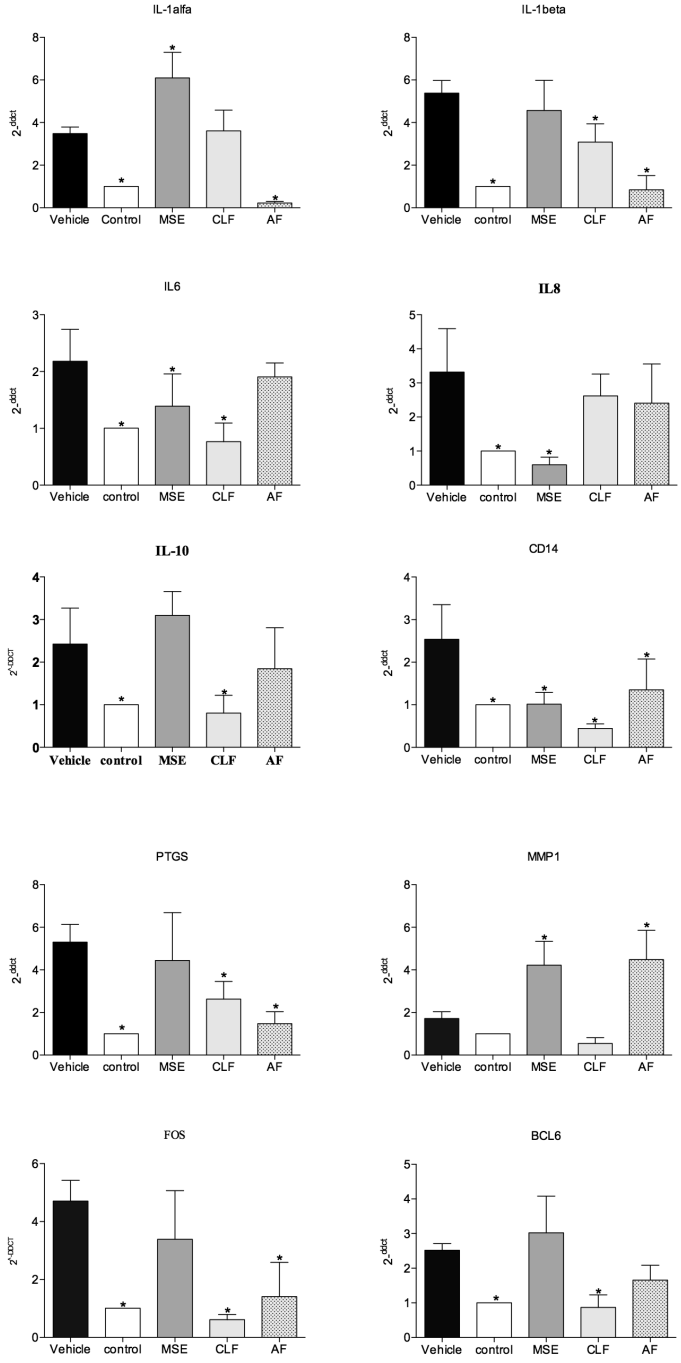

Fig 4. Gene expression analysis of lower chamber co-culture invasion assay. The expression levels of IL-1alfa, IL-1beta, IL-6, IL-8, IL-19, CD14, PTGS, MMP-1, FOS, BCL6 were evaluated and compared to the control (non-infected cells). Quantification of the relative transcript amounts was performed using qPCR with 50 ng of each cDNA. Data quantification was performed using the $2^{\Delta\Delta}$CT method. Statistical analysis included one-way ANOVA followed by Dunnet's post-hoc tests. A significance level of $p < 0.05$ (*) indicates differences from the vehicle group.

The results showed that cells invaded by *A. actinomycetemcomitans* and treated with MSE had statistically significant down-regulation of the genes IL6, IL8, CD14 compared to the control group (non-infected cells) ($p<0.05$). The chloroform fraction down-regulated the gene expression of IL-1beta, IL6, IL10, PTGS, CD14, PTGS, FOS and BCL6 ($p<0.05$) compared to the control group. The group treated with AF showed a down-regulation of transcript levels for the genes IL-1alpha, IL-1beta, CD14, PTGS and FOS ($p<0.05$) (Fig. 4).

Cytokine assay

The concentrations of IL-1 alpha, IL-1beta, IL-6, IL-8, IL-10 and GM-CSF were quantified by ELISA to confirm whether the proteins encoded by the down-regulated genes were found at reduced levels in the supernatant. MSE reduced the expression of IL-6 in the infected cells compared to the vehicle control group ($p<0.05$). Significant differences in the levels of a number of cytokines were found between the CLF and control groups. Specifically, reduced expression levels were observed for the cytokines IL-1beta, IL-6, IL-8 and GM-CSF (reductions of 32%, 80, 3%, 30, 58 and 4% compared to the vehicle control, respectively). Infected cells expressed low levels of IL-1 alpha when treated with the AF, a decrease of 31% compared to the vehicle control group (Fig. 5).

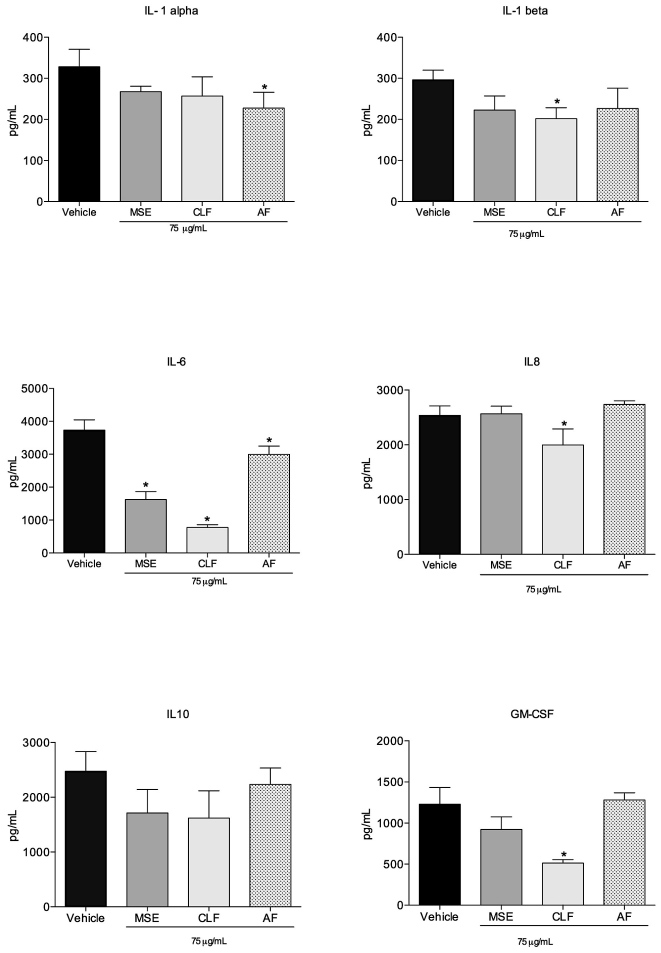

Fig. 5 Cytokine assay. Quantification of IL-1alpha, IL-1beta, IL-6, IL-8, IL-10 and GM-CSF in the co-culture supernant after 24 h of *A. actinomycetemcomitans* invasion. Cells were treated with 75 μg/mL of MSE and fractions and bacteria inocula were established at $2x10^6$ CFU/mL. Data are expressed as mean±SD, n=6. Symbols indicate statistical differences ($p<0.05$, Dunnet's test). # indicates $p<0.05$ compared to non-treated group; * indicates $p<0.05$ compared to vehicle group.

Discussion

Periodontal disease is an oral infectious inflammatory disease and the most common human chronic disorder [23]. The relationship between this disease and many systemic diseases (including cardiovascular disease, diabetes, adverse pregnancy outcomes and others) is well recognized [24], [25]. Based on current knowledge of inflammation

pathways, it appears that natural products may be a good source for developing multi-target drugs with activity against the microorganisms responsible for periodontal disease [26], [27].

For this study, we evaluated the toxicity, antimicrobial and anti-inflammatory activity of compounds naturally occurring in the plant *M. sylvestris* [28]. A screening assay simulating the effect of *A. actinomycetemcomitans*, a species known to be associated with periodontal disease, was used to model the infection of epithelial and subepithelial cell lines [22]. These results confirmed the internalization of the bacteria, indicating the possible activation of the membrane and intracellular receptors [29]. Transcriptional factors and cytokines identified in the infection process suggested signaling and host response pathways were involved in the bacteria challenge and during the treatment with *M. sylvestris* extract and fractions.

The antimicrobial susceptibility test showed that MSE and CLF had activity not only against *A. actinomycetemcomitans* but also against other periodontopathogens (*F. nucleatum, P. gingivalis* and *P. intermedia*) that are implicated in the development and virulence of periodontal disease [1]. In addition, this demonstrates that *M. sylvestris* works against both microaerophiles and anaerobes. In the literature, it has been shown that the ethanolic extract of *M. sylvestris* is effective as a bacteriostatic agent against methicillin-resistant *S. aureus* ($I_{50} \leq 32$ μg/ml) [30], and moderate to low activity was reported against strains of *Helicobacter pylori* (MIC ranged from 0.625 to >5.0 mg/mL)[31]; moreover, the aqueous fraction was reported to have anti-fungal activity, though not against *Candida albicans* [32]. Overall, though antimicrobial effects of *M. sylvestris* have been reported in the literature for a few microorganisms [30],[31],[32] these studies used different extract preparations and the majority were based on agar-diffusion tests, making inter-study comparisons difficult.

Our findings also demonstrate that the bioguided fractionation was successful and may be a model for bioprospecting new drugs, as long as the active fraction (CLF) presented enhanced antimicrobial activity relative to the unfractionated extract. In addition, our data describe the cytotoxicity of the extract and fractions *in vitro* to provide better estimation of the potential of the compound as favorable therapeutic agent. The viability test showed that the CLF fraction was non-toxic at concentrations up to 100 μg/mL and AF had no toxic effects at any of the concentrations tested. The LD_{50} of the extract for cell lines OBA-9 and HGF (250 μg/mL and 210 μg/mL, respectively) gave insight into the safe concentrations for use in the biological assays. *M. sylvestris* is widely known as a food or condiment and has been used for millennia in traditional medicine; however, only one *in vivo* test of its toxicity has been reported in the literature [33].

The bacterial products from *A. actinomycetemcomitans* affected the cell immune response and increased the production of local cytokines. All the treatments tested affected different signaling pathways. Upon treatment with the aqueous fraction, both the IL-1alpha gene and protein expression levels were reduced. The pro-inflammatory cytokine IL-1 and tumor necrosis factor alpha (TNF alpha) are modulators of the host response to microbial infection. It has previously [34] been demonstrated that IL-1 specific marker is a strong indicator of susceptibility to severe periodontal disease in adults. Furthermore, it has been established that IL-1 is involved in the induction of bone resorption by promoting the differentiation of osteoclast precursors in active osteoclasts [35].

A statistical reduction of IL-6 gene expression and protein levels were found after treatment with the chloroform fraction (CLF). The higher expression levels of IL-6 in untreated periodontal disease might induce an increase in matrix metalloproteinases (MMPs) that are related to tissue destruction [36], [37]. IL-6 has been reported as a principal regulator

in the acute phase of inflammation and may promote osteoclastogenesis by increasing tRANKL expression [38].

In addition, the CLF treatment regulated the expression of other immunomodulatory genes (CD14, MMP1 and FOS), which indicates an effect on more than one signaling pathway and may result in a good therapeutic outcome. Finding compounds that trigger CD14 or toll-like receptors (TLRs) is potentially useful in periodontal disease. The binding of lipopolysaccharides (LPS) with CD14 might induce the temporary activation of many protein kinases and the phosphorylation of intracellular proteins essential for LPS activation in monocytes/macrophages [39].

The MSE could regulate the transcription of IL-8 but not the same cytokine expression. The answer to the question of how genomic information can be processed differently to produce a specific cellular proteome to date remains unanswered [40], [41]. The literature has been demonstrated that *M. sylvestris* may regulated the expression of cytokines in the inflammatory process. In a pre-clinical study, important anti-inflammatory action of the hydroalcoholic extract was found to interfere with the production of IL-1beta and consequently block leukocyte migration [42]. Furthermore, the aqueous extract of *M. sylvestris* was found to have an immunomodulatory property, acting as a macrophage activators and promoting both IL-12 and (IFN) interferon transcripts [42]. Overall, the literature and present data highlight the biological activity of *M. sylvestris* in treating inflammation.

The phytochemical investigation of *M. sylvestris* showed a high occurrence of phenolic compounds in all studied extracts and fractions. This is consistent with a previous report [14], in which 4-hydroxybenzoic acid, 4-methoxybenzoic acid, 4-hydrocycinnamic acid and tyrosol were isolated from *M. sylvestris*. Furthermore, the interest in phenolic compounds has increased in recent years due to their possible implications for human heath, such as in

treating and preventing cancer, cardiovascular disease and other pathologies [11]. Overall, phenolic compounds are particularly potent natural products with a wide range of biological properties known in the literature that could be used extensively in dentistry.

The results of the present study showed that the low-polarity fraction CLF has relevant dual activity, simultaneously controlling infection and inflammation processes. Thus, *M. sylvestris* may be considered as a potential drug candidate for use as a new therapeutic approach in the treatment of the periodontal disease.

Conclusion

In our study we found that *Malva sylvestris and its* chloroform fraction were able to minimize the infection and inflammation process in oral human cells by a putative pathway that may involve the antimicrobial effect and modulation of cytokines and receptors. Therefore, this natural product may be considered as a successful dual anti-inflammatory–antimicrobial candidate.

