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AVALIAÇÃO DO EFEITO GASTROPROTETOR DAS SEMENTES DE *Persea americana* Mill.

BRENA RAMOS ATHAYDES

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Brena Ramos Athaydes

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Dissertação apresentada à Universidade Federal do Espírito Santo, como requisito parcial para a obtenção do título de Mestre em Ciências Farmacêuticas, do Programa de Pós-Graduação em Ciências Farmacêuticas.

Orientadora: Profª. Drª. Rita de Cássia Ribeiro Gonçalves

Co-orientador: Prof. Dr. Rodrigo Rezende Kitagawa

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Brena Ramos Athaydes

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BANCA EXAMINADORA:

Prof. Dr. Warley de Souza Borges / UFES

Prof. Dr. Marcio Fronza / UVV

Profª. Drª. Rita de Cássia Ribeiro Gonçalves (orientadora) / UFES

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(Tiago 1:5 e 6)

RESUMO

A úlcera péptica é uma das doenças mais comuns que afetam a população mundial. É caracterizada por um desbalanço entre os fatores protetores (produção de muco e bicarbonato, antioxidantes e prostaglandinas) e agressores (estresse oxidativo, anti-inflamatórios não-esteroidais e *Helicobacter pylori*) da mucosa gástrica. O processo inflamatório ulceroso induz a liberação de citocinas pró-inflamatórias e o estresse oxidativo, que cronicamente podem levar ao câncer gástrico. No Brasil, há relatos do uso das sementes de *Persea americana* Mill. (abacateiro) para o tratamento de doenças gástricas, no entanto, sem evidências científicas. Algumas pesquisas também já destacaram seus efeitos antioxidante e antimicrobiano. Nesse contexto, avaliamos a atividade antioxidante, anti-*H. pylori*, o efeito imunomodulador e o efeito antitumoral em células de adenocarcinoma gástrico, do extrato hidroalcoólico (SCE) e das frações acetato de etila (SEAP) e hexânica (SHP) obtidos a partir das sementes de abacate. SEAP apresentou os melhores resultados; portanto, também realizamos o perfil químico e o estudo dos efeitos gastroprotetores em modelo agudo de úlcera gástrica induzida por indometacina da SEAP, através de análise histológica e quantificação dos parâmetros bioquímicos da inflamação. SEAP e SHP foram eficientes em inibir o crescimento de células tumorais gástricas e na atividade anti-*H. pylori*, confirmada por alterações na morfologia bacteriana. A SEAP apresentou os melhores resultados na captura de $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, HOCl e inibição da enzima HRP, além de modular a inflamação por inibir significativamente a produção de IL-6. O estudo cromatográfico por ESI FT-ICR MS da SEAP revelou a presença de flavonoides, fenilpropanoides e taninos, como ácido cafeioilquínico, catequina e epicatequina (confirmados por CLAE-DAD) e derivados de quercetina e kaempferol. SEAP reduziu as características das lesões gástricas nas análises macroscópica e histológica, além de aumentar a produção de muco. Nos parâmetros do estresse oxidativo, houve redução significativa dos níveis de AOPP e MDA com aumento da atividade da SOD. Estes resultados mostram que as sementes de *P. americana* Mill. são capazes de inibir as vias envolvidas na formação de úlcera e câncer gástrico devido à presença de composto fenólicos, sendo uma alternativa estratégica no tratamento de doenças gástricas.

Palavras-chaves: Helicobacter pylori, Estresse Oxidativo, Persea americana, Polifenois, Gastroproteção.

ABSTRACT

Peptic ulcer is one of the most common diseases affecting the world's population. It is characterized by an imbalance between protective (mucus and bicarbonate production, antioxidants and prostaglandins) and aggressor factors (oxidative stress, non-steroidal anti-inflammatory drugs and *Helicobacter pylori*) of the gastric mucosa. The inflammatory ulcer process induces the release of pro-inflammatory cytokines and oxidative stress, which can chronically lead to gastric cancer. In Brazil, there are reports of the use of *Persea americana* Mill. seeds (avocado) for the treatment of gastric diseases, however, without scientific evidence. Some research also highlights its antioxidant and antimicrobial effects. In this context, we evaluated the antioxidant and anti-*H. pylori* activity, the immunomodulatory effect and the antitumor effect in gastric adenocarcinoma cells of the hydroalcoholic extract (SCE) and ethyl acetate (SEAP) and hexane (SHP) partitions from avocado seeds. SEAP obtained better results; therefore, we also performed its chemical profile and the study of its gastroprotective effects in the acute indomethacin-induced gastric ulcer model, through histological analysis and quantification of the biochemical parameters of the inflammation. SEAP and SHP efficiently inhibited gastric tumor cells and *H.* pylori growth, confirmed by bacterial morphology changes. SEAP presented better results in the capture of $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, HOCl and inhibition of HRP enzyme, besides modulating inflammation by inhibiting IL-6 production significantly. SEAP chromatographic study by ESI FT-ICR MS showed the presence of flavonoids, phenylpropanoids and tannins, such as caffeioylquinic acid, catechin and epicatechin (confirmed by HPLC-DAD) and quercetin and kaempferol derivatives. SEAP reduced gastric lesions characteristics in macroscopic and histological analysis, besides increasing mucus production. In oxidative stress parameters, there was a significant reduction of AOPP and MDA levels with increase of SOD activity. These results show that *P. americana* Mill. seeds are capable to inhibit the pathways involved in the formation of ulcer and gastric cancer due to the presence of phenolic compounds, being a strategic alternative in the treatment of gastric diseases.

Keywords: Helicobacter pylori, Oxidative stress, Persea americana, Polyphenols, Gastroprotection.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

$ABTS^{\bullet+}$ - *[2,2'-azino-bis(3etilbenzotiazolina-6-ácido sulfônico)]*

AINEs - Anti-inflamatórios não esteroidais

AOPP - Produtos de oxidação proteica

CagA - Citotoxina associada ao gene A

CIM - Concentração inibitória mínima

CLAE-DAD - Cromatografia líquida de alta eficiência acoplada a um detector de arranjo de diodo

COX - Ciclo-oxigenase

DNA - Ácido desoxirribonucleico

$DPPH^{\bullet}$ - *2,2-diphenil-1-picrilhidrazila*

ERO - Espécies reativas de oxigênio

ESI FT-ICR MS - Espectrometria de Massas com Ionização por eletrospray e Ressonância Ciclotrônica de Íons por Transformada de Fourier (Electrospray Ionization Fourier Transform Ion Cyclotron Ressonance Mass Spectrometry)

H^+ - Íons hidrogênio

H_2O_2 - Peróxido de hidrogênio

HOCl - Ácido hipocloroso

HP-NAP- Proteína de *Helicobacter* ativadora de neutrófilo

HRP - *Horseradish peroxidase*

IBP - Inibidor de bomba de prótons

IL- 6 - Interleucina 6

IL- 8 - Interleucina 8

IL-1β - Interleucina 1 beta

IL-10 - Interleucina 10

INCA - Instituto Nacional de Câncer

iNOS - Óxido nítrico-sintase induzível

LDL - Low Density Lipoprotein (Lipoproteína de baixa densidade)

MALT - Linfoma de tecido linfoide associado à mucosa

MDA - Malondealdeído

NADPH - Nicotinamida adenina dinucleotídeo fosfato

NF-κB - Fator de transcrição nuclear κB

NO - Óxido nítrico

$O_2^{\bullet-}$ - Aniôn superóxido

OH• - Radical hidroxil

$ONOO^-$ - Peroxinitrito

PGE2 - Prostaglandina 2

pH - Potencial hidrogeniônico

RENISUS - Relação Nacional de Plantas Medicinais de Interesse ao SUS

RNA - Ácido ribonucleico

SCE - Extrato bruto hidroalcoólico das sementes de *Persea americana* Mill.