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2.2 ARTIGO[2]

Anti-inflammatory, anti-osteoclastogenic and antioxidant effects of *Malva sylvestris* extract and fractions: *in vitro* and *in vivo* studies

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Abstract

Given their medical importance, natural products represent a tremendous source of drug discovery. *Malva sylvestris* is a plant cited extensively in the ethnopharmacological literature and is known worldwide. The aim of this study was to investigate the extract (MSE) and fractions (HF, CLF, EAF and AF) of *M. sylvestris* for anti-inflammatory, anti-osteoclastogenic, antioxidant effects and a chemical identification of the bioactive fraction. The *in vivo* experiments consisted of the quantification of neutrophil migration to the peritoneal cavity, paw edema and cytokine release. *M. sylvestris* extract (MSE) and fractions at 3, 10 and 30 mg/kg were administered orally. Macrophages were cultured by cell viability assay to determine the concentration of MSE and fractions for all cell-based experiments. Transcriptional factors were quantified by qPCR and the expression of the following genes were studied: carbonic anhydrase II (CAII), cathepsin K and tartrate-resistant acid phosphatase (TRAP). Gel zymography with collagen as the substrate was used to identify the latent and the active gelatinase MMP-9 secreted in the media stimulated with LPS (*E. coli*) in RAW 264.7 cells. TRAP staining was employed to evaluate osteoclast (OC) formation and TRAP-positive multinuclear macrophages with more than three nuclei were counted as OCs. Antioxidant activities measured for all extract and fractions for the two most common radical scavenging assays using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The chemical analysis was performed using the MS/MS technique. The aqueous fraction (AF) was identified as the bioactive fraction, with the oral treatment significantly reducing the neutrophil migration to the peritoneal cavity, antiedematogenic and IL-1B cytokine level (54% reduction). The viability tests showed a concentration-dependent effect, where the MSE and fractions at concentrations equal to 10 µg/mL were not toxic for the cells. In the TRAP gene expression analysis, all the treatments tested presented a downregulation of the transcription levels. CLF (chloroform fraction) and

AF treatments had the ability to reduce the osteoclastogenesis on RAW 264.7 cell lines ($p<0.05$) measured in the TRAP staining assay. In our study, the activity of MMP-9 decreased when treated with the AF and EAF, with a reduction of 69% and 75%, respectively. Moreover, the bioactive fraction had the ability to regulate the oxidation pathway, eliminating the radicals of ABTS and DPPH method. Mass spectrometry identified rutin as the bioactive compound in the AF. The AF of *M. sylvestris* presented *an*ti-inflammatory, anti-osteoclastogenic and antioxidant abilities in different *in vitro* and *in vivo* methods. In addition, we suggest that given its multi-target activity the bioactive fraction may be a good candidate in the therapy of chronic inflammatory diseases.

Introduction

Inflammation is a biological process that involves vascular and cellular events coordinated by mediators such as prostaglandins, leukotrienes and cytokines (Perretti et al., 2015). This is an organism's essential and protective mechanism in response to injury, infection and trauma (Ward, 1974). Thus, inflammation appears to be an inherently self-perpetuating event in terms of possible transformation due to the largely biological chemokine attraction (Souza and Lerner, 2013).

A prolonged inflammation process may lead to chronic diseases such as periodontal disease and rheumatoid arthritis, which are associated with tissue injury and bone resorption (Crotti et al., 2015). In some of these chronic inflammations, pro-inflammatory mediators and reactive oxygen species (ROS) can promote osteoblast apoptosis and bone resorption through the activation of the NF-κB signaling pathway, which plays an important role in osteoclastogenesis (Conner and Grisham, 1996). The classic NF-κB pathway stimulation includes the receptor activator of nuclear factor kappa-B ligand (RANKL), the osteoclastogenic cytokine, as well as TNF-α and other inflammatory mediators (Takeshita et

al., 2000). These cytokines may induce bone resorption, affecting the production of the essential osteoclast differentiation (Henderson et al., 1996).

Osteoclast differentiation and the activation of bone resorption function by mature osteoclasts are events that require RANKL and its permissive macrophage colony-stimulating factor (M-CSF) to induce the expression of RANK, a receptor for RANKL. RANKL plays an essential role in the differential, recruitment, activation and survival of osteoclasts by binding to its receptor (RANK) on osteoclasts or progenitor cells (Ohshiba et al., 2003). A number of the RANK-induced signaling pathways in osteoclasts ultimately induce the expression of several genes, including TRAP, cathepsin K and carbonic anhydrase, which are enzymes involved in the regulation of the dissolution of mineral and collagen (Franco et al., 2011; Zhang et al., 2011).

In addition, matrix metalloproteinase (MMP) is a family of proteolytic enzymes involved in the role of extracellular matrix degradation that includes a variety of tissues and bone (Ohshiba et al., 2003). In the group of MMPs, MMP-9 is an important proteinase that osteoclasts express in high levels. Moreover, there are studies showing the relation of MMP-9 activity in bone destruction, including in some diseases such as rheumatoid arthritis (Franco et al., 2011; Takeshita et al., 2000).

Traditionally, anti-inflammatory therapy has focused on controlling cytokine and adhesion molecule expression, including non-steroidal drugs and glucocorticoids (Georgakopoulou and Scully, 2014; Rainsford, 2007). However, in the past few years it has been recognized that the inflammation resolution may be based on multi-target drugs (Koeberle and Werz, 2014). Multiple signaling pathways are a way to improve the pro-inflammatory, immunomodulatory and proresolving cascades, which define the aspects of the inflammation (Kohli and Levy, 2009). Thus, natural products have played an important role *in*

the development of new sources in the treatment of inflammatory diseases (Cragg et al., 2014).

The screening of extracts from natural sources has historically led to the discovery of many clinical drugs in current therapy (Molinari, 2009). Since natural products do not have a standard composition, there is interest in identifying biological therapeutic potential in new plant extracts (Harvey, 2008). The ethnopharmacological literature has reported a wide use of *Malva sylvestris* since ancient times for its emollient, antioxidant and anti-inflammatory properties (Gasparetto et al., 2012). Given its widespread and medicinal importance, the aim of this study was to investigate the extract and fractions of *Malva sylvestris* for anti-inflammatory, anti-osteoclastogenic, antioxidant and chemical identification of the bioactive fraction.

Material and Methods

Preparation of the extract and fractions

Malva sylvestris leaves were collected in the inner region, municipality of "Princesa Isabel", state of Paraiba, in northeastern Brazil. The leaves were extracted with absolute ethanol at room temperature and then filtered to obtain the ethanol extract of *M. sylvestris* (MSE). The MSE was further fractioned using liquid-liquid extraction. The fractions obtained were: hexane (HF), chloroform (CLF), ethyl acetate (EAF) and aqueous (AF), and these were monitored with thin-layer chromatography (TLC) using the anisaldehyde reagent (4-methoxybenzaldhyde, acetic acid, sulfuric acid, 1.0:48.5:0.5), followed by heating at 100^{o}C for 5 min. Fluorescent substances were visualized under ultraviolet (UV) at wavelengths of 254 and 366 nm. All the extract and fractions were re-suspended in 1% ethanol and used in the biological assays [17].

Anti-inflammatory analysis

Animals

Male Balb/c albino mice (20–25g), SPF, were purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil). The mice were maintained in a room with a controlled temperature (22 ± 2°C) for a 12 h light/12 h dark cycle, humidity 40-60%, with food (standard pellet diet) and water provided *ad libitum*. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and had received prior approval from the local Animal Ethics Committee (CEUA, Ethics Committee on Animal Use/UNICAMP, process number 2790–1).

Neutrophils migration in the peritoneal cavity

To determine the neutrophil migration into the peritoneal cavity of the MSE and fractions, 3, 10 and 30 mg/kg were administered orally and 2 mg/kg dexamethasone was administered by subcutaneous (s.c.) injection 1h before administration of inflammatory stimulation by intraperitoneal (i.p.) injection of carrageenan at 500 μg/cavity. The vehicle (0.9% NaCl) was used as the negative control. The mice were euthanized 4 h after the challenge (carrageenan administration) and the peritoneal cavity cells were harvested by washing the cavity with 3 mL of phosphate-buffered saline (PBS) containing EDTA. The volumes recovered were similar in all experimental groups and equal to approximately 95% of the injected volume. In order to count the total number of cells, a Neubauer chamber was used. Smears were prepared using a cytocentrifuge (Cytospin 3; Shandon Lipshaw), stained with a Panoptic staining kit and neutrophils cells were counted (until 100 cells) using an optical microscope (1000X). The results are presented as the number of neutrophils per cavity [18].

Carrageenan-induced paw edema

A paw edema was induced by subplantar injection of 0.05 mL of lambda carrageenan (1% w/v in 0.9% of saline) into the left hind paw in the mice. An equal volume of vehicle was injected into the contralateral paw. The volume of both hind paws up to the ankle joint was measured with a plethysmometer (UGO Basile, Model 7140) immediately before the 0, 1, 2, 3, 4 and 5 hours post-carrageenan. The difference in the volumes between the hind paws was a measure of the edema (mL). The MSE and the bioactive fraction previously selected in the neutrophil migration model were administered by oral treatment (30 mg/kg), the reference drug, indomethacin (10 mg/kg), or the vehicle (10 mL/kg of 0.9% of saline), were given intraperitoneally 1/2 h or orally 1 h before the subplantar injection of the phlogistic agent [19].

Cytokines quantification

Based on a previous test (neutrophil migration assay), the MSE and the bioactive AF were selected for the quantification of proinflammatory cytokines produced in the peritoneal cavity. The mice were treated with the MSE or the AF (30 mg/kg, oral) 1h before the administration of inflammatory stimulation by intraperitoneal (i.p.) injection of carrageenan at 500 μg/cavity. After 4 h, the animals were euthanized and the samples were homogenized in 500 μL of the appropriate buffer containing protease inhibitors (Sigma, St. Louis, MO, USA). Levels of TNF-α and IL-1β were determined by ELISA using protocols supplied by the manufacturers (Peprotech, Rocky Hill, NJ, USA) from both the experiments.

Osteoclasteogenic assays

Cell culture

RAW 264.7 cells were purchased from the Rio de Janeiro cell bank (Rio de Janeiro, Brazil) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Gibco, Life Technologies, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Life Technologies, CA). Cells were maintained in a humidified incubator at 37°C in 5% CO_2 [7].

Cell viability

A cell-based assay to screen the MSE and fractions (HF, CLF, EAF, AF) to measure the enzyme activity as a marker of viable cells. RAW 264.7 cells were seeded *(*~ $1x10^5$ cells/mL) in a 96-well plate and incubated for 24 h at 37ºC with 5% CO_2. The MSE and fractions (0.1-1000 µg/mL) were added to the cell culture and incubated for 24 h. After the incubation time, the supernatant was discarded and the cells were washed with PBS. Fresh medium with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) 0.5 mg/mL were then incubated for an additional 4 h. After the incubation time, the cell growth medium was replaced by ethanol and colorimetric measurements were performed with a microplate reader at 570 nm [20]. The extract MSE and all fractions and aqueous were re-suspended in 1% ethanol and used in the biological assays.

Analysis of gene expression

Quantitative PCR (qPCR) was performed to evaluate the possible effects of MSE and fractions on the expression of predominant osteoclast marker genes. In addition, we also aimed to evaluate whether our natural products could control the transcription of genes involved in bone metabolism. The genes analyzed were: carbonic anhydrase II (CAII),

cathepsin K, tartrate-resistant acid phosphatase (TRAP) and glycerol 3 phosphate dehydrogenase (GAPDH). The primers sequences were: CAII (forward: TGGTTCACTGGAACACCAA, reverse: CACGCTTCCCCTTTGTTTTA), cathepsin K (forward: CAGCTTCCCCAAGATGTGAT, reverse: AGCACCAACGAGAGGAGAAA), TRAP (forward: CCCTCTGCAACTCTGGACTC, reverse: TAGAGGCGAACAGGAAGGAA), GAPDH (forward: AACTTTGGCATTGTGGAAGG, reverse: ACACATTGGGGGTAGGAACA). RAW 264.7 cells (~ $1x10^6$ cells/mL*)* were seeded into 24-well plates for 24 h and treated with a 10 µg/mL concentration of the MSE and fractions in serum-free medium for 24 h and the stimulatory response was induced by 1 µg/mL LPS (Sigma Aldrich, St. Louis, Mo). Cultures were washed twice with PBS and RNA was subsequently isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. RNA was treated with DNase Set (Qiagen, Valencia, CA, USA). The cDNA was synthesized from total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and random primers, as previously described [17]. Quantification of the relative transcript amounts performed by qPCR with 10 ng of each cDNA and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reactions were performed in the instrument StepOnePlus™ (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an endogenous control. The relative gene copy number was calculated using the $2^{\Delta\Delta}$CT method and o primer-dimers were generated during the applied 40 real-time PCR amplification cycles.

TRAP staining

To examine the effect of the MSE and fractions on sRANKL-induced osteoclastogenesis in RAW 264.7 macrophage cells, a quantitative measurement was conducted. Osteoclast formation was measured by the quantification of TRAP+

multinucleated osteoclasts per well, using light microscopy. RAW 264.7 cells were seeded in 96-well plates (~ $5x10^3$ cells/mL) and stimulated with sRANKL (50 ng/mL). Treatments were MSE and fractions at 10 µg/mL, and the cell culture medium was α-MEM with 10% fetal bovine serum (FBS). After 6 days, cells were fixed with 4% paraformaldehyde, washed with PBS, and stained for TRAP (Sigma Aldrich, St. Louis, MO, USA). TRAP-positive multinucleated (>3 nuclei) cells were counted as osteoclast-like cells [7].

Gelatin zymography

RAW 264.7 cells (~ $1x10^6$ cells/mL) were seeded into 24-well plates for 24h. Inflammatory response was induced by 1 µg/mL LPS for 48 h (Sigma Aldrich, St. Louis, Mo). The test concentrations of the MSE and the HF, CLF, EAF and AF were 10 µg/mL for 48 h. The supernatant was collected and the amount of total protein was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). An equal amount of protein was designed by electrophoresis in Tris-Glycine Gels (Novex®, Life Technologies, Carlsbad, CA) under non-reducing conditions. The protein separated in the gel was developed using Developing Buffer supplied by the manufacture (Novex®, Life Technologies, Carlsbad, CA). Subsequently the developed gelatin gel was stained with Coomassie R-250 Stain [7].

Antioxidants assays

DPPH

The reaction mixture consisted of 0.5 mL of extract and fractions, 3.0 mL of pure ethanol, and 0.3 mL of DPPH radical in a 0.5 mM ethanol solution, which was incubated at room temperature for 45 min, and the activity was expressed in µmol Trolox/g of sample per dry weight. The calibration curve was constructed with the standard Trolox in the concentration range of 0 to 200 µM Trolox. Several MSE and fractions concentrations were

used, and readings were monitored at 517 nm using a spectrophotometer (Shimadzu, Japan). The antioxidant activity measured by the DPPH free radical method can be expressed as IC_{50}, *i.e.*, the antioxidant concentration required to reduce the initial DPPH radical by 50%. The sample concentration required to reduce the initial DPPH radical by 50% (Alencar *et al*., 2007).