SEAP - Fração acetato de etila das sementes de *Persea americana* Mill.

SHP - Fração hexânica das sementes de *Persea americana* Mill.

SOD - Superóxido dismutase

SUS - Sistema único de saúde

TNF-α - Fator de necrose tumoral alfa

***Vac*A** - Citotoxina de vacuolização A

WHO - World Health Organization (Organização Mundial da Saúde)

SUMÁRIO

I. INTRODUÇÃO GERAL E JUSTIFICATIVA DO ESTUDO

No intuito de melhorar a qualidade de vida, o homem tem usado as plantas como forma de alimento, subsídio para construção de moradias, vestimentas, uso cosmético e terapêutico. Algumas plantas são conhecidas por conterem substâncias com ações farmacológicas que são usadas desde a antiguidade para a cura de doenças. O conhecimento de seu potencial medicamentoso deriva da sabedoria popular que desde a pré-história é transmitida de geração para geração, com o seu preparo variando de acordo com a cultura e a diversidade ambiental de cada região (SILVA et al., 2012).

A redução de efeitos colaterais, a ampliação das possibilidades terapêuticas, o menor custo de produção em relação aos medicamentos sintéticos e o intenso uso popular fazem com que a inclusão da Fitoterapia seja recomendada inclusive pela Organização Mundial de Saúde (OMS). No Brasil, o Sistema Único de Saúde (SUS) tem colaborado para o desenvolvimento de práticas de saúde pública na intenção de impulsionar a disponibilização de fitoterápicos para a população (Ministério da Saúde, 2016).

Algumas plantas já estão sendo usadas pelo SUS e distribuídas como fitoterápicos para o tratamento de doenças simples. Entre as mais conhecidas, estão: *Maytenus ilicifolia* (Espinheira-santa), *Mikania glomerata* (Guaco), *Aloe vera* (Babosa), *Rhamnus purshiana* (Cáscara-sagrada), utilizadas para gastrites, expectorante, queimaduras e constipação intestinal, respectivamente (Ministério da Saúde, 2012). Dessa forma, com a finalidade de ampliar e orientar a pesquisa nesta área, o Ministério da Saúde publicou em 2009 a Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS), um documento que contém 71 espécies e/ou gêneros de vegetais de grande potencial e tradicionalmente utilizados na medicina popular no Brasil. Nesta lista encontra-se o objeto de estudo desse trabalho, a *Persea americana* Mill.

Persea americana Mill., popularmente conhecida como “abacateiro”, é uma planta dicotiledônea da família Lauraceae, nativa do centro-sul do México, mas que também pode ser encontrada em diversos países tropicais, pois prefere solos férteis e úmidos, e clima ameno e quente. Sua fruta fresca é amplamente consumida em pratos culinários, embora as indústrias de alimentos, cosmético e farmacêutico processem sua polpa para aumentar a comercialização e dar maior valor agregado

ao abacate (MELGAR et al., 2018). Somente no México, o maior consumidor, a produção pode chegar a mais de 700.000 toneladas por ano, sendo que apenas 3-5% é industrializada (RAMOS-JERZ, 2007).

A planta é utilizada na medicina tradicional para o tratamento de diferentes doenças por possuir atividades vasorrelaxante, analgésica, anti-inflamatória, antioxidante, anti-hepatotóxica, anti-hipertensiva, anticonvulsivante (YASIR; DAS; KHARYA, 2010), antitrombótica (RODRIGUEZ-SANCHEZ et al., 2015) e no auxílio da redução de níveis glicêmicos (LIMA et al., 2012). No entanto, grande parte destes estudos foi realizada com o fruto e as folhas e pouco se sabe sobre a semente do abacate. A semente gera toneladas de resíduos, como é observado no México, onde o consumo da fruta é elevado e a semente é descartada (RAMOS-JERZ, 2007). Mas ao mesmo tempo, a semente pode ser de grande interesse no âmbito econômico, já que a recuperação de seus compostos bioativos tem sido o principal foco de muitos estudos científicos (AYALA-ZAVALA et al., 2011; CHEL-GUERRERO et al., 2016). Já foram encontradas atividades terapêuticas para as sementes, como por exemplo, redução dos níveis de triglicerídeos, colesterol total e sua fração LDL (NWAOGUIKPE; BRAIDE, 2011; OYEYEMI; OYEYEMI, 2015), redução de níveis glicêmicos e da pressão arterial, e atividades antimicrobiana, antioxidante e anticâncer (DABAS et al., 2013; YASIR; DAS; KHARYA, 2010). No Brasil, as sementes de *P. americana* têm sido popularmente usadas no tratamento de doenças gástricas, mas sem evidências científicas. De acordo com relatos, parecem ser efetivas contra infecções e inflamações do trato gastrointestinal.

Diversas caracterizações químicas mostraram que as sementes são ricas em uma mistura complexa de compostos polifenólicos, sendo os derivados de (epi)catequinas os componentes majoritários. Também apresentam flavonoides (quercetina e derivados de glicosídeos de kaempferol e isorhamnetina), ácidos fenólicos derivados de ácido clorogênico e cumarínico, derivados de ácido hidroxicinâmico e hidroxibenzoico, como ácido cafeoilquinico e coumaroilquínico (KOSIŃSKA et al., 2012; MELGAR et al., 2018), dímeros de procianidinas e outros taninos condensados (Figura 1) (LÓPEZ-COBO et al., 2016). Grande concentração desses compostos encontra-se na fração acetato de etila, onde já foi isolada uma proantocianina (GEISSMAN; DITTMAR, 1965; RODRIGUEZ-CARPENA et al., 2011). Também já foram quantificados alcaloides, triterpenos, esteróis, ácidos graxos, antocianinas e saponinas (ARUKWE et al., 2012; LEITE et al., 2009;

NWAOGUIKPE; BRAIDE, 2011; RODRIGUEZ-CARPENA et al., 2011). A presença destes metabólitos secundários pode estar relacionada com o uso popular no tratamento de desordens gástricas.