ABTS•+

The antioxidant activity by the ABTS•+ method (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) was assessed according to the method described by Re et al. (1999) with modifications. The ABTS radical was formed through the reaction of 7 mM ABTS•+ solution with 140 mM potassium persulfate solution, incubated at 25 °C in the dark for 12–16 h. Once formed, the radical was diluted with ethanol P.A. to an absorbance of 0.700 ± 0.020 at 734 nm. Three different dilutions of each vegetable extract were prepared in triplicate. After that, 30 μL of the MSE and fraction dilution were transferred to test tubes with 3.0 mL of ABTS radical in the dark. The absorbance was read at 734 nm after 6 min of reaction using ethanol as a blank. Trolox, a synthetic water-soluble antioxidant analogue of vitamin E, was used as the reference at concentrations ranging from 100 to 2000 μM and the results were expressed as μM Trolox/g sample.

Chemical analysis

HPLC analysis

A Shimadzu Prep 6AD LC system equipped with SPD-M10Avp photodiode array detector (PDA), a 10AF auto injector and FRC-10A fraction collector were used to perform the high-performance liquid chromatography (HPLC) analysis. For the analytical test, diluted solutions of the AF were filtered (Millipore – 0.22 μm), and 10 μL aliquots were injected into a Shimadzu reverse-phase analytical column of 250 mm × 4.6 mm × 5 μm (particle size). For

the mobile phase, we used water (solvent A) and methanol (solvent B) at a constant flow rate of 1 mL/min. The gradient started with 80–90% for solvent B at 15 min returning to 80% at 30 min. The fraction was detected according to characteristic UV-vis spectra (spectral range of 200–450 nm) and retention times [21].

Mass spectrometric analysis of the bioactive fraction

The tandem mass spectrometry (MS/MS) system employed to confirm analyte identities was a Quattro LC triple quadrupole (Micromass, Manchester, UK) fitted with a Z-electrospray (ESI) interface operating in negative ion modes. The temperatures of source block and desolvation gas were set at 100 °C and 450 °C, respectively. Nitrogen was used as both desolvation (nearly 380 L hr-1) and nebulizer (nearly 38 L hr-1) gas, while argon was used as the collision gas. The voltages employed in the ESI source during the analysis were 40 V for the cone, 3 kV for the capillary and 3 V for the extractor. For identity confirmation, the analysis were carried out in the multiple-reaction monitoring (MRM) mode using collision energies of 15-25 eV. For this analysis, 23.8 mg of the AF were diluted with 1 mL MeOH: 0.1% formic acid (1:1, v/v) and injected into the LC-MS/MS system under a flow of 20 μL min^{-1}. Table 1 presents the ion transitions under MRM mode employed to monitor for rutin in the sample.

Table 1. Ion transitions under MRM mode employed to monitor for rutin in the sample.

Compounds	**Ionization mode**	**Molecular ion $[M+H]^+$ or $[M–H]^-$ or $[M+Na]^+$**	**Fragments/Product ions**
		(*m/z*)	
Rutin	Positive	611	464, 303, 147, 129
	Positive	633	486, 331, 324, 133
	Negative	609	301, 271, 179, 151

Statistical analysis

Continuous variables are presented as mean values + SD. The Shapiro–Wilk test was used for the assessment of normality. All reported p- values are compared to a significance level of 0.05. For multiple group comparisons, the data were subjected to one-way analysis of variance (ANOVA). To determine overall difference between the group means and Tukey's significant difference for pair-wise differences for within group comparisons. the Bonferroni post-test indicated a significant difference between the controls. Data were analysed using STATA™ (Version 10.0, Stata Corporation, College Station, TX, USA) and GraphPad (Version 5.0, GraphPad Software Inc., San Diego, CA).

Results

Neutrophils migration in the peritoneal cavity

The MSE and fractions were primarily tested for anti-inflammatory effects using a neutrophil migration model as an in vivo model to screen anti-inflammatory compounds. The results showed that the oral administration of 30mg/kg of the MSE decreased the migration of neutrophils into the peritoneal cavity compared to the carrageenan group ($\rho<0.05$).

The fractionation method used for the MSE was effective, showing that the CLF, EAF and AF concentrated the biological activity and reduced the neutrophil influx in all doses tested. However, the AF yielded the best results, showing in all doses tested with a significant reduction of neutrophil migration and the lowest percentages of inflammatory cells compared to the other fractions (Figure 1).

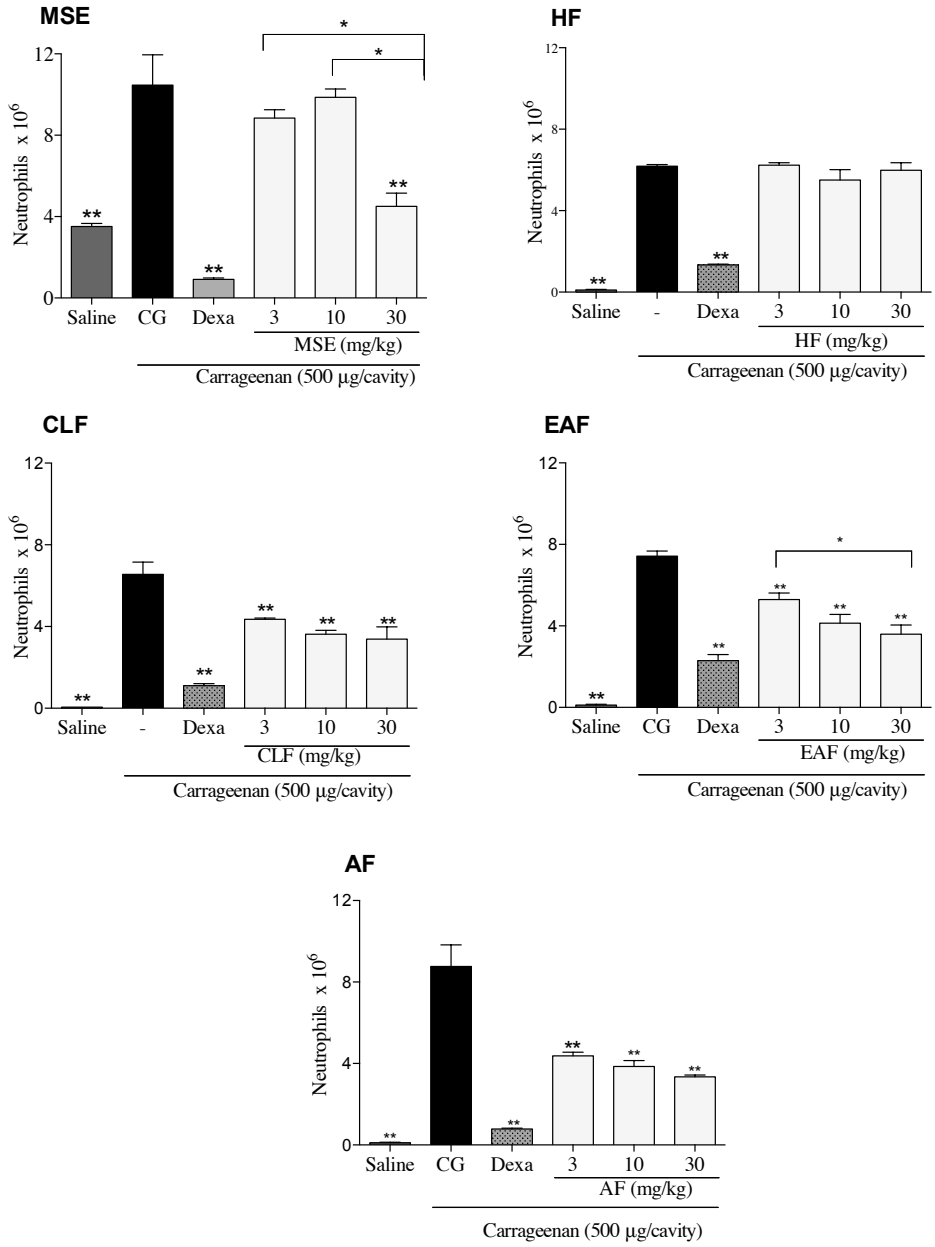

Figure 1. Inhibitory effect of the MSE, HF, CLF, EAF, AF on neutrophil migration into the peritoneal cavity induced by carrageenan. Neutrophil migration was determined 4 h after the injection of carrageenan 500 µg/cavity. Mice previously treated with the vehicle, MSE, HF, CLF, EAF, AF. The data are expressed as mean ± SD, n = 6. Symbols indicate statistical difference ($p < 0.05$, ANOVA, Tukey post test) ** compared to the carrageenan group; * comparison between the concentrations tested 3, 10 and 30 mg/kg.

Carrageenan-induced paw edema

The MSE and the bioactive AF were verified for antiedematogenic activity. The results for the AF demonstrated a biological activity in the first hour analyzed. AF was comparable to the positive control indomethacin for second and third hours analyzed as shown in (Table 2).

Table 2. Effect of MSE and the AF on carrageenan-induced paw edema on mice.

Treatment (mg/kg)	**Time (h) after injection of carrageenan**				
	1	2	3	4	5
Carrageenan	0.08±0.02	0.13±0.02	0.17±0.01	0.12±0.02	0.12±0.02
Indo 10	0.05±0.03	0.05±0.03*	0.08±0.02*	0.08±0.02*	0.08±0.02*
MSE 30	0.06±0.02	0.08±0.02*	0.07±0.01*	0.11±0.03	0.11±0.03
AF 30	0.04±0.01*	0.06±0.02*	0.07±0.02*	0.10±0.02	0.09±0.02

The data are expressed as mean ± SD, n = 6. Symbols indicate statistical difference ($p < 0.05$, ANOVA, Bonferroni post test) * compared to the carrageenan group.

Cytokines assay

The administration of the AF at a dose of 30 mg/kg significantly reduced the level of cytokine IL-1β (54%) compared to the control group vehicle ($p<0.05$). Thus, non-significant differences were found at TNF-α expression levels (Figure 2).

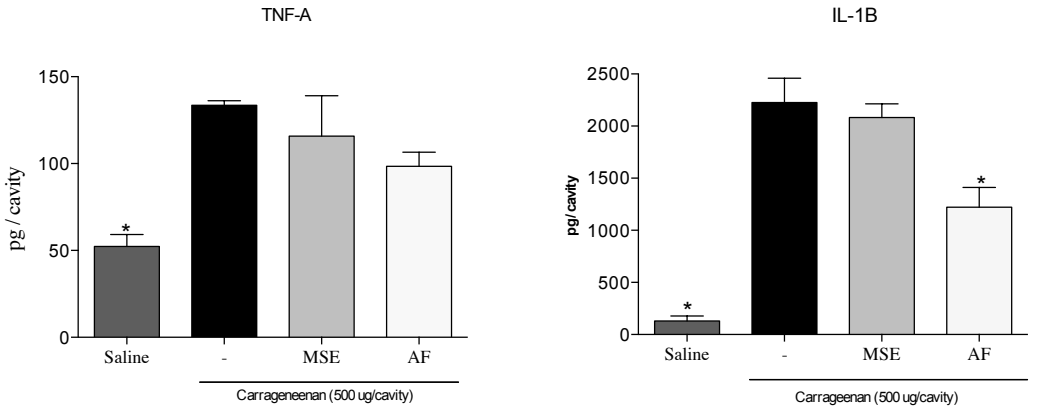

Figure 2. Quantification of TNF-α and IL-1 β in the peritoneal cavity. Mice were previously treated with the vehicle, the MSE and the AF at a dose of 30 mg/kg 1h before the carrageenan injection. Data are expressed as mean±SD, n=6. Symbols indicate statistical difference ($p<0.05$, ANOVA, Dunnett's test); *$p<0.05$ compared to the carrageenan group.

Cell viability assay

The viability test showed a concentration-dependent effect, where the MSE, HF, and CLF at concentrations higher than 10 µg/mL were toxic for the cells. The AF and EAF did not affect the cell viability at any of the concentrations tested when compared to the control group (non-treated) ($p>0.05$)(Figure 3).

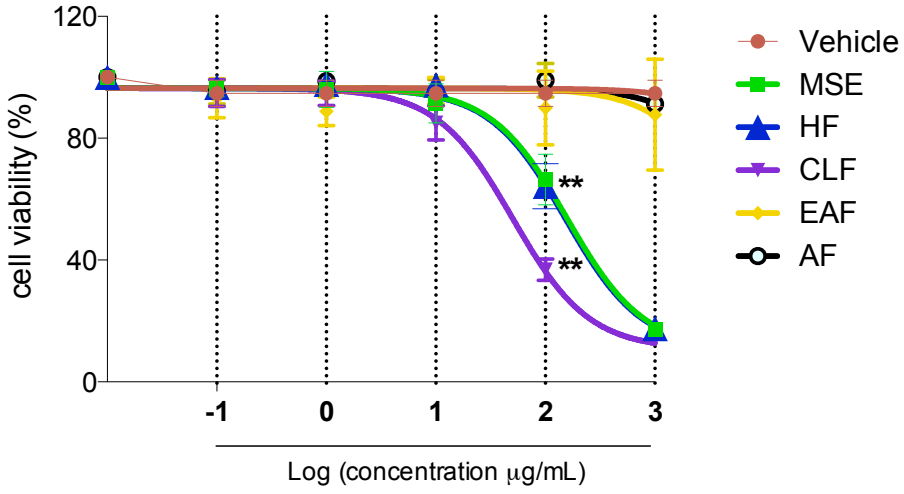

Figure 3. Effect of MSE and fractions on cell viability. Log dose response of MSE, HF, EAF, and AF on RAW 264.7 cells**.** Significant difference between fractions at the indicated dose ($p<0.05$). **Significant difference between EC50 values ($p < 0.05$). Data were expressed as mean±SD, n=9.

Analysis of gene expression

Gene expression analysis showed that the AF was the only treatment that had the ability to downregulate all the study genes: ACII, cathepsin K and TRAP ($p>0.05$) (Figures 4, 5, 6). The MSE, CLF, EAF and AF downregulated the gene expression of TRAP ($p>0.05$) (Figure 6). The gene transcription of cathepsin K was controlled by the treatments with the AF and EAF ($p>0.05$) (Figure 5).

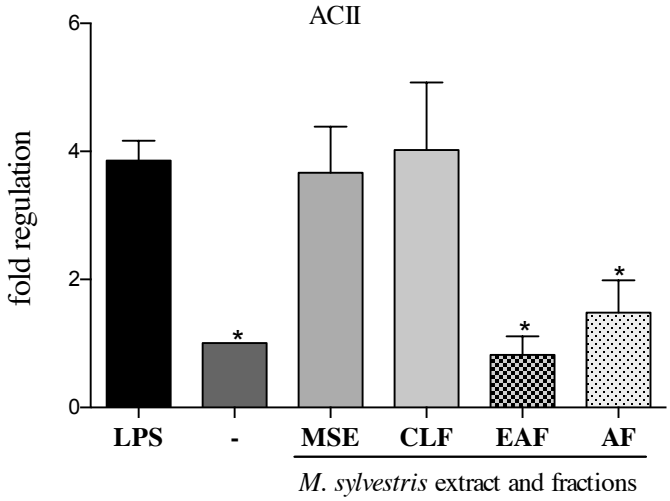

Figure 4. Effect of MSE and fractions on CAII (*c*arbonic anhydrase) expression levels. Quantification of the relative transcript amounts performed by qPCR with 10 ng of each cDNA. Fold regulation was calculated in comparison to the LPS control group. Statistical analysis were performed by one-way ANOVA followed by Dunnett's post-hoc tests. *$p < 0.05$ significantly different from LPS-stimulated cells.

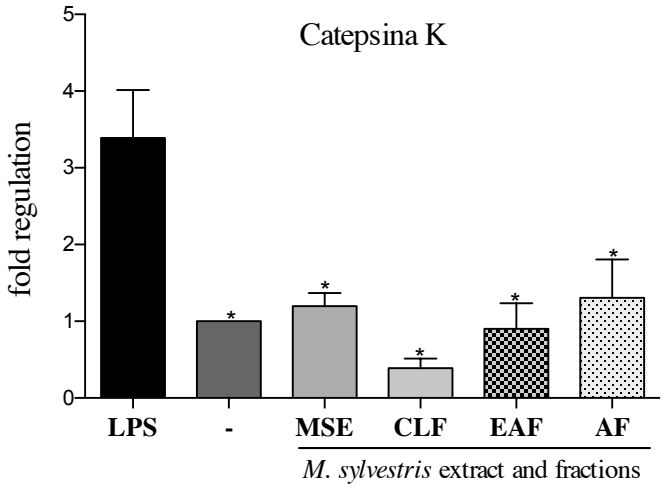

Figure 5. Effect of MSE and fractions on cathepsin K expression levels. Quantification of the relative transcript amounts performed by qPCR with 10 ng of each cDNA. Fold regulation was calculated in comparison to the LPS control group. Statistical analysis was performed by one-way ANOVA followed by Dunnett's post-hoc tests. *p < 0.05 significantly different from LPS-stimulated cells.

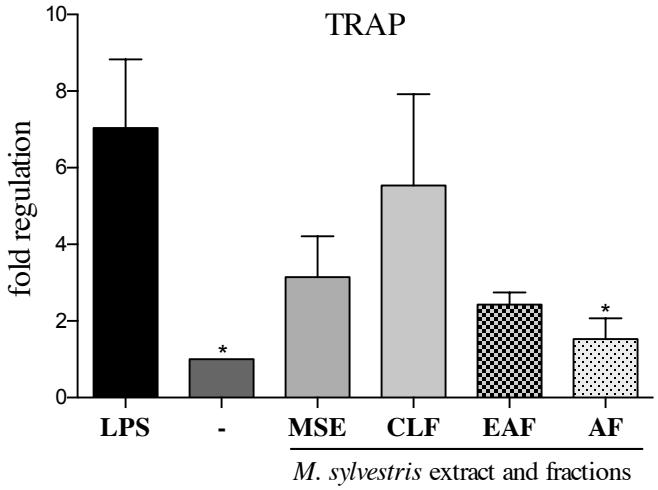

Figure 6. MSE and fractions effect on TRAP expression levels. Quantification of the relative transcript amounts performed by qPCR with 10 ng of each cDNA. Fold regulation was calculated in comparison to the LPS control group.Statistical analysis were performed by one-way ANOVA followed by Dunnett's post-hoc tests. *p < 0.05 significantly different from LPS-stimulated cells.

TRAP staining

The number of TRAP-positive cells levels had high levels of expression in the stimulated control group ($p<0.05$). The CLF and AF treatments had the ability to reduce the osteoclastogenesis on RAW 264.7 cell lines compared to the vehicle control group ($p<0.05$) (Figure 7).

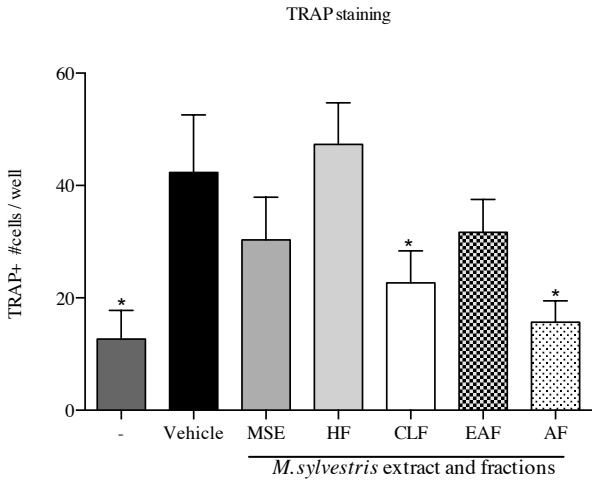

Figure 7. Activity of *M. sylvestris* and fractions on RANKL-mediated osteoclast differentiation *in vitro*. RAW 264.7 cells were stimulated with sRANKL (50 ng/ml) for 6 days. Cells were fixed and stained for TRAP. TRAP+ multinuclear cells were counted. Data represent mean ± SD of three cultures. * $p < 0.05$; significantly lower than sRANKL-stimulated group.

Gelatin zymography

The results showed that two different fractions caused the reduction of gelatinolytic activity: the AF and EAF at the 10 μg/*mL* concentration. The non-treated group and the AF, EAF were not significantly different ($p>0.05$). In our study, MMP-9 activity decreased 69% and 75% (AF and EAF, respectively). In contrast, HF and CLF increased the enzyme activity (Figure 8).

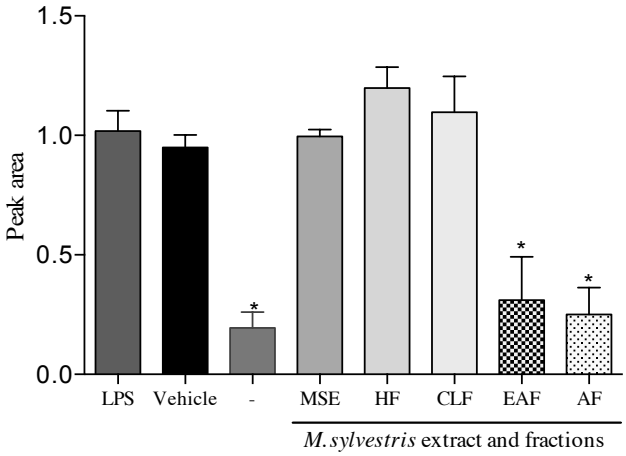

Figure 8. Effect of MSE and fractions on MMP-9 expression levels. Proteolysis activity was stimulated by LPS *(E. coli)* 1 μg/mL. Supernatant was mixed 1:1 with sample buffer and then applied to the gels. Quantification was performed by peak area and normalized by the protein ladder band. Data quantification was performed using ImageJ software. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. *p < 0.05 significantly different from LPS-stimulated cells.

DDPH and ABTS$^{\bullet+}$ assay

The results are presented in Table 3 and show that using the ABTS•+ method, the AF and EAF had the highest antioxidant activity (1.3 μmol Trolox/g) and (1.1 μmol Trolox/g), respectively.

Based on the DPPH method results, the samples where the concentration to reduce the initial amount of DPPH radical by 50% were the EAF (0.94 g/L), followed by the AF (1.01 g/L), CLF (1.78 g/L). The lowest IC_{50} values can be considered good results in terms of antioxidant activity, given that a low concentration is required to reduce the DPPH free radical by 50%.

Table 3. Antioxidant activity of MSE and fractions using $ABTS^{\bullet+}$ and DPPH method.

	Antioxidant activity	
Groups	E_{50} DPPH (g/L)	ABTS•+ (μmol Trolox/g)
MSE	2.62±0.08[a]	0.34±0.03[a]
HF	6.01±1.78[b]	0.25±0.02[a]
CLF	1.78±0.20[c]	0.64±0.10[a]
EAF	0.94±0.04[d]	1.10±0.20[b]
AF	1.01±0.06[d]	1.30±0.20[b]

* Averages of triplicates ± SD / means followed by letters showing the columns that differ statistically ($p < 0.05$). Tukey's multiple comparison test. DPPH: 2,2-diphenyl-1-picryl-hydrazine; ABTS: 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid.

Chemical analysis

HPLC analysis

The HPLC analysis was used for the bioassay-guided fractionation and the major compound of the AF was identified and represented (Figure 9). The peak corresponding to rutin was identified at retention time 47.58 min and UV absorbance at 350 nm.

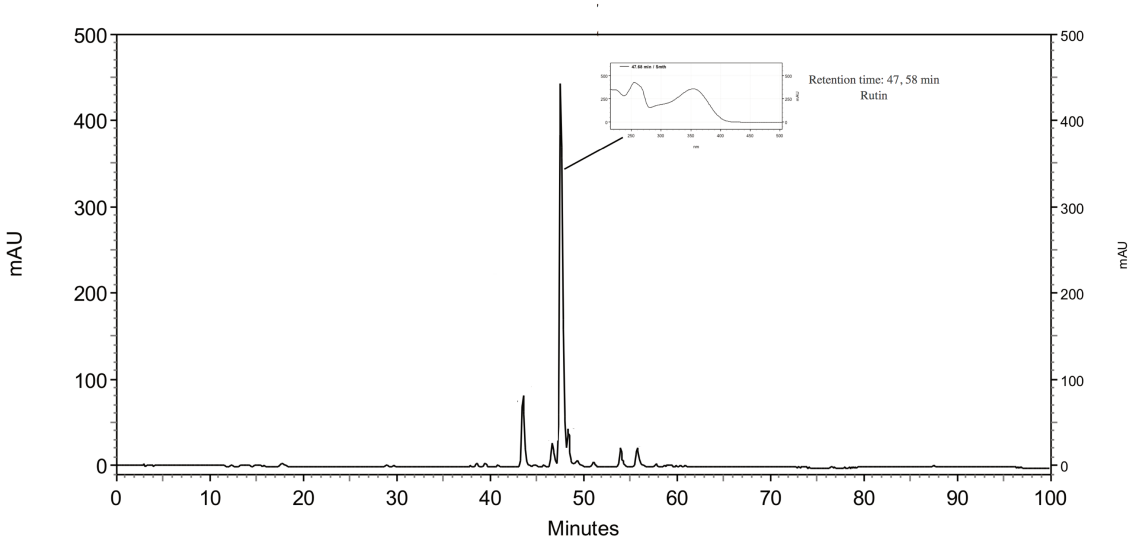

Figure 9. Analytical HPLC-PAD chromatogram of the AF of the ethanolic extract of *M. sylvestris* recorded at UV 350 nm.

Mass Spectrometric Analysis of Bioactive Fraction (Aqueous Fraction)

For identity confirmation of rutin, ESI positive and negative modes were employed. For ESI positive mode, protonated ion $[M+H]^+$ (m/z 611, Figure 10) and sodiated ion $[M+Na]^+$ (m/z 633, Figure 11) as well as their respective product ions were monitored. For ESI negative mode, the deprotonated ion $[M-H]^-$ (m/z 609, Figure 12) and its respective product ions were monitored.

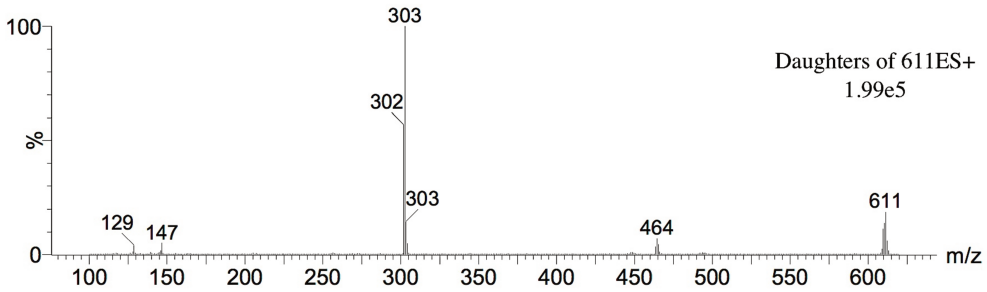

Figure 10. Mass spectrum on ESI positive mode under MRM monitoring for molecular ion m/z 611.

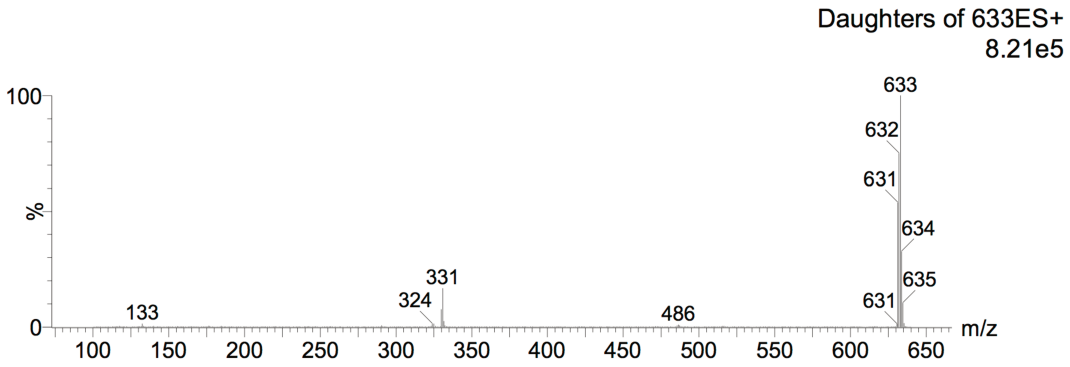

Figure 11. Mass spectrum on ESI positive mode under MRM monitoring for molecular ion m/z 633.