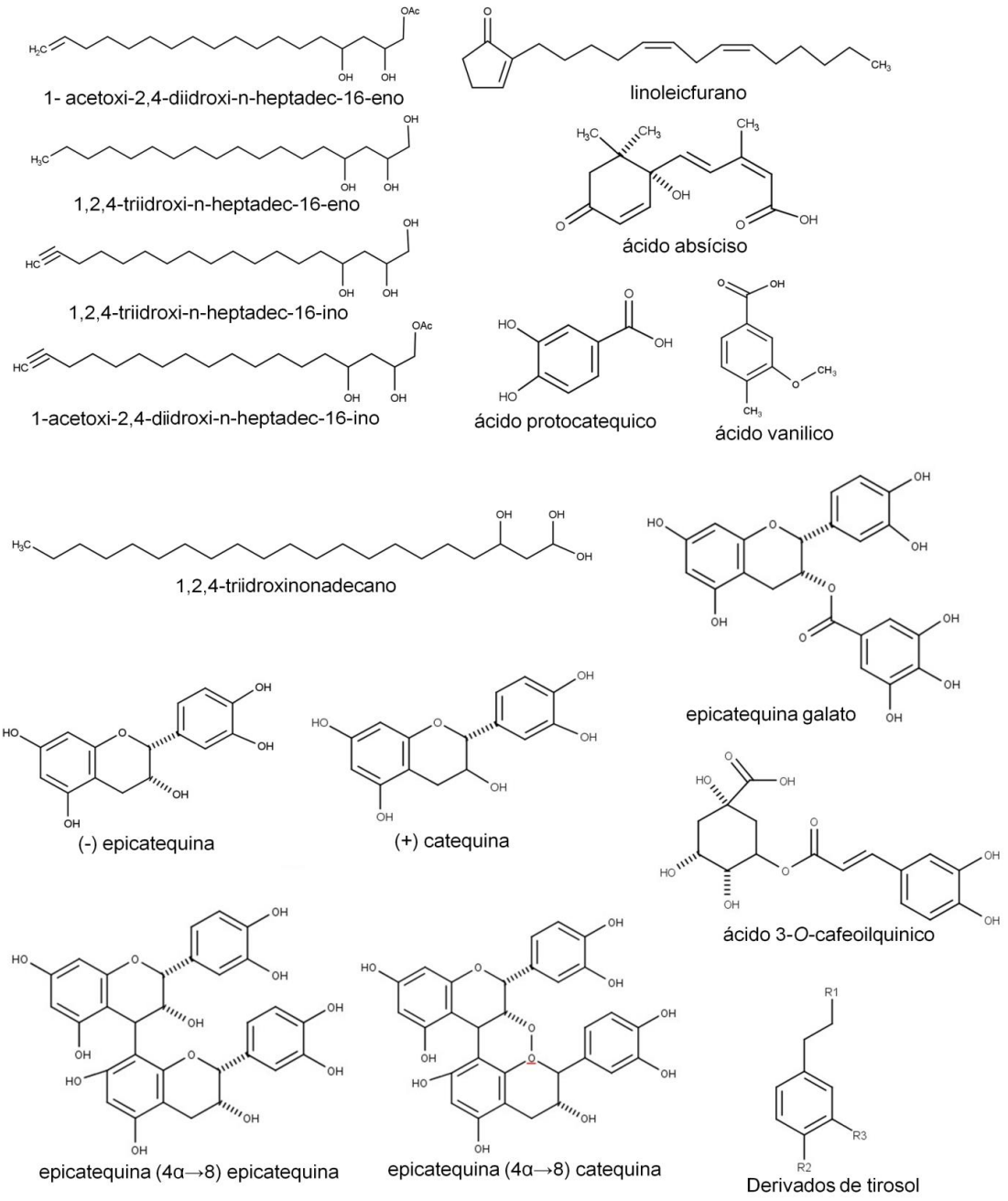

Figura 1 – Estruturas químicas dos polifenois encontrados em sementes de abacate (*Persea americana* Mill.). Fonte: Adaptado de Dabas et al. Avocado (*Persea americana*) seed as a source of bioactive phytochemicals. **Current Pharmaceutical Design**, v. 19, p. 6133-6140, 2013.

As principais doenças inflamatórias gástricas que afetam a população mundial atualmente são gastrite e úlcera, que podem evoluir para o câncer gástrico. A origem destas patologias é complexa e controversa, no entanto, é geralmente aceita como

um resultado do desequilíbrio entre os fatores protetores e agressores da mucosa gástrica. Os principais mecanismos protetores são o muco, a secreção de bicarbonato no ambiente estomacal, aumento e manutenção dos níveis de antioxidantes e a produção de prostaglandinas; e os fatores agressores estão relacionados ao estresse oxidativo, fatores psicossociais como o consumo de álcool, fumo e uso abusivo de drogas como os anti-inflamatórios não esteroidais (AINEs), além de doenças crônicas e infecciosas, principalmente a presença da bactéria *Helicobacter pylori* (BOEING et al., 2016; FARZAEI; ABDOLLAHI; RAHIMI, 2015; YUAN; PADOL; HUNT, 2006).

A gastropatia por AINEs é considerada uma "epidemia silenciosa" e, portanto, tem sido uma área de intensa pesquisa. Cerca de 15-30% dos pacientes usuários normais de AINEs têm uma ou mais úlceras quando examinados por endoscopia, e 3 a 4,5% têm sintomas clínicos significantes. Seu uso prolongado eleva os riscos de formação de úlcera gástrica devido aos seus efeitos colaterais resultantes da inibição das enzimas ciclo-oxigenases 1 (COX-1) e 2 (COX-2). As prostaglandinas derivadas de COX-1 estão envolvidas na manutenção da integridade da mucosa gástrica, estimulação da secreção de muco e bicarbonato e fornecimento de fluxo sanguíneo adequado na mucosa gástrica, enquanto as prostaglandinas provenientes de COX-2 protegem a mucosa da adesão de leucócitos e suportam a renovação do epitélio via proliferação epitelial (BARRISON; WOLFE, 1999; SULEYMAN et al., 2010; WALLACE, 2008).

Os AINEs induzem lesão da mucosa gástrica principalmente pela deterioração dos mecanismos endógenos de defesa através da inibição da síntese de prostaglandinas. No entanto, o processo ulceroso induzido por AINEs pode ocorrer independente da inibição da prostaglandina, onde a resposta inflamatória e a produção excessiva de espécies reativas de oxigênio (ERO) são os fatores patogênicos mais importantes. Durante a reação inflamatória, ocorre adesão de leucócitos ao endotélio vascular causando distúrbios microcirculatórios, considerado um evento inicial crítico na gastropatia por AINEs. A infiltração de neutrófilos ativados aumenta a produção de citocinas pró-inflamatórias e de enzimas relacionadas ao estresse oxidativo da mucosa, caracterizado por um desbalanço entre o sistema oxidante e antioxidante. O aumento de espécies reativas é resultado da produção descontrolada e excessiva de ERO, como ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2) e radical hidroxil ($OH^{\bullet}$), causando oxidação de

proteínas, DNA e lipídios, com a diminuição da atividade de antioxidantes como a enzima superóxido dismutase (PÉREZ et al., 2017; SULEYMAN et al., 2010; TAKEUCHI et al., 1991; YADAV et al., 2012). Em conjunto, esses fatores levam ao risco aumentado de câncer gástrico (BHATTACHARYYA et al., 2014; PÉREZ et al., 2017). Entre os AINEs comumente usados, a indometacina possui o maior potencial ulcerogênico para humanos (YUAN; PADOL; HUNT, 2006). Além disso, por possuir estrutura ácida como a maioria dos AINEs, a indometacina mantém sua forma lipofílica indissociável em ambiente estomacal ácido, podendo lesionar a barreira hidrofóbica de muco e desestabilizar o revestimento extracelular de fosfolipídeos de membrana (FORNAI et al., 2011: WALLACE, 2008). Por esses motivos, a indometacina tornou-se um modelo amplamente utilizado para indução de lesões gástricas em animais.