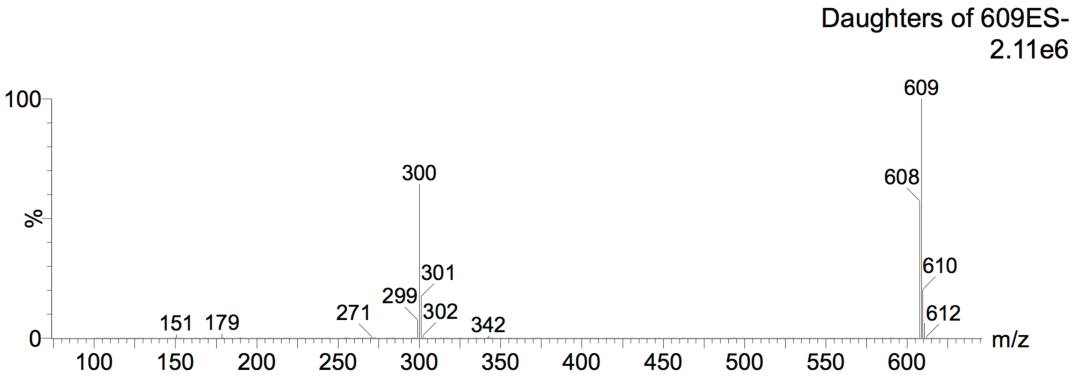

Figure 12. Mass spectrum on ESI negative mode under MRM monitoring for molecular ion m/z 609.

Discussion

The complexity of non-resolving inflammation and the lack of understanding it render pharmacological therapy difficult [1]. Multipronged therapeutic approaches are needed to treat chronic inflammatory diseases, and the most potent drug interventions may increase the risk of adverse effects [22]. Glucocorticoids are commonly used as a reference drug when treating chronic inflammation, and their major adverse effect is additional bone loss [23].

Many epidemiological and experimental studies suggest that natural products may have the ability to decrease oxidative and inflammatory process, thereby helping to prevent

chronic diseases as a dietary product [24]. The MSE and fractions were primarily tested for anti-inflammatory effects using the neutrophil migration model as an *in vivo* activity screening model. Our findings show that the extract and all the fractions, except the HF, had the capacity to reduce the number of neutrophil migrating into the carrageenan-induced peritonitis. However, with the bioguided method of fractionation, we were able to establish that the AF concentrated the active compound and presented the most significant anti-inflammatory reduction. The mechanisms involved in the inhibition of neutrophil migration to the damage site are accompanied by the reduction in inflammatory markers and oxidative stress [25]. The AF promoted the significant reduction of cytokine IL-1β, which plays a role in the release of prostanoids [26]. The release of pro-inflammatory cytokines is related to the migration process, and induces the rolling and adhesion of neutrophils in the vascular endothelium and transmigration to the inflammatory site [1]. In this way, the putative mechanism associated with this activity may be inhibition of the synthesis of cytokines such as IL-1β, which are involved in cell migration [27].

The carrageenan paw edema was accessed to verify the potency of the AF in the inflammation process [28]. The biphasic nature of edema is key in the role of the inflammatory response [29]. In the first phase (0-3h) there is an increase in histamine, serotonin and chemical mediators, and these are related to the greater vascular permeability and production of cytokines such as IL-1β and TNF-α (Winter et al., 1962). The second phase is sensitive to most clinically anti-inflammatory drugs and related to the release of prostaglandins, protease, and lysosome [30]. The results of the AF (30 mg/kg, orally.) showed an anti-inflammatory activity statistically equal to the positive control indomethacin (10 mg/kg i.p.). In this way, the anti-inflammatory effect of the fraction may be due to the suppression of cyclooxygenases involved in the formation of prostaglandins.

This study demonstrated that the AF and EAF had a significant inhibitory effect on MMP-9 accessed by zymography. MMP-9 (gelatinase B, 92 KDa Type IV) is produced in the cell environment and is activated after release into the extracellular space [7]. This protein is involved in the breakdown of the extracellular process of bone development and may be found in many pathological conditions, such as arthritis and tumor metastasis [31]. The use of natural products to inhibit MMPs may contribute to attenuating the proteolysis of the extracellular matrix and the role in bone osteoclastic resorption [32].

Osteoclasts are formed by the fusion of hematopoietic cells of monocyte-macrophage lineage during the differentiation process [33]. RAW 264.7 cells are precursors and can express phenotype marker genes such as TRAP and cathepsin K, which represent the expression of mature osteoclasts [34]. The AF was able to reduce the expression of both genes and the enzyme anhydrase carbonic II that is overexpressed during the bone resorption process. Despite the lack scientific information about natural products and bone-protective effects, it is known that flavonoids, a common class of natural products, have shown promise in the area of health promotion related to dietary components like calcium and vitamin D [35]. Flavonoids have been related to the activity of the signaling pathways that influence the osteoblast in an osteoclastic difference. Consistent with our study, authors have tested an osteoclastic differential *in vitro* model on RAW 264.7 cells and found a therapeutic potential in a Chinese traditional herb for reducing the TRAP, MMP-9, cathepsin K genes in this way, showing an ability to directly interact with the bone cells (osteoblasts, osteoclasts, osteocytes) [36].

Some tests that evaluate radical scavenging capacity may contribute to the etiology of various degenerative diseases, particularly those related to the chronic inflammation process [22]. When sequestering, radical antioxidants can regulate the oxidation pathway [37]. The ABTS and DPPH assays conducted on *M. sylvestris* and fractions screened

the biologically antioxidant activity and related it to the bone remodeling effect. The bioactive AF and EAF had a significantly ($p<0.05$) better capacity eliminate radicals. In agreement with our study, other investigations have reported the highest antioxidant activity for the AF using the β-carotene-linoleic acid method [16]. In particular, the literature shows that extracts obtained from leaves, as ours were, have a very strong antioxidant property and the activity is tied to the region where the plant has been collected, with the strongest obtained from *M. sylvestris* extracts being found in northeastern Portugal (Barros *et al*., 2010).

The chemical analysis of the AF identified the compound rutin. This compound is a common flavonoid used in plant-derived beverages, food and folk medicine. Studies have reported diverse pharmacological activities of rutin, including promoting health and reducing risk of chronic diseases. In particular and consistent with our study, research has found rutin to be an anti-inflammatory drug candidate with a unique mechanism for selective COX-2 [38]. Authors have also reported that rutin could inhibit more than 20 genes coding for critical pro-inflammatory factors including TNF-α, IL-1β, IL-1 and IL-8, and migration inhibitory factor [39]. Rutin appears to be a potential phytochemical ingredient for chronic inflammatory treatment or even a promising functional food for the market.

Conclusion

The AF of *M. sylvestris* presented the capacity for anti-inflammatory, anti-oesteoclasteogenic and antioxidant activities. In addition, we suggest that the bioactive fraction and its compounds rutin with its multi-target activity may be a good candidate for drug discovery in the therapy of chronic inflammatory diseases.

Acknowledgement

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2.3 ARTIGO[3]

Evaluation of *Malva sylvestris* as inhibitor of HIV-1 BaL in a dual chamber *in vitro* model

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Abstract

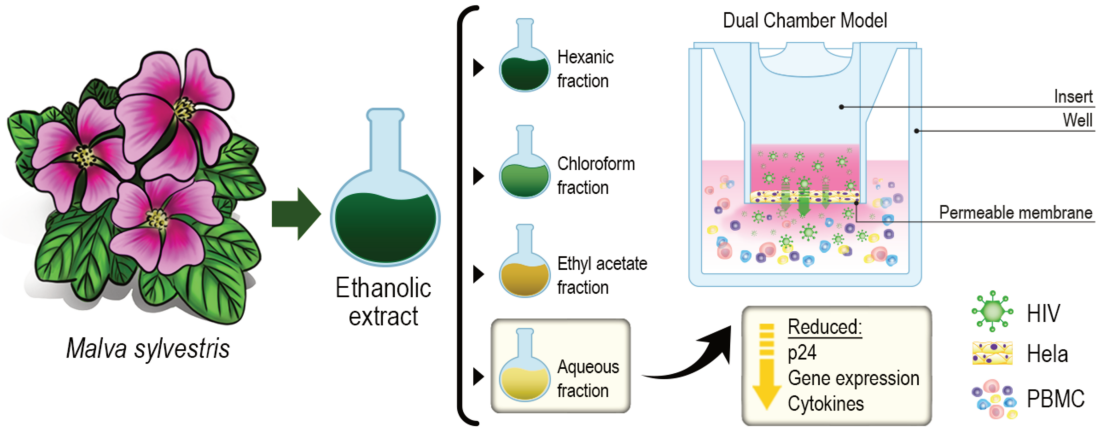
Ethnopharmacological relevance- Emphasis is now being placed on identifying novel molecular agents, including microbicides that can be applied topically and protect against sexually transmitted infections, especially HIV. Natural products are a good source of drug discovery.

Aim of the study- to investigate the potential anti-HIV activity of aqueous fraction of *Malva sylvestris* on cells infected by HIV-1BaL using a dual-chamber model.

Material and Methods- Extract and fractions were screened for anti-HIV activity measuring the antibody-mediated neutralization of HIV-1. The *in vitro* cytotoxicity of the aqueous fraction (AF) was assessed using a fluorescent assay for the TZM-bl, PBMC and HeLa cell lines. Antiviral activity was supported by p24 quantification in the supernatant of a dual-chamber model, cytokine release, and transcription of specific targets CD4, TRIM5 and Bcl-2 and tested against HIV-RT.

Results- The AF demonstrated potential anti-HIV activity on the TZM-bl cell line, showing a reduction score higher than 60% infectivity. Results showed no cytotoxicity effect for AF in all cell lines studied ($p > 0.05$). Quantification of p24 in the supernatant of the dual-chamber model demonstrated a reduction in viral particles after AF treatment ($p < 0.05$). Cytokines were quantified and AF reduced the expression of all signaling related to the inflammatory process, such as IL1-alpha, IL-beta, IL-6, IL-8 and GM-CSF ($p<0.05$) compared to the control group. In particular, IL-6 had low levels of expression when compared to the AZT positive control group. The molecular pathways used by AF to regulate the HIV-1BaL infection showed a controlled transcription of the genes CD4, Bcl-2 and TRIM5 and HIV-RT.

Conclusions- *M. sylvestris* contains highly potential anti-HIV components, non toxic effects and anti-inflammatory properties, thereby characteristics to be considered as a potential microbicide.



1. *Malva sylvestris* action in a HIV infectivity model *in vitro*.

Introduction

Human immunodeficiency virus (HIV) is responsible for the acquired immunodeficiency syndrome (AIDS). The first cases of AIDS were reported in 1981 and today, more than 34 years later, it is one of the world's most serious health problems. According to estimates by the World Organization, approximately 78 million people have been infected with HIV worldwide and about 39 million people have died of HIV. Sub-Saharam Africa remains most severely affected, with 1 in every 20 adults living with the virus (WHO, 2010). The epidemic continues to expand in countries and regions where HIV treatment is insufficient coverage (Maartens et al., 2014).

The advent of antiretroviral therapy modified the epidemiology of HIV leading to decreased mortality and risk of transmission (Simon et al., 2006). Currently, therapy consists of a combination of three classes of drugs that transformed the infection disease into a chronic event: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase

inhibitors and protease inhibitors (Pomerantz and Horn, 2003). However, a significant number of therapies are still not curative and financial obstacles may limit access by some populations to prevention techniques and treatment (Cutler and Justman, 2008). The focus has been placed on discovery and development of novel agents, including microbicides that can be applied topically and protect against sexually transmitted infections (STIs), especially HIV(Pirrone et al., 2012). The future distribution of microbicides may prove to have an important social impact, reducing health care costs and risk of HIV infection (D'Cruz and Uckun, 2004).

Natural products are the most productive source of drug discovery and the bioactivity-guided fractionation of crude extracts may generate new molecules with biological activity, especially with anti-HIV activity (Cragg et al., 2014). Many natural compounds have a specific biological activity and low toxicity effects working on a particular target that may act to complement the traditional antiviral drugs (Yang et al., 2001). Studies have shown that a natural product native to Europe, North Africa and Asia named *Malva sylvestris* has the potential to treat inflammation, and has antimicrobial properties and antioxidant, activity (Gasparetto et al., 2012). Its use in folk medicine is widespread and given its medical importance it is a good candidate for drug discovery. Therefore, the aim of this study was to investigate the potential anti-HIV activity of the aqueous fraction of *M. sylvestris* on cells infected by HIV-1BaL using a dual-chamber model.

Material and Methods

Preparation of the extract and fractions

M. sylvestris leaves were registered in the herbarium of the University of Sao Paulo (USP, Piracicaba, Sao Paulo, Brazil) under an identification number (ESA #1214403). All material was collected in northeast Brazil (07º44'12" S and 37º59'36" W) and the extract

was prepared with absolute ethanol using constant shaking (100 rpm, 1 week, in the dark at room temperature) and exhaustive maceration technique. The ethanolic extract of *M. sylvestris* (MSE) was homogenized, lyophilized, weighed for the fraction using liquid-liquid partitioning by chemical polarity gradient with hexane, chloroform and ethyl acetate solvents. The final residue obtained was totally soluble in water and thus was called the aqueous fraction (AF) (Benso et al., 2015b).

Cell lines

The cell lines included in this study were: TZM-bl obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH and peripheral blood mononuclear cells (PBMC) (Astarte Biologics, LLC, Redmond, USA, Catalog number 1001, Lot number 2536DE13) obtained from a healthy donor and HeLa from ATCC (CCL-2).

Virus strain

HIV-1BaL was originally explanted from a primary culture of cells from infant explanted lung and obtained through NIH (AIDS Reagent Program, Division of AIDS, NIAID, NIH). All virus samples were stored at -80ºC and serial dilutions were conducted (1:10). After 3 days, the multiplicity infection of HIV-1BaL was verified using the enzyme-linked immunosorbent assay (ELISA) assay to establish the tissue culture infective dose (TCID)(Pasetto et al., 2014). The multiplicity of infection (MOI) was obtained and corrected to MOI=1, for all assays. The positive control treatment was Zidovudine (AZT) (Sigma Aldrich, St Louis, USA). For all assays the vehicle was (1% DMSO, v/v; Sigma Aldrich, St Louis, USA).

Anti-HIV activity to evaluate microbicide candidate

Cytotoxicity activity

Cell viability assay was conducted using the CellTiter-Blue® reagent (Promega, Corp, Madison, WI, USA) that provides an estimate of viable cells through a dye indicating the metabolic capacity. Cells were cultured (200 µL, $1x10^5$ cells/mL) in a 96-well plate and incubated for 24 h at 37ºC with 5% CO_2. The AF at the biological activity concentrations (25-50 µg/mL) were added to cell culture and incubated for 24 h. Subsequently, the culture medium was discarded and the cells washed with PBS (Lonza, Walkersville, MD, USA) and a fresh new medium with 20 µL of CellTiter-Blue was added and incubated for 4 h (Pasetto et al., 2014). The contents were transferred to a new microplate and the fluorescence reading conducted in a microplate reader (SpectraMax M5, Molecular Devices®) with 550 nm excitation, 585 emission.

Anti-HIV activity in infected TZM-bl cells

The *in vitro* activity of AF was conducted on the TZM-bl cell line. The assay was previously described by (Ochsenbauer-Jambor et al., 2006). These cells are genetically engineered from a HeLa cell that expresses CD4, CXCR4, and CCR5 and contains reporter genes for luciferase (Luc) upon infection and therefore enables the measurement of relative HIV-1 infectivity by quantifying luminescence units. Cells were cultured in a 96-well plate ($1x10^5$ cells/well) and infected with HIV-1BaL (MOI=1) for 48 h. The cytotoxicity activity was measured by fluorescence as previously described. The cells were washed with PBS and treated with lysis buffer. In the following step 20 µL/well luciferase reagent was added. The luminescence reading was conducted in a microplate reader (SpectraMax M5 Molecular Devices) with 500 ms of integration time.

Dual-chamber model

Quantification of HIV-1 p24 antigen by ELISA

Aiming to mimic the epithelium of the female genital tract and to verify the potential of AF as a microbicide, the dual-chamber model was developed(Gali et al., 2010; Pasetto et al., 2014). Transwell assay inserts with 8 μM diameter pores and 0.3 cm^2 of culture surface (Greiner Bio-One®) were positioned in the wells of a 24-well plate. The first layer, (1×10^5 cells/mL) HeLa cells were culture into each transwell insert/apical chamber. PBMC cells (1×10^5 cells/mL) were seeded into the basal chambers below the inserts, and the plates were incubated at 37°C, 5% CO_2. To assess confluence, the transepithelial electrical resistance (TEER) of each HeLa cell layer was measured with a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA,) before and after AF treatments (Gali et al., 2010). Cell layer confluence in the transwell inserts was measured daily until the optimal TEER (>150 Ohm/cm2) was reached on day 4. The plate was incubated at 37°C, in humid air containing 5% CO_2. After 24 h (day 5), the cell viability and luciferase assays were performed to analyze the toxicity and anti-HIV activity of the AF.

Transcription analysis of genes involved in the role of HIV-1 disease

RNA was isolated from the cell culture (day 5) of PBMC cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quantification was measured in the NanoPhotometer P360 (Implen®, Westlake Village, CA, USA). Reverse transcription of RNA to cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Three primers CD4, Bcl-2 and TRIM5 were selected to comprehend generally the mechanism of action of the AF in the infection caused by HIV-1BaL. The threshold was manually adjusted within the logarithmic curve above the

background level and below the plateau phase. A comparative Ct method was used to calculate the relative gene number. The relative gene copy number was calculated using the $2^{\Delta\Delta}$CT method.

Quantification of cytokines

Cytokines were measured for all samples using an ELISA assay with a specific kit (Qiagen, Valencia, CA). The cytokines were measured by standard ELISA protocol using a panel of 6 inflammatory cytokines (IL-1alpha, IL-1beta, IL-6, IL-8, IL-10, GM-CSF).

Anti- HIV reverse transcriptase activity

Reverse transcriptase assay (RT) is an *in vitro* colorimetric enzyme immunoassay for screening anti-viral agents. The (RT) is a crucial enzyme for retrovirus replication, and its presence in the virion is indispensable for infectiity. In a microcentrifuge tube, 20 μL of recombinant HIV-1-RT (Roche Diagnostics, Indianapolis, IN, USA) was diluted in lysis buffer and 20 µL of the appropriate AF dilutions or vehicle control was added and incubated at 37°C. After 1 hour, 60 µL of each of sample was added into a microplate (Roche Diagnostics, Indianapolis, IN) precoated with streptavidin and incubated at 37°C for 1 hour. The wells were washed with washing buffer and 200 μL of anti-DIG-POD (antibody to digoxigenin conjugated to peroxidase) working solution was added to each well and then incubated at 37°C to bind to the digoxigenin-labeled DNA. After 1 hour, the wells were washed and 200 μL of peroxidase substrate ABTS solution was added to each well and was incubated at room temperature for 30 min. The resulting signal intensity is directly proportional to the RT activity and was determined by the absorbance using a microplate reader at 405 nm.

Results

Anti-HIV activity to evaluate microbicide candidate

Cytotoxicity activity

The cytotoxicity test of the AF was conducted on TZM-bl, HeLa, PBMC and cell lines. AF presented non-toxic effects on cell viability at the concentrations 25 and 50 μg/mL. The AZT positive control (60 μM) and vehicle control (1% DMSO) did not cause any cell toxicity (Figure 1).

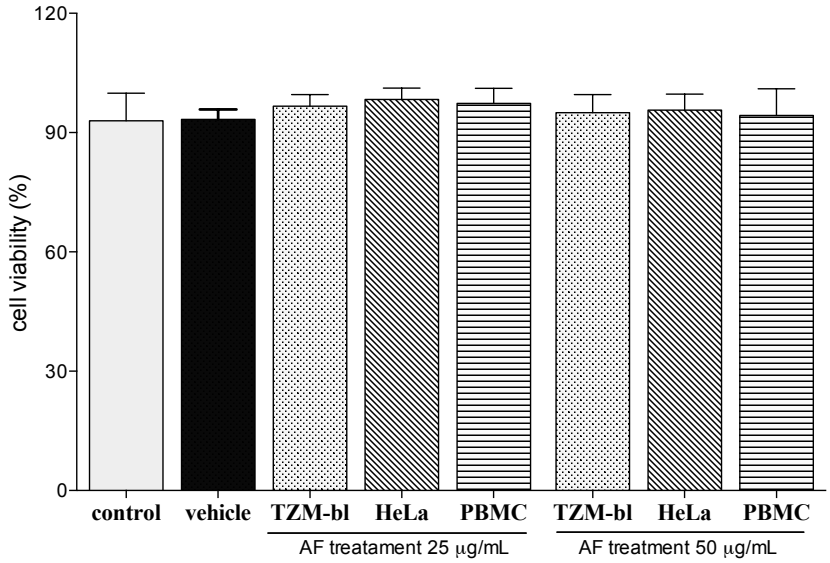


Figure 1. AF cell viability tested on TZM-BL, HeLa and PBMC cell lines. Data are expressed as mean ± SD using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests compared to the vehicle control. The level of statistical significance was set at 0.05.

Anti-HIV activity in infected TZM-bl cells

AF decreased significantly the HIV-1 infectivity on TZM-bl cells ($p<0.05$), blocking 67% of HIV-1BaL infection at the 25 μg/mL concentration. The inhibition effect of between the 25 and 50 μg/mL concentrations were not statistically different ($p>0.05$). The positive control (AZT 60 μM) inhibited 80% the HIV-1BaL infection, and the vehicle control (1% DMSO) did not affect the infectivity of the HIV-1BaL (Figure 2). The cell viability was assessed and non-toxic effects were found in the test groups ($p>0.05$).

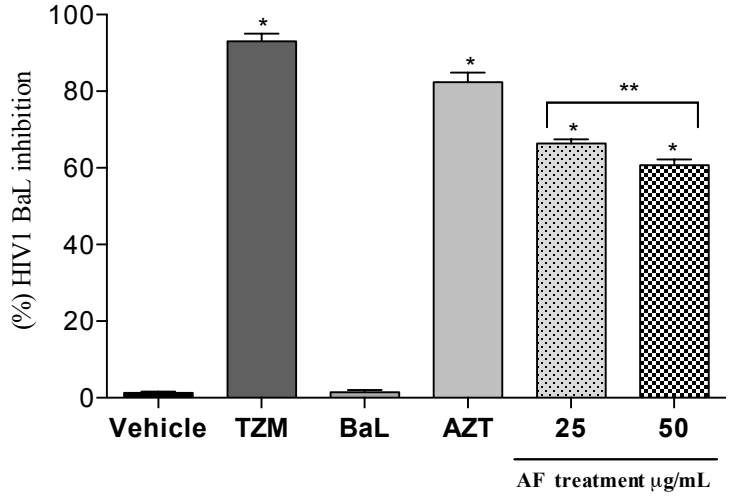


Figure 2. Anti-HIV-1 activity of AF on TZM-bl cell line infected by HIV-1BaL. Data are expressed as mean ± SD using a one-way ANOVA followed by Tukey's multiple comparison tests. Symbols indicate statistical difference ($p<0.05$) * compared to the vehicle group; ** comparison between the concentrations tested 25 and 50 μg/kg.

Dual-chamber model

Quantification of HIV-1 p24 antigen by ELISA

The results of p24 quantification by ELISA after PBMC cells were treated with the AF showed a statistically significant reduction in the infection ($p<0.05$). The AZT positive control and AF 50 μg/mL are not statistically different ($p>0.05$) (Figure 3).

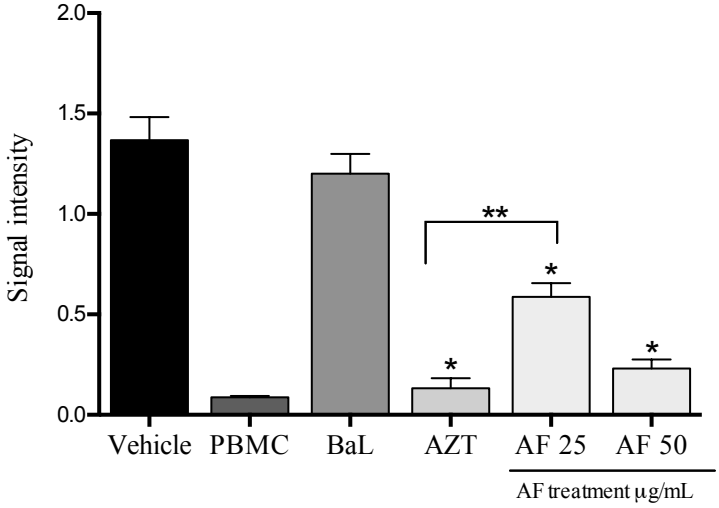


Figure 3. AF-mediated inhibition of HIV-1BaL infection in PBMC cells. Raw data is showed. Data are expressed as mean ± SD using a one-way ANOVA followed by Tukey's multiple comparison tests. Symbols indicate statistical difference ($p<0.05$) * compared to the vehicle group; ** comparison between the AF treatment and the AZT group.

Transcription analysis of genes involved in the role of HIV-1 disease

The AF showed a significant downregulation expression ($p<0.05$) of CD4, TRIM5 and Bcl-2 when compared to the control group (vehicle) (Figure 4).

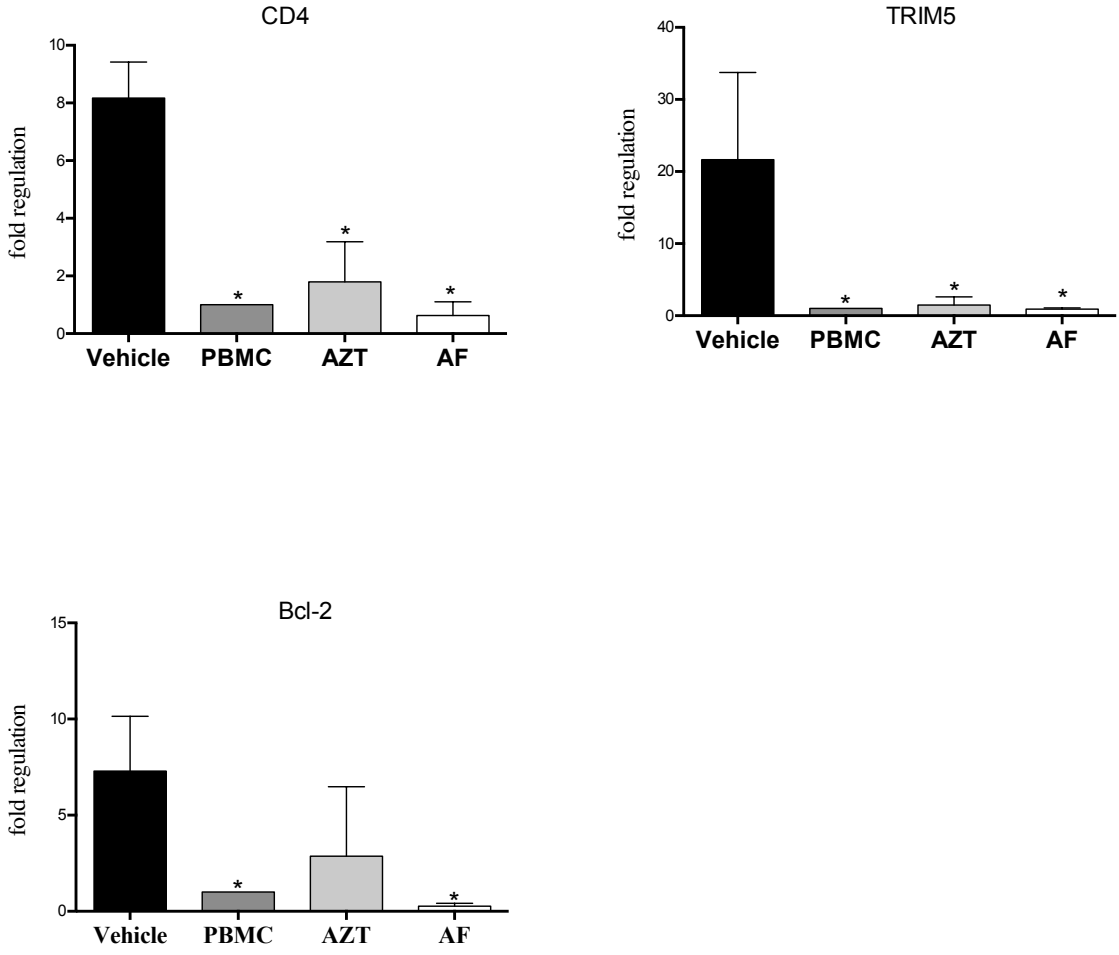


Figure 4. Gene expression quantification by qPCR technique. The AF and its effects on CD4, TRIM 5 and Bcl-2 gene expression levels. Quantification of the relative transcript amounts performed by qPCR with 20 ng of each cDNA. The AF treatment was at the 50 μg/mL concentration. Data quantification was performed using 2(-DeltaDeltaC(T)). Statistical analysis were performed by a one-way ANOVA followed by Dunnett's post-hoc tests. *$p < 0.05$ was significantly different.