A infecção por *Helicobacter pylori* também está associada a doenças digestivas severas, incluindo gastrite crônica, úlcera péptica, além de ser um importante fator na patogênese do câncer gástrico (WATARI et al., 2014). A *H. pylori* coloniza 50% da população mundial, onde mais de 70% dos pacientes infectados são assintomáticos, e desse número, 10-20% podem desenvolver a doença durante sua vida (KENNETH; MCCOLL, 2010), fazendo com que ela seja um dos patógenos mais prevalentes no homem. A persistência bacteriana é o principal fator de risco em 75% dos casos de câncer gástrico, estando relacionado principalmente ao tumor do tipo adenocarcinoma e linfoma de tecido linfoide associado à mucosa (MALT) (ABADI; KUSTERS, 2016; BAE et al., 2018; HERRERA; PARSONNET, 2009; JEMAL et al., 2011; TALAIEZADEH; HAJIANI; TARSHIZI, 2013). No Brasil, o perfil de mortalidade por câncer gástrico entre todos os tipos de câncer é de 9,2% em homens e 5,8% em mulheres (WHO, 2014), aparecendo em terceiro lugar na incidência entre homens e em quinto, entre as mulheres (INCA, 2016).

Helicobacter pylori é um bacilo gram-negativo, microaerófilo, de forma espiralada com 4-6 flagelos unipolares, que habita a camada de muco que recobre as células epiteliais gástricas humanas (DUNN; COHEN; BLASER, 1997). As características do estômago, como oscilações relacionadas à disponibilidade de nutrientes, temperatura, presença de ERO e principalmente, o ambiente com pH ácido, dificultam qualquer possibilidade de sobrevivência e apenas micro-organismos com certo grau de adaptabilidade conseguem se colonizar de forma bem-sucedida e persistente. A presença da *H. pylori* neste ambiente só é possível

devido à presença de fatores de virulência que combatem tais adversidades, entre eles, a eficiente produção da enzima urease, que metaboliza a ureia presente no suco gástrico liberando dióxido de carbono e amônia, isolando a acidez estomacal. Outros dois fatores que explicam esse fenômeno é o contato íntimo com a camada de muco que cobre a mucosa gástrica ao passar pelo lúmen gástrico, formando um nicho protegido contra o ácido gástrico, e a presença de flagelos, que permite que ela passe através do muco viscoso e espesso e alcance um pH mais favorável para conseguir se aderir às células epiteliais subjacentes (ABADI, 2017; PÉREZ-PÉREZ; BLASER, 1996).

As cepas de *Helicobacter pylori* também possuem diversas variações genômicas que codificam fatores de virulência relacionados à sua permanência e ao aumento da sua capacidade invasora e destrutiva. A ilha de patogenicidade *cag*-PAI, um componente do genoma da *H. pylori*, contém genes homólogos aos de outras bactérias capazes de codificar componentes do Sistema de Injeção IV da bactéria, atravessando suas membranas como uma agulha e injetando a citotoxina associada ao gene A (*cag*A) e outras proteínas codificadas pela *cag*-PAI no citosol da célula hospedeira, modulando vias do metabolismo e a expressão de proto-oncogenes dessa célula. Ainda, sabe-se que cepas que contém a *cag*-PAI intacta têm maior capacidade de indução da resposta inflamatória de células epiteliais, e pacientes infectados por cepas que expressam o gene *cag*A têm maior probabilidade de desenvolver câncer gástrico do que aqueles que são infectados por cepas que não expressam o gene (BUTCHER et al., 2017; DING et al., 2007; NAITO; YOSHIKAWA, 2002; WEN; MOSS, 2009; WROBLEWSKI; PEEK; WILSON, 2010).

Dois outros fatores que podem contribuir para a permanência da infecção e o desenvolvimento da ulceração crônica são a citotoxina de vacuolização A (*vacA*) e o produto do gene da proteína de ativação de neutrófilos para *H. pylori* (*HP-NAP*). A *vacA*, presente na maioria das cepas de *H. pylori*, tem papel crucial na patogênese da bactéria por modular a resposta inflamatória e desestabilizar a função da barreira epitelial gástrica interrompendo as atividades celulares basais, causados principalmente pela extensa capacidade de induzir a vacuolização citoplasmática, levando à morte celular (WANG, 2014; WROBLEWSKI; PEEK; WILSON, 2010). O *HP-NAP*, expresso pelos genes *cagA* e *vacA*, induz a aderência de neutrófilos às células epiteliais durante o processo inflamatório, ativando a NADPH oxidase presente externamente nas membranas celulares que dá início a cascata de

produção de espécies reativas (ALGOOD; COVER, 2006; DE BERNARD; D'ELIOS, 2010).

A NADPH oxidase ativada transfere um elétron da última camada da NADPH para moléculas de oxigênio, formando o radical $O_2^{\bullet-}$, o qual por dismutação espontânea ou enzimática catalisada pela superóxido dismutase, é rapidamente convertido a H_2O_2. O H_2O_2 é convertido facilmente em radical $OH^{\bullet}$, considerado como o radical mais deletério ao organismo, pois devido à sua alta reatividade, causa danos irreversíveis a proteínas e aminoácidos, danos e mutações ao DNA e RNA, e ainda podem iniciar a peroxidação lipídica em ácidos graxos. O H_2O_2 também pode ser consumido na presença de íons cloreto, pela ação da mieloperoxidase, resultando na formação de um potente oxidante, o ácido hipocloroso (HOCl) (BARREIROS; DAVID; DAVID, 2006; BHATTACHARYYA et al., 2014; NAITO; YOSHIKAWA, 2002).

Além da geração de ERO, os pacientes infectados pela *H. pylori* apresentam alta expressão da enzima óxido nítrico-sintase induzível (iNOS) e a consequente ativação da via de formação de ERO, gerando óxido nítrico (NO) (NEUMANN et al., 2016; RAFIEI et al., 2012). O NO reage com os intermediários de oxigênio formados. A sua reação com $O_2^{\bullet-}$ resulta na formação de peroxinitrito ($ONOO^-$), um poderoso oxidante de proteínas. Ao ser protonado na presença do íon hidrogênio (H^+), o $ONOO^-$ também gera o radical hidroxil (BECKMAN; KOPPENOL, 1996).

Porém, para impedir a erradicação da bactéria pela geração de espécies reativas, a *H. pylori* apresenta mecanismos de resistência aos danos oxidativos de uma inflamação crônica: I) é capaz de expressar as enzimas catalase e superóxido dismutase que estabilizam as espécies reativas formadas em resposta à inflamação, além de promoverem atividade lítica dos macrófagos e neutrófilos; II) também é capaz de induzir apoptose das células de defesa e reduzir o efluxo do antioxidante glutationa (via proteína *vac*A); e III) produzir a enzima arginase que bloqueia a produção de óxido nítrico e inibe a proliferação de células de defesa. A bactéria também modula o complexo enzimático da NADPH oxidase, regulando a produção das espécies reativas liberadas em defesa do organismo. Através desses mecanismos ela inibe a proliferação celular e induz apoptose das células de defesa do organismo hospedeiro (ALGOOD; COVER, 2006; ALLEN et al., 2005).