Quantification of cytokines

The AF at the 50 μg/mL concentration was able to reduce significantly all the cytokines analyzed: IL-1alpha, IL-1beta, IL-6, IL8, IL10, GM-CSF ($p<0.05$). The comparison between the AF groups and the AZT group showed no difference ($p<0.05$), except in the cytokine quantification of IL-6, where AZT had no reduction activity (Figure 5).

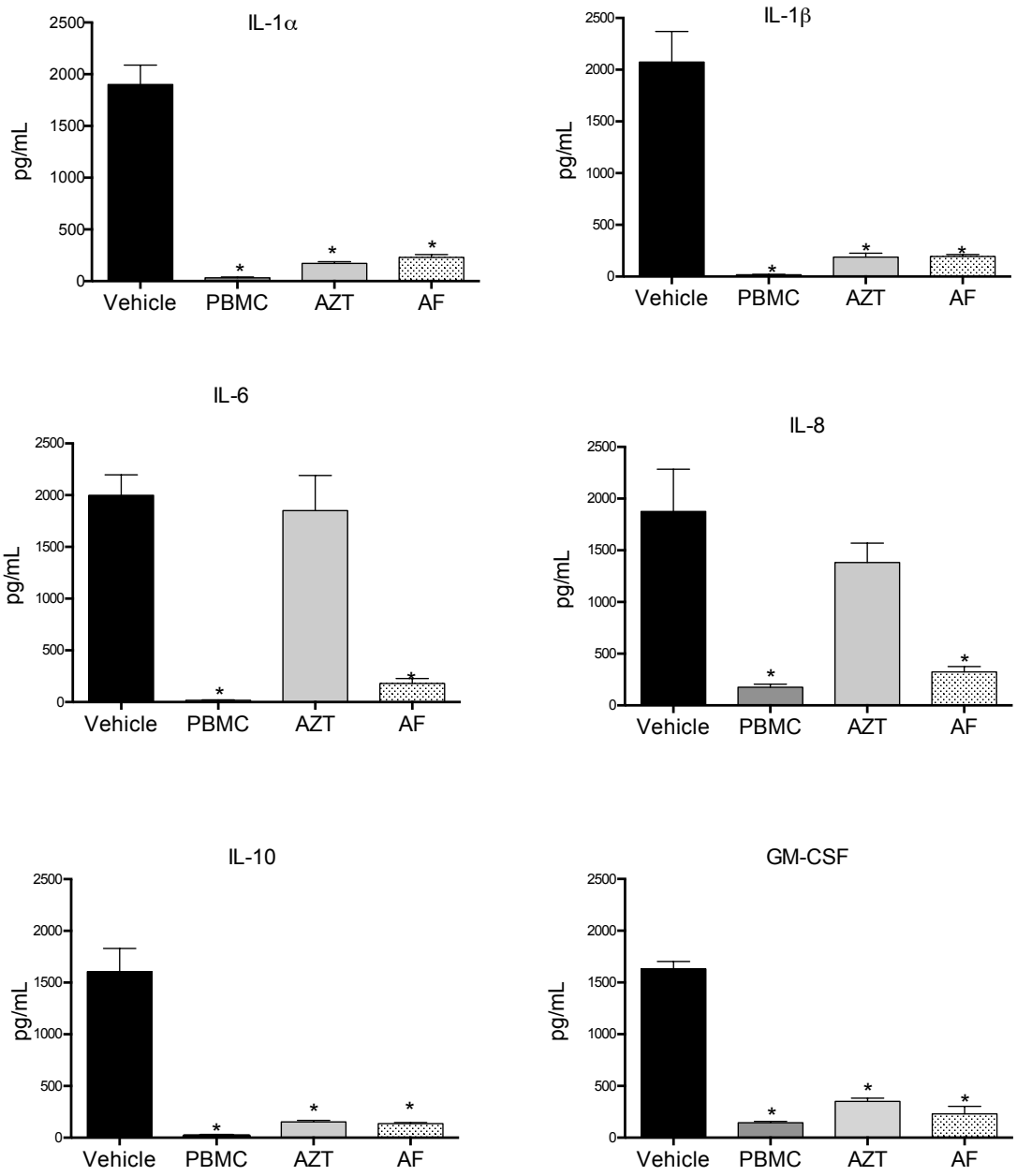


Figure 5. Cytokine quantification in the supernatant released from the cells in the dual-chamber model. The AF treatment was at the 50 μg/mL concentration. Data are expressed as mean±SD. Symbols indicate statistical differences ($p<0.05$, ANOVA, Dunnett's test); * indicates $p<0.05$ compared to the vehicle group.

Anti- HIV reverse transcriptase activity

Quantification of the inhibitory effect on HIV reverse transcriptase was done for AF (25-50 μg/mL) and the results are shown in Fig. 6. The inhibitory activity of AF against HIV-1 reverse transcriptase decreased signicantly compared to the vehicle control group.

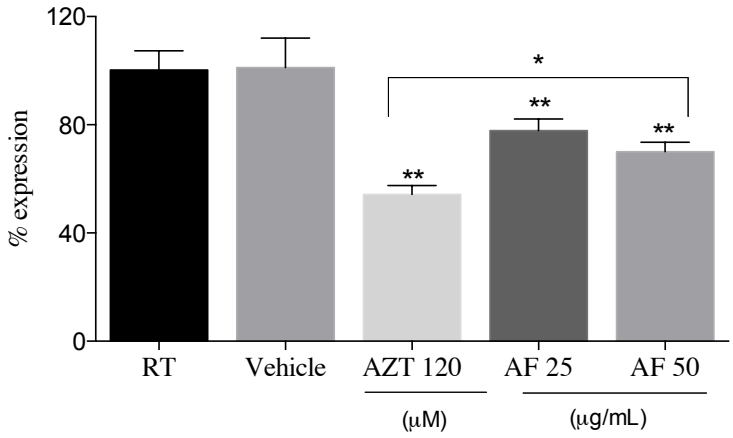


Figure 6. Anti HIV-1–RT effect of AF. The data are expressed as mean ± SD, n = 6. Symbols indicate statistical difference ($p < 0.05$, ANOVA, Tukey post test) ** compared to the reverse transcriptase group (RT 4 ng); * comparison between the treatments AF (50) and (AZT 120).

Discussion

Natural products and derivates have been considered potential candidates for drug discovery (Cragg et al., 2014) and in particular new insights into the treatment of HIV-1 (Pasetto et al., 2014). Plant extracts with strong activity acting in the nanomolar/picomolar range can be used to enhance the activity of synthetic compounds and work as anti-HIV agents (Singh and Bodiwala, 2010).

The development of potent and safe topical formulations of anti-HIV and against HIV-associated pathogens, referred to as microbicides, has become a major priority in HIV

research (NIH, 2012). According to WHO, the microbicibe must have low citotoxicity, anti-viral activity and not induce inflammatory response of the epithelial monolayer (Mauck et al., 2001).

Following the guidelines, our first evaluation of the cytotoxicity of *M. sylvestris* extracts. The AF, showed no cytotoxicity in different cell lines (TZM, HeLa and PBMCs). Other authors supported our findings, showing that *M. sylvestris* extract from Iran had no lethal dose possible to estimate in several concentrations tested. The anti-HIV activity of AF and the relative potency was based on effective dose (IC_{50}). In a dose dependent manner, AF (IC_{50}=18 μg/mL) displayed anti HIV effect against HIV-1 strain BaL and maintained the number of viable cells. Some naturally occurring products demonstrated to be a important source of anti-HIV activity in this same *in vitro* model. The extract prepared from the stem of *Acacia catechu* inhibited HIV infection in low concentrations and the IC_{50} values were found in concentrations lower than 2 μg/mL (Nutan *et al*., 2013). The compound Myrcetin revealed high therapeutic index (137.4) acting with a non cytotoxicity effects, indicating a potential and safe biological activity (Pasetto, et al, 2014).

The AF potential microbicide activity was assessed in a dual chamber model using epithelial and blood cells lines. The purpose of the model is to measure anti-HIV activity, cell viability, and inflammatory response of the epithelial monolayer; this was of particular importance for microbicides that tended to increase rather than decrease HIV transmission rates (Smith-McCune et al., 2015). The AF acts against HIV, the amount of p24 protein expressed in the cell culture supernatant decreased significantly by AF treatment. Additionally, the putative pathway by which AF affect HIV-1 infection involve the modulation cell chemokine receptor interaction (CD4), modulates the apoptosis process affecting virus survival (BCL-2) and affect the human retrovirus restriction factor (TRIM 5) (Pierson et al., 2000).

The cytotoxicity profile, and their potential to induce an inflammatory response were assessed. The AF did not induce the inflammatory response and reduced the expression of all cytokines studied - IL1-alpha, IL-beta, IL-6, IL-8 and GM-CSF - compared to the vehicle control group. In particular, IL-6 had low expression levels compared to the negative control group. In addition, cytokines modulate the monocyte function as well as HIV replication with these cells and they are considered to be major reservoirs of HIV-1, playing an important role in the pathogenesis of AIDS (Badley et al., 2013).

To elucidate the mechanism of the anti-HIV effects of the AF, the compound's inhibition of HIV-1 reverse transcriptase enzymatic activity was tested. The HIV-1 RT is unique to the virus, and is the enzyme that controls HIV-1 replication infected cells (Tao et al., 2007). We found that AF inhibited HIV-1-RT activity. Phytochemical investigations AF of *M. sylvestris* resulted in the identification of potentially bioactive flavonoid rutin (Benso et al., 2015a). It is important to note that flavonoids, beyond action against reverse transcriptase, also modulate several steps of HIV-1 life cycle, including entry, integration and maturation phases (Li et al., 2000).

In 2007, (Tao et al., 2007) found no anti-HIV activity for rutin in the TZM-bl HIV-1BaL infection model; however, for the modified structure sodium rutin sulfate, a polyanionic compound, a significant virus inhibition IC_{50} 8.5 μM (5.19 μg/mL) was demonstrated. The authors correlated the activity with the presence of sulfated polysaccharides in the structure, such as dextran sulfate, that may preferably bind to the V3 loop of X4 gp120 rather than that of R5 gp120.

Reports have shown a variety of pharmacological activities for the flavonoid group and the association between chemical structure and biological activity (Asada et al., 2013; Soto-Cabrera et al., 2015). Moreover, the biochemical effects are caused by the ability to inhibit a number of enzymes, such as reductase, lipoxygenase, cyclooxygenase and different

hormones (Rathee et al., 2009). Quercetin, rutin and catechin are flavonoids that possess antiviral action, which may be related to the non-glycosidic compounds and hydroxylation at the 3-position permitting the inhibition activity. Overall, they are proven to have biological activity and they have been important components in traditional medicine for many years (Gerdin and Svensjö, 1983).

The discovery of therapeutic agents acting as microbicide is promising in preventing interventions in AIDS research. AF has shown three important requirements for a compound to become a microbicide, as according to WHO regulatory guidelines for microbicide development (Mauck et al., 2001). The first requirement is low toxicity. AF showed low cytotoxicity on eukaryotic cell, indicating that it is safe. The second requirement is the inhibitory activity on HIV infection. AF demonstrated anti-HIV-1 activity in the dual-chamber model. The third requirement is low potential to induce inflammatory response. The AF did not induce proinflammatory cytokines production on HeLa cell monolayer.

Therefore, this study, having determined the cytotoxicity and anti-HIV activity of AF against HIV-1. Nevertheless, further studies with AF of *M. sylvestris* are still needed with different HIV-1 strains in order to identify the molecular targets.

Conclusion

M. sylvestris contains highly potential anti-HIV components, non toxic effects and anti-inflammatory properties, thereby characteristics to be considered as a potential microbicide.

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3 DISCUSSÃO

Para facilitar a análise do estudo apresentado na forma de três artigos, esta discussão reunirá os resultados de acordo como os objetidos específicos da tese de forma a tornar mais claro a compreensão dos achados para cada proposta, a saber: (1) As atividades antimicrobiana e anti-inflamatória do extrato de *M. sylvestris* (MSE) e frações em células infectadas por *Aggregatibacter actinomycetemcomitans*; (2) A atividade do MSE e frações quanto a capacidade anti-inflamatória anti-osteoclástica, antioxidante, e finalmente, identificar quimicamente o composto ativo; e (3) A ação anti-HIV da fração aquosa de *M. sylvestris* em células infectadas por HIV-BaL.

(1) As atividades antimicrobiana e anti-inflamatória do extrato de *M. sylvestris* (MSE) e frações em células infectadas por *Aggregatibacter actinomycetemcomitans;*

A *Malva sylvestris* é uma fonte promissora na descoberta de moléculas bioativas com potencial aplicação farmacológica em diversas áreas (Gasparetto et al., 2012). Os dados apresentados neste trabalho sugerem que a *M. sylvestris* e suas frações bioativas possuem constituintes de interesse para o desenvolvimento de novas formulações com atividade antimicrobiana e antiinflamatória de interesse em Odontologia e em Medicina.

Os agentes antimicrobianos podem contribuir na prevenção e controle de doenças orais, tais como: cárie e doença periodontal (Wu, 2009). Os resultados do extrato e da CLF de *M. sylvestris* demonstraram atividade inibitória para os microrganismos *F. nucleatum, P. gingivalis* e *P. intermedia*, que estão diretamente relacionados ao mecanismo de virulência na doença periodontal (Pihlstrom et al., 2005). A atividade antimicrobiana pareceu não ser exclusiva a microrganismos periodontapatogênicos, pois a literatura mostra resultados inibitórios para o extrato etanólico de *M. sylvestris* em cepas de *Staphylococcus aureus* resistentes a meticilina e ainda em cepas de *Helicobacter pylori*, no entanto, outros resultados demonstram atividade para fração de maior polaridade, a aquosa, em diferentes cepas fúngicas exceto *Candida albicans* (Cogo et al., 2010; Quave et al., 2008). Embora a especificidade do mecanismo de ação do MSE e frações não estar elucidado, é conhecido que o grupo de compostos fenólicos, presentes em grande quantidade no extrato e frações de *M. sylvestris* tem a habilidade de produzir metabólitos quelantes de ferro. Estes quelantes formam complexos estáveis com íons metálicos e consequentemente reduz o número de

espécies bacterianas, o que poderia explicar, em parte, a ação inibitória dos compostos estudados (Xia et al., 2010).