O processo inflamatório instalado seja devido à interação da *H. pylori* ao epitélio gástrico ou ao uso indiscriminado de AINEs, além de induzir o estresse

oxidativo, levam a uma liberação aumentada de citocinas pró-inflamatórias, como TNF-α, IL-1β, IL-6 e IL-8 (DE BERNARD; D'ELIOS, 2010; YADAV et al., 2012), devido à infiltração de neutrófilos e liberação de fatores derivados de neutrófilos para a mucosa gástrica. A IL-6 é essencial na inicialização do desenvolvimento de tumores, sendo ela estimulada pela prostaglandina PGE2, que está elevada em macrófagos presentes na inflamação (FEDERICO et al., 2007). O aumento dos níveis de IL-6 em pacientes com câncer gástrico e sua forte associação com o risco de desenvolvimento deste tipo de câncer foi identificado no estudo de Sánchez-Zauco et al. (2017).

O aumento dos níveis de TNF-α é um importante fator na iniciação, progressão e persistência da inflamação e ulceração gástrica mediada pela indometacina. Mesmo que a indução inicial de TNF-α atue como mecanismo de defesa, em estágios posteriores pode ajudar no recrutamento de monócitos, macrófagos e de várias outras citocinas pró-inflamatórias para sustentar e promover a inflamação (PÉREZ et al., 2017; YADAV et al., 2012). O TNF-α estimula a expressão da enzima iNOS em células inflamatórias e epiteliais através da ativação da via do NF-κB (GEORGE et al., 2018; SOL; DÍAZ-MUÑOZ; FRESNO, 2007).

Essas citocinas pró-inflamatórias aparecem em níveis aumentados em humanos infectados com *H. pylori* em comparação a não infectados, sendo que a presença de polimorfismos que resultam em níveis elevados de IL-1β e TNF-α e reduzidos de IL-10 (citocina anti-inflamatória) estão associados a um maior risco de desenvolvimento de gastrite crônica e câncer (ALGOOD; COVER, 2006; BOCKERSTETT; DIPAOLO, 2017).

Através desses dados, podemos perceber que a infecção por *Helicobacter pylori* e o uso abusivo de AINEs têm efeitos sinérgicos no dano da mucosa gástrica através das diferentes vias do processo inflamatório. Mesmo que alguns mecanismos sejam realizados independentemente, ambos aumentam o risco de ulceração e dano à mucosa gástrica, como pode ser visto na figura 2. Juntos, a infecção por *H. pylori* e o uso de AINEs representam aproximadamente 90% dos casos de úlcera péptica (YUAN; PADOL; HUNT, 2006).

O tratamento padrão para o manejo da infecção por *H. pylori* consiste em regimes com múltiplos medicamentos. O regime de primeira linha é a terapia tríplice convencional que consiste na combinação de um inibidor de bomba de prótons (IBP) uma vez ao dia, mais dois antibióticos (amoxicilina e claritromicina ou

metronidazol/levofloxacina no lugar da amoxicilina) duas vezes ao dia, durante 7 dias. O sucesso do tratamento por esse esquema pode falhar em até 30% dos pacientes, além disso, as taxas de erradicação têm caído nos últimos tempos, em parte, devido à resistência da bactéria à claritromicina. A terapia quádrupla, que é estabelecida em regiões com altos níveis de resistência à claritromicina, contém IBP, bismuto e dois antibióticos (tetraciclina e metronidazol) durante 7 a 10 dias, possuindo taxas de erradicação igual ou superior a terapia tripla (COELHO et al., 2013).

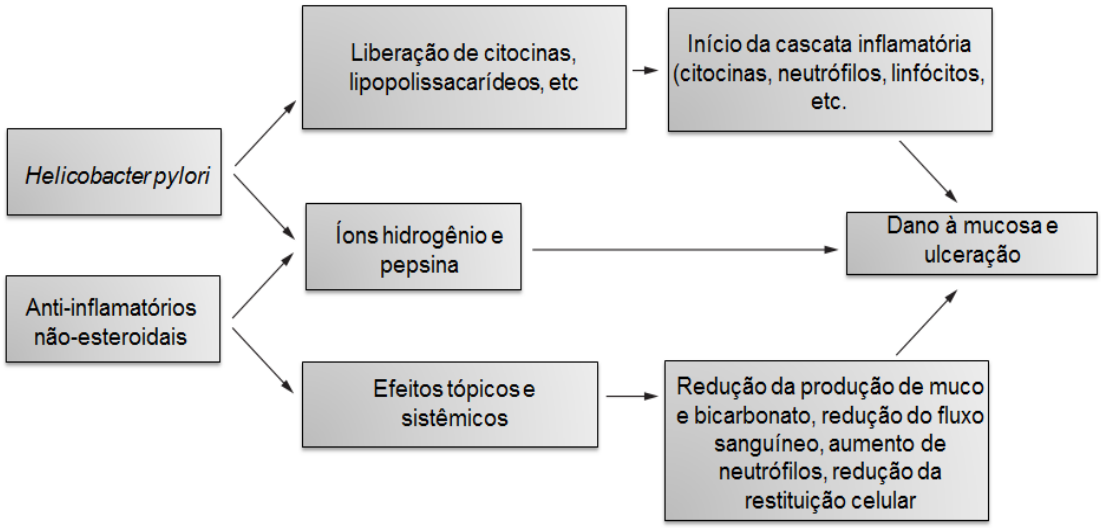

Figura 2 - *Helicobacter pylori* e anti-inflamatórios não esteroidais atuam de forma sinérgica no desenvolvimento de úlceras gástricas. Fonte: Adaptado de YUAN, Y.; PADOL, I. T.; HUNT, R. H. Peptic ulcer disease today. **Nature Clinical Practice Gastroenterology & Hepatology**, v. 3, n. 2, p. 80–89, 2006.

Para lesões gástricas induzidas por AINEs, os medicamentos normalmente utilizados são IBP (lanzoprazol, pantoprazol, e omeprazol), bloqueadores muscarínicos M1 (lelenzepina, pirenzepina), antagonistas de receptores de histamina H2 (cimetidina, ranitidina) que diminuem a secreção de ácido, e agentes citoprotetores como carbenoxolona e sucralfato, que promovem a defesa da mucosa gástrica. Esses fármacos equilibram os fatores defensivos (rotação celular, fluxo sanguíneo mucoso, secreção de mucina, secreção de bicarbonato e muco celular) e fatores agressivos (sais biliares, pepsina e ácido) da mucosa gástrica (GOEL; BHATTACHARYA, 1991; TARIQUE et al., 2016).

Embora os medicamentos sintéticos atualmente disponíveis contra úlceras gástricas sejam bem aceitos, a maioria apresenta efeitos colaterais e, dependendo da causa, a terapia pode ser longa e dispendiosa, dificultando a adesão do paciente

(YUAN; PADOL; HUNT, 2006), além da possibilidade de falha terapêutica por resistência bacteriana associada aos antibióticos (LI et al., 2018). Ainda, há incidências de perigo de interações medicamentosas e recidivas durante a terapia de úlcera por fármacos sintéticos. Em conjunto, esses fatores dificultam a erradicação da *H. pylori* e dos problemas gástricos relacionados ao uso abusivo de AINEs, que consequentemente, dificultam a redução dos casos de câncer gástrico (AMIN et al., 2013; LOPES et al., 2014).

Segundo Szabo (2014), a gastroproteção continua sendo um assunto relevante, sendo de vital importância a constante pesquisa em busca de novas substâncias que atuem como alternativas ao tratamento atual dos distúrbios gástricos. As plantas medicinais podem ser uma valiosa alternativa no tratamento de distúrbios gástricos com poucos efeitos adversos (AWAAD; EL-MELIGY; SOLIMAN, 2013; BI; MAN; MAN, 2014).

Entre os mecanismos gastroprotetores de extratos vegetais destacamos o aumento de fatores defensivos, como a secreção de mucina, muco celular, secreção de bicarbonato, fluxo sanguíneo mucoso, renovação do epitélio gastrointestinal (KANGWAN et al., 2014) e a diminuição de fatores agressivos, como a eliminação da bactéria *H. pylori*. Algumas espécies vegetais estudadas, como o extrato hidroalcoólico de cascas de *Persea major* (SOMENSI et al., 2017), alilpirocatecol, o principal composto fenólico das folhas de *Piper betel* (BHATTACHARYA et al., 2007), a fração enriquecida de flavonoides a partir das folhas de *Maytenus ilicifolia* (BAGGIO et al., 2007) e o flavonoide crisina (GEORGE et al., 2018) comprovaram esse efeito na prevenção de lesões gástricas induzidas agudamente por indometacina em modelos animais. Ainda, por serem antioxidantes, as espécies vegetais reduzem a peroxidação lipídica, auxiliando na prevenção da inflamação que leva à formação de úlceras gástricas. Numerosos estudos também mostram a ação anti-*H. pylori* de espécies vegetais, como exemplo podemos citar, extratos de *Annona cherimola*, *Guaiacum coulteri*, *Passiflora incarnata*, *Melissa officinalis*, entre outros (WANG, 2014).

Nenhum estudo anterior comprova a ação gastroprotetora das sementes de *P. americana* Mill., mas existem estudos evidenciando sua eficácia antimicrobiana. Rodríguez-Carpena et al. (2011) mostraram inibição do crescimento de *Bacillus cereus* e *Listeria monocytogenes*, além da cepa de *Escherichia coli* pelo extrato acetona:água das sementes do abacate, fato explicado pela presença de

flavonóides e ácidos fenólicos. Esse mesmo estudo comprovou que, por possuir maior variedade e maior quantidade de compostos fenólicos, a semente apresenta atividades antioxidantes e antimicrobianas mais intensas que a própria polpa do fruto. Idris, Ndukwe e Gimba (2010) também mostraram o efeito de extratos da semente de *P. americana* sobre diferentes micro-organismos, onde a fração acetato de etila apresentou melhores halos de inibição e menores Concentrações Inibitórias Mínimas (CIM) para micro-organismos como *S. aureus*, *S. pyogenes*, *B. subtilis* e *E. coli*.

Além do interesse na pesquisa de novos alvos terapêuticos para o tratamento antiúlcera e antimicrobiano, compostos com atividade antitumoral são igualmente importantes, já que o câncer gástrico está estreitamente relacionado à inflamação persistente devido à infecção por *H. pylori* (PEEK; BLASER, 2002; WROBLEWSKI; PEEK; WILSON, 2010). Na pesquisa de novos agentes anticâncer de origem natural, testes de citotoxicidade *in vitro* são necessários para avaliar o nível de toxicidade basal, que é a capacidade intrínseca de um composto causar morte celular por consequência de ser danoso às funções celulares basais. Tais compostos devem ser de baixa toxicidade em células normais enquanto são inibidores seletivos do crescimento de células tumorais (EISENBRAND et al., 2002).

Nesse contexto, sabendo que o tratamento de úlceras pépticas por AINEs e a erradicação da *H. pylori* com as terapias atuais têm suas limitações, surge o interesse na pesquisa em compostos vegetais para erradicar a infecção e fornecer meio eficaz de reduzir a inflamação gástrica e o câncer. Dessa forma, o objetivo desse estudo foi avaliar os efeitos gastroprotetores das sementes de *P. americana* Mill, investigando suas propriedades anti-*H. pylori,* antioxidante, seu potencial efeito sobre fatores que auxiliam na patogênese da lesão gástrica e câncer, em experimentos *in vitro* e seu potencial na prevenção de lesões gástricas induzidas agudamente por indometacina em modelo *in vivo*, utilizando ensaios bioquímicos e avaliação histopatológica. E por fim, já que a combinação de estudos farmacológicos e analíticos melhora a compreensão do uso de plantas medicinais, bem como seus possíveis efeitos terapêuticos e adversos, nós investigamos a composição de polifenois por métodos cromatográficos de alta eficiência, para melhor compreender a relação entre essas substâncias e suas atividades gastroprotetoras.

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Capítulo 1

Artigo 1: Effect of avocado (*Persea americana* Mill.) seeds on *Helicobacter pylori* infection and gastric adenocarcinoma cells

Original article

Effect of avocado (*Persea americana* Mill.) seeds on *Helicobacter pylori* infection and gastric adenocarcinoma cells

Brena Ramos Athaydes[1], Raffaela Zunti Carminati[1], Rodrigo Rezende Kitagawa[1,2], Rita de Cássia Ribeiro Gonçalves[1,2]*

[1]Graduate Program in Pharmaceutical Sciences, Federal University of Espirito Santo, Vitoria, Brazil.

[2]Department of Pharmaceutical Sciences, Federal University of Espirito Santo, Vitoria, Brazil.

*Corresponding author: Department of Pharmaceutical Sciences, Federal University of Espirito Santo, Av Marechal Campos, 1468, CEP 29043-900, Maruipe/ES, Brazil; E-mail address: rita.goncalves@ufes.br

Abstract

Persea americana Mill. (avocado), is used as an antiulcer in traditional medicine, however, not much is known about its seed, discarded as waste. *Helicobacter pylori* is a risk factor for the development of ulcers and gastric cancer, because its persistent infection stimulates the immunomodulatory system and causes oxidative stress. We evaluated the gastroprotective effect against *H. pylori* infection and anticancer potential of hydroalcoholic extract from avocado seeds (SCE), and its fractions, ethyl acetate (SEAP) and hexanic (SHP). SEAP presented better anti-*H. pylori* and antioxidant activity and decreased IL-6, probably resulting of higher contents of polyphenolic compounds. Treatment with SEAP and SHP shows the presence of blebs, membrane deformations, coccoid and filamentous forms in microscopy analysis. SHP and SEAP presented great inhibition of gastric adenocarcinoma cells. Avocado seeds appear as a natural alternative in the treatment of gastric diseases caused by *H. pylori*, modulating different pathways involved in this process.

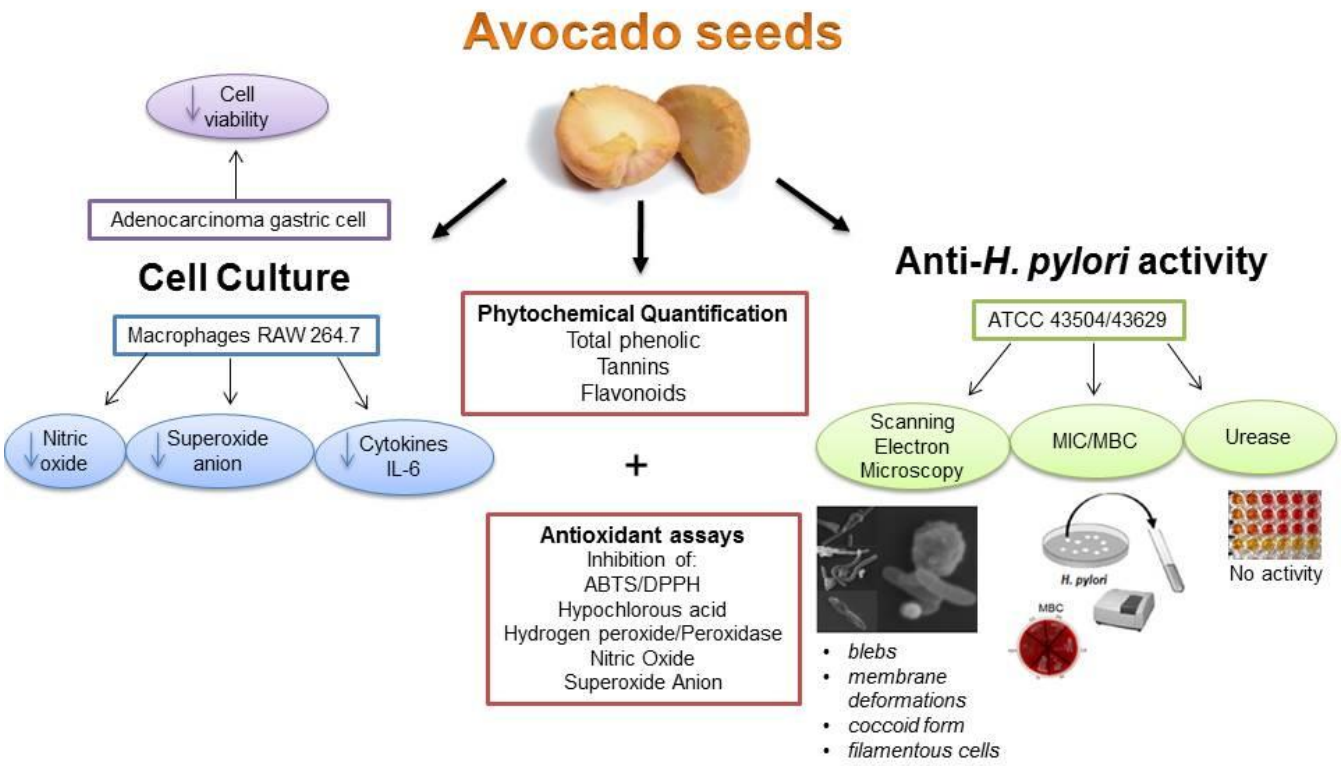
Key words: *Persea americana; Helicobacter pylori; Oxidative stress; Immunomodulator; Antitumor; Gastroprotection.*

Abbreviations: ABTS, [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)]; AGS, human gastric adenocarcinoma cells; cagPAI, cytotoxin-associated gene to *H. pylori* pathogenicity island; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5-5'-dithio-bis (2-nitrobenzoic) acid; ELISA, enzyme-Linked Immunosorbent assay; HpFabZ, *Helicobacter pylori* beta-hydroxyacyl-acyl carrier protein; HP-NAP, *Helicobacter pylori* neutrophil-activating protein; HRP, Horseradish peroxidase; IL-6, 8 and 10, interleukins; iNOS, inducible nitric oxide synthase; L-NAME, N(ω)-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MALT, mucosa-associated lymphoid tissue; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide**;** $NaNO_2$, sodium nitrite; NBT, nitrotetrazolium blue chloride; NF-κB, nuclear factor kappa B; NPS, nitroprusside; PBP, penicillin binding proteins; PMS, phenazine methosulfate; SCE, seeds crude extract; SEAP, seeds ethyl acetate partition; SEM, scanning electron microscope; SHP, seeds hexane partition; TNB, 5-thio-2-nitrobenzoic acid; TNF- α, tumor necrosis factor; VacA, vacuolating cytotoxin A.

Highlights:

- Avocado seeds have high antioxidant activity due to its phenolic compounds.
- The seeds presented immunomodulatory activity, mainly by inhibiting IL-6 cytokine.
- Avocado seeds has gastric antitumor activity *in vitro* with low toxicity.
- Samples showed good potential against *H. pylori* with different mechanisms of action.

Graphical Abstract:



1. Introduction

Persea americana Mill. (Lauraceae), popularly known as "avocado", is a native species from Mexico where the leaves and fruit pulp are used as food technology, also in traditional medicine for treatment of different diseases due its vasorrelaxant, analgesic, anti-inflammatory, antioxidant and antiulcer activities and antihypertensive, hypoglycemic and anticonvulsant effects (Yasir, Das & Kharya, 2010). The industrial processing of avocado has generated a significant amount of by-products from the seed with high value-added product, since a high proportion of bioactive substances with pharmacological activities remain in this residue (Ayala-Zavala et al., 2011).

The seed extracts of *P. americana* Mill. demonstrated to be efficient in the reduction of triglycerides, glycemic and blood pressure levels. It has also antimicrobial and antioxidant activities and is used for dermatological purposes (Dabas, Shegog, Ziegler & Lambert, 2013). These biological activities have been justified by the presence of polyphenols, such as tannins like catechins and procyanidins, besides hydroxybenzoic and hydroxycinnamic acid derivatives, flavonoids and alkaloids (Melgar et al., 2018; Rodriguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011).

In Brazil, avocado seeds have been known to be used for gastric disease, but without scientific evidence. According to some reports, the seeds provide very effective results in the treatment of infection and inflammation of gastrointestinal tract.

Nowadays, a lot of gastric diseases are known to be caused by the microorganism *Helicobacter pylori*: a spiral and flagellated gram-negative bacillus, which causes localized conditions such as gastritis, peptic ulcers, besides being an important factor in the pathogenesis of gastric cancer (Algood & Cover, 2006). Currently, half of the world's population is infected by *H. pylori*, and more than 70% of those infected individuals are asymptomatic. It is also associated with 75% of the gastric cancer cases, mainly developing adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma tumor types (Bae et al., 2018; Herrera & Parsonnet, 2009).

The establishment of *H. pylori* infection in the stomach acidic environment is part of its virulence factors, among them the production of urea by urease protein, which metabolizes the available urea, isolating the bacteria from the stomach acidity.

During this process, the human immune system also is capable of creating a robust innate and adaptive immune response to the infection, however it usually fails to eliminate *H. pylori* completely resulting in a persistent infection. The constant stimulation of host defense cells and the inflammatory process activation induced by cytokines, such as IL-6, IL-8 interleukin and tumor necrosis factor (TNF-α), increases the production of reactive oxygen and nitrogen species, contributing to the local inflammation being determinant in the chronicity of gastric ulceration that leads to gastric cancer (Abadi, 2017; Butcher, Hartog, Ernst & Crowe, 2017; Naito & Yoshikawa, 2002).

According to the International Guidelines, the first line treatment includes a combination of a proton pump inhibitor, clarithromycin and amoxicillin or metronidazole/tinidazole. However, adverse effects, treatment length and therapy costs may cause difficulties for its adherence and frequently lead to bacterial resistance (Siddique, Ovalle, Siddique & Moss, 2018), emphasizing the needs for new antimicrobials or alternative therapies with new pathways of mechanism of action. Reducing side effects, increasing therapeutic possibilities and sometimes economically more advantageous than conventional treatments, natural food has been increasingly used in the treatment of health-related conditions. Moreover, these benefits generate pharmaceutical industry interest in the discovery of new natural drugs (Wang, 2014).

In this context, the research of phytonutrients with gastroprotective activity that could prevent diseases promoted by *H. pylori* is a promising alternative to the standard treatment, due to a therapeutic and an economic point of view. Therefore, the aim of the present study was to investigate anti-*H. pylori* properties of seeds from *Persea americana* Mill., as well as its gastric antitumor activity and investigate its effects and the possible mechanisms and factors involved in gastric lesion pathogenesis.

2. Materials and Methods

2.1 Reagents and Equipment

All the reagents used in antioxidant assays were purchased from Sigma-Aldrich (Saint Louis, USA). Brain heart infusion (BHI) was acquired from Merck (Darmstadt, Germany), Dulbecco's modified eagle medium (DMEM) and fetal bovine

serum were acquired from Vitrocell (São Paulo, Brazil). *Helicobacter pylori* ATCC 43504 and ATCC 43629 were obtained from Fiocruz (Rio de Janeiro, Brazil). Murine macrophages RAW 264.7 (ATCC TIB 71) were kindly provided by professor Marcio Fronza from Vila Velha University (UVV, Vila Velha, Brazil) and human gastric adenocarcinoma cells AGS (ATCC CRL-1739) were obtained from Cell Line Service (Rio de Janeiro, Brazil). All the other reagents were analytical grade and commercially available. Kit for cytokines assay by immunoenzymatic method (ELISA Ready-SET-Go!) was purchased from eBioscience (San Diego, USA). Absorbance measurements were performed on iMark^{TM} Bio-Rad (Washington, USA) microplate reader and Ephoch 2 microplate reader, Biotek Instruments (Highland Park, USA). The scanning electron microscope used was JEOL®, JSM-6610LV (Tokyo, Japan).

2.2 Plant material

The seeds of *Persea americana* Mill. were collected in Cariacica city, Espírito Santo, Brazil, located: (coordinates) -20.378669 of south latitude and -40.370717 of west longitude (20 22'43.2 "S; 40 ° 22'14.6" W), in march 2016. An exsiccate with a flowering branch was carried out for botanical identification and deposited in the Central Herbarium VIES of the Federal University of Espirito Santo (number 38.282).

2.3 Preparation of avocado seed extract and fractions

The seeds of *P. americana* Mill. were grated and dried at 40ºC during 5 days until the complete elimination of the humidity. The hydroalcoholic extract called seed crude extract (SCE) was obtained by turbolysis at 10% w/v with 70% ethylic alcohol. The resultant solution was filtered, submitted to evaporation at 50ºC under reduced pressure in a rotary evaporator until the complete elimination of ethanol, and subsequently fractionated with hexane and ethyl acetate. The two partitions were dried in room temperature yielding hexanic (SHP) and ethyl acetate (SEAP) partitions of the seed.

2.4 Phytochemical analysis

2.4.1 Phytochemical screening

The qualitative phytochemical characterization of the extract and fractions was based on the classic identification assays relative to the following compounds:

flavonoids, saponins, cardiotonic glycosides, anthraquinones, tannins, alkaloids, polyphenols, terpenes and steroids.

2.4.2 Total phenolic content and total tannins determination

The total polyphenol content and tannins were determined using the methodology described by Nunes, Jamal, Kitagawa and Gonçalves (2016). Gallic acid was used as standard and the results were expressed in milligrams of gallic acid equivalent per gram of sample (mgEGA/g).

2.4.3 Total flavonoid content determination

Total flavonoid content was determined using the methodology of Marques et al. (2012) with adaptations. In a microplate were added 20 µL of extract (100 µg/mL) diluted in 99 µL of distilled water, 6 µL of glacial acetic acid, 100 µL of 20% pyridine and 25 µL of 6.5% aluminum chloride-methanol solution. Thirty minutes later, spectrophotometric reading was performed on a microplate reader at 415 nm. Quercetin was used as standard and the results were expressed in milligrams of quercetin equivalent per gram of sample (mgEQ/g).

2.5 Antioxidant activity assays

Samples used for the assays were prepared in DMSO (or methanol) and tested at concentrations ranging from 3.125 to 100 µg/mL. Lower concentrations of standard and SEAP were tested for the DPPH and ABTS assays (0.009375 to 100 µg/mL). Trolox, quercetin and gallic acid were used as antioxidant controls, depending on the assay. The results were expressed in IC_{50}, the concentration that inhibits 50% of the absorbance, and percentage of inhibition (%).

2.5.1 DPPH assay

In this assay the mixture consisted in 100 µL of each sample or standard at various concentrations and 200 µL of DPPH solution 0.004% (w/v) dissolved in ethanol solution. The absorbance was measured at 540 nm after 10 minutes of incubation in room temperature in the dark. The maximum absorption reference was obtained with 200 µL DPPH solution and 100 µL of ethanol (control) (Gülçin, Berashvili & Gepdoremen, 2005).

2.5.2 ABTS assay

The radical was prepared by an aqueous mixture of ABTS (7 mM) and potassium persulfate (2.45 mM) in room temperature and in absence of light for 16 hours before use. The ABTS solution was diluted in ethanol to determine the absorbance of 0.7 to 734 nm. In a microplate, 297 µL of ABTS solution and 3 µL of different sample concentrations were added and after 15 minutes of incubation in the dark, the absorbance was measured at 750 nm (Re et al., 1999).

2.5.3 Hypochlorous acid scavenging assay

This method was based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB) by hypochlorous acid (HOCl), resulting in the formation of 5-5'-dithio-bis (2-nitrobenzoic) acid (DTNB). In this assay was used the modified method described by Ching, Jong and Bast (1994). The concentration of HOCl solution was determined from its molar extinction coefficient of the absorbance at 295 nm. The TNB solution was prepared by the mixture of DTNB (1 mM), ethylenediamine tetra acetic acid (EDTA, 5 mM) and sodium borohydride ($NaBH_4$, 20 mM), quantified from its molar extinction coefficient of the absorbance at 412 nm until 140 µM. The reaction was prepared with 20 µL of HOCl (25 µM), 60 µL of phosphate buffer 50 mM, pH 6.6 and 20 µL of samples, gallic acid or quercetin. After 2 minutes was added 100 µL of TNB solution (final concentration of 70 µM) and absorbance was measured at 415 nm.

2.5.4 Hydrogen peroxide assay

This assay was based on Pick and Keisari method (1980). Hydrogen peroxide (H_2O_2) is broken down by peroxidase to generate a reactive intermediate that oxidizes phenol red to a yellow compound that at basic pH becomes purplish red and can be quantified at 610 nm. The concentration of H_2O_2 solution was determined from its molar extinction coefficient of the absorbance at 230 nm. The reaction solution contained 20 µL of samples (3.125-100 µg/mL) or trolox (0.78-100 µg/mL), 40 µL of H_2O_2 (2 mM), 16 µL of a solution containing NaCl (140 mM), phenol red (0.1 mg/mL), dextrose (5.5 mM) diluted in potassium phosphate buffer (10 mM, pH 7.0, 37ºC), 104 µL of potassium phosphate buffer and 20 µL of peroxidase