O tratamento com o MSE e as frações CLF e AF afetou diretamente na resposta do hospedeiro por meio de diferentes mediadores inflamatórios. AF teve a capacidade controlar a expressão de citocinas IL-1alfa e TNF-alfa que são moduladores da resposta do hospedeiro frente a um desafio infeccioso (Graves et al., 2011). A capacidade de reduzir a expressão de citocinas pró-inflamatórias, a exemplo, TNF-alfa foi demonstrada *in vivo* por polifenóis provenientes do chá verde que atuaram bloqueando diretamente a expressão de NF-KB (Yang et al., 1998). Esta atividade biológica, além de atenuar o processo inflamatório tem a capacidade de promover redução nos níveis de mediatores resposáveis pela osteoclastegênse envolvidas na doença periodontal (Gennaro et al., 2015).

A literatura tem identificado metabóltios de *M. sylvestris* e provado a sua ação anti-inflamatória (Gasparetto et al., 2012). Alguns desses compostos pertencem a classe das antocianinas, incluindo o marcador químico malvidina-3-glucosídeo, que demonstrou potencial biológico pelo mecanismo de redução nos níveis de expressão de citocina IL-6 (Prudente et al., 2013). Em acordo com estes resultados, o presente estudo demonstrou que o tratamento de CLF em células infectadas por *A. actinomycetencomitans* promoveu controle da transcrição gênica e da expressão da citocina IL-6, e ainda, reduziu a colonização bacteriana em células epiteliais e gengivais. A IL-6, tem papel fundamental na instalação da doença periodontal, pois atua na indução de atividade proteolítica das matriz de metaloproteinases (MMPs). Além disso, sua atividade promove a degradação de matriz celular e consequentemente destruição tecidual e a significativa expressão durante a fase aguda do processo inflamatório promovendo osteoclasteogênese pelo aumento da expressão de tRANKL (Graves et al., 2011; Kang et al., 2014).

O MSE reduziu a transcrição gênica de IL-8 que é considerada um dos maiores ativadores de eventos celulares da resposta inflamatória devido ao seu mecanismo de ação ser responsável pelo início de eventos celulares que atraem leucócitos para o local da inflamação (Pihlstrom et al., 2005). Esse potencial biológico é considerado de interesse no desenvolvimento de novos alvos terapêuticos, pois inibidores de IL-8 atuam em em diferentes níveis celulares como macrófagos, endotélio e células epiteliais promovendo um decrécimo na ativação e atuando em uma série de doenças de origem inflamatória aguda e crônica (Perretti et al., 2015).

Os resultados demonstraram que o MSE e AF presentaram potencial farmacológico modulando genes e citocinas envolvidas no processo de regulação da resposta inflamatória. No entanto, fração de baixa polaridade CLF, apresentou uma relevante dupla atividade controlando o processo inflamatório e infeccioso ao mesmo tempo, condição necessária nas atuais abordagens terapêuticas na doença periodontal.

(2) A atividade do MSE e frações quanto a capacidade anti-inflamatória, anti-osteoclástica, antioxidante, e finalmente, identificação química do composto ativo;

Os resultados do primeiro estudo demonstraram potencial farmacológico para o extrato e frações de *M. sylvestris* atuando no controle de transcrição e expressão de citocinas reguladoras do evento inflamatório. Neste segundo estudo, avaliou-se a capacidade do extrato e frações em eventos inflamatórios *in vivo*, metabolismo ósseo e ação no seqüestramento de radicais livres *in vitro*, fenômenos que caracterizam doenças inflamatórias crônicas de difícil abordagem terapêutica.

A atividade anti-inflamatória *in vivo,* mostrou a ação do extrato e frações sobre o modelo de migração de neutrófilos para a cavidade peritoneal. Os resultados, mostram que o MSE e todas as frações, com exceção da hexânica, reduziram o número de neutrófilos no processo inflamatório. Este modelo de fracionamento bioguiado permitiu concentrar o composto ativo na fração de maior polaridade, a fração aquosa, que apresentou as melhores porcentagens de redução de migração de neutrófilos. A migração de células para cavidade peritoneal é pode depender da via do óxido nítrico que expressa moléculas de adesão. Utilizando o mesmo modelo *in vivo* pesquisadores analisaram a atividade anti-inflamatória da fração aquosa do extrato de geoprópolis que apresentou mecanismo de ação de regulação do óxido nítrico e consequetemente redução no número de leucócitos que migraram na cavidade peritoneal (Dal Secco et al., 2006; Franchin et al., 2013).

A inibição da inflamação no modelo induzido por carragenina tem sido mostrada como preditivo para atividade de drogas anti-inflamatórias e as doses utilizadas dos anti-inflamatórios não esteroides podem ser correlacionadas com doses efetivas em pacientes. Foi demonstrado que o modelo de edema de pata é um modelo bifásico que tem importante papel na resposta inflamatória (Posadas et al., 2004). A primeira fase, ou inicial (0-3h), é o período que há aumento de histamina, serotonina e mediadores químicos, estes relacionados com o aumento da permeabilidade vascular e a produção de citocinas IL-1β and TNF-α. Na segunda

fase, ou tardia (3-5h), há aumento de prostaglandinas, proteases e lisossomos, neste momento atuam a maior parte das drogas anti-inflamatórias que estão no mercado (Levy, 1969; Morris, 2003). A fração bioativa AF foi avalida neste modelo e apresentou uma resposta mais rápida que o controle positivo indometacina reduzindo significativamente a resposta inflamatória já na primeira hora avalida, esta atividade farmacológica que foi mantida durante toda a fase inicial do modelo. O efeito anti-inflamatório da fração pode estar relacionado a supressão da citocina quantificada IL-1β e ao mecanismo de cicloxigenases, pois ambos os precursores de prostaglandinas e traboxanos são derivados da via do ácido araquidônico e drogas inibidoras desta via tem mostrado efetividade neste modelo e potencial uso clínico (Morris, 2003).

O presente estudo ainda demonstrou que AF e a EAF tem atividade inibidora de MMP-9 no modelo de de zimografia que caracteriza o processo de proteólise (Franco et al., 2011). Essas enzimas proteolíticas estão classicamente envolvidas no processo de degradação extracelular e no metabolismo ósseo, sendo encontradas em diversas patologias, especialmente relacionados a doenças inflamatórias crônicas (Cataldo et al., 2003). Os osteoclastos são formados pela fusão de células hematopoiéticas de monócitos durante o processo de diferenciação (Syggelos et al., 2013). Células RAW 264.7 são precursoras e podem expressar genes marcadores como TRAP e Catepsina K que representam a expressão de osteoclastos maduros (Kim et al., 2012). O tratamento com AF reduziu a expressão de ambos os genes citados anteriormente e a enzima AC II que é super expressa durante o processo de reabsorção óssea (Al Mamun et al., 2015). Pouco se conhece sobre o mecanismo protetor dos produtos naturais na saúde óssea, no entanto, estudos tem demonstrado que a classe fitoquímica dos flavonóides tem melhorado o metabolismo ósseo por ser fonte de componentes alimentares fornecedores de cálcio e vitamina D (Wu et al., 2015). Estes compostos conseguem atuar nas vias de sinalização durante o processo de diferenciação do osteoclasto em osteoblasto, isto foi demonstrado no trabalho com o extrato de *Yukmijihwang-tang*, origem chinesa, em avaliação *in vitro* em células Raw 264.7 e a regulação dos genes TRAP, MMP-9, catepsina K com atuação direta na interação das células envolvidas no metabolismo ósseo que são os osteoblastos, osteoclastos e osteócitos (Shim et al., 2011).

Evidências científicas tem relacionado a atividade biológica de eliminação de radicais livres com a atuação em diversas doenças degenerativas, especialmente relacionadas a inflamação crônica, isso porque, os antioxidantes atuam sequestrando os radicais livres e podem regular a via de oxidação (Bocci and Valacchi, 2013). Essa relação pode ser observada

no estudo da *M. sylvestris* sendo que a fração bioativa foi a mesma nos modelos de reabsorção óssea e atividade antioxidante. Pesquisadores relataram atividade antioxidante na fração aquosa de *M. sylvestris* e relacionam a presença de atividade ao tipo de solo de sua produção, pois os melhores resultados foram encontrados na região nordeste de Portugal. A *M. sylvestris* apresenta atividade promissora de investigação clínica, especialmente como alimento funcional, pois estudos sugerem que os alimentos com a capacidade de modular processo oxidativo combinado a atividade de remodelação óssea e anti-inflamatória pode contribuir a prevenção de doenças crônicas (Barros et al., 2010; Gasparetto et al., 2012).

A identificação da rutina como composto majoritário na fração aquosa confirma os achados da literatura que a identificam como flavonóide com importantes atividades farmacológicas incluindo antioxidante e anti-inflamatória, controlando a transcrição de genes e proteínas pró-inflamatórios (Choi et al., 2014). Estudos recentes, demonstram potencial no tratamento de doenças crônicas como diabetes, hipertensão e hipercolesterolemia (Moukette et al., 2015; Nafees et al., 2015). Desta forma, a rutina apresenta potencial farmacológico de interesse a serem investigado no tratamento de doenças crônicas, ou ainda, como um alimento funcional para o mercado (Chua, 2013).

(3) A ação anti-HIV da fração aquosa de *Malva sylvestris* em células infectadas por HIV-BaL.

Os produtos naturais são fontes de compostos bioativos que podem atuar estimulando o sistema imunológico (Cragg et al., 2014). Compostos naturais com atividade anti-HIV são reportados, em especial, no grupo dos flavonóides. Estes compostos atuam por mecanismos de inibição da transcriptase reversa, inativação da protease viral, ou ainda, na indução da síntese de interferons que promovem a ativação de linfócitos e estimulam o sistema imune (Wang et al., 1998; Yu and Zhao, 2012). O melhoramento da atividade farmacológica de compostos sintéticos ou a descoberta de novos agentes pode contribuir para que novos fármacos estejam disponíveis no mercado, e ainda, aumentar a eficiência no tratamento anti-HIV (Yang et al., 2001).

A OMS fornece pareces técnicos das exigências e orientações para o desenvolvimentos de novas formulações com ação anti-HIV. Para agentes microbicidas os critérios são: apresentar atividade antiviral, baixa citotoxicidade e não induzir resposta inflamatória aos tecidos (Gilks et al., 2006). Desta forma, o primeiro ensaio conduzido neste trabalho foi a análise de viabilidade celular e os resultados demonstraram que a AF não

apresentou atividade citotóxica nas linhagens celulares testadas. No entanto, os possíveis efeitos tóxicos do uso do extrato ou frações de *Malva sylestris* são pouco explorados na literatura. Apenas um estudo *in vivo (Microtox bioassay)* foi conduzido para avaliar a toxicidade do extrato etanólico da planta e os autores concluiram que o efeito encontrado foi próximo ao máximo estabelecido para o teste- 20% de inibição de bioluminescência (Razavi et al., 2011).

O potencial efeito anti-HIV da fração aquosa foi demonstrado em método *in vitro* pela quantificação da proteína viral p24 no sobrenadante de células infectadas por HIV-1 BaL. Os compostos naturais que apresentam forte atividade biológica e em baixas concentrações podem contribuir para potencializar o efeito de compostos sintéticos e atuar como agentes anti-HIV (Singh and Bodiwala, 2010). Na literatura podem ser identificados compostos com características desejáveis, a exemplo, o extrato preparado das folhas de *Acacia catechu* que apresentou atividade antiviral em baixas concentrações 2 μg/mL e o flavonoide Miricetina com alto índice terapêutico (137.4) e baixa citotoxicidade (Nutan et al., 2013; Pasetto et al., 2014).

A entrada do vírus no hospedeiro e a replicação envolve uma série proteínas e a produção de inúmeros subprodutos que podem interferir a infecção viral a nível celular (Demers et al., 2013). A AF apresentou atividade regulatória de citocinas pró-inflamatórias, estas possuem papel no controle da homeostase do sistema imune e os seus efeitos podem ser inibitórios, estimulatórios ou bifuncional (Marsili et al. 2012). Isto indica que a infecção e a replicação podem acontecer continuamente pela regulação de um complexo de citocinas e por uma variedade de células (Altfeld and Gale, 2015). Os mecanismos moleculares envolvidos na regulação da AF na infecção viral demonstraram regulação na transcrição dos genes CD4 Bcl-2 e TRIM5 que são responsáveis por codificar fatores de sobrevivência e a latência do virus durante o processo infeccioso (Asaoka et al., 2005; Hu et al., 2013).

Desta forma, a fração bioativa de *M. sylvestris* AF possui compostos com potencial atividade anti-HIV, baixa citotoxicidade, e a capacidade de modular a expressão de citocinas, o que implica em características de interesse no desenvolvimento de novas formulações com aplicabilidade clínica.

4 CONCLUSÃO

Os resultados deste estudo permitiram concluir que:

a) A fração clorórmica da *M. sylvestris* possui compostos bioativos com atividade promissora como novos agentes terapêuticos com atividade antimicrobiana e anti-inflamatória;

b) A fração aquosa da *M. sylvestris* apresentou atividade anti-inflamatória, anti-osteoclástica e antioxidante em modelos experimentais *in vitro* e *in vivo*, desta forma, a fração bioativa apresenta potencial biológico para desenvolvimentos de novas terapias no tratamento de doenças crônicas;¡

c) A fração aquosa apresentou atividade anti-HIV, não foi citotóxica em modelo *in vitro* e modulou da expressão de citocinas, o que implica em características de interesse para o desenvolvimento de novas formulações de aplicabilidade clínica.

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Anexo 1 – Correspondência periódico PlosOne

De: plosone <plosone@plos.org>
Data: 9 de dezembro de 2015 09:43:17 BRST
Para: "brunabenso@hotmail.com" <brunabenso@hotmail.com>
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Anexo 2 - Certificado do Comitê de Ética em Animais

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp
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C E R T I F I C A D O

Certificamos que o projeto **"Avaliação do potencial antimicrobiano, anti-inflamatório, remodelação óssea, antioxidante e antineoplásico da Malva sylvestris"** (protocolo nº **2790-1**), sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Bruna Benso**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **10 de dezembro de 2012**.

Campinas, 10 de dezembro de 2012.

Profa. Dra. Ana Maria A. Guaraldo
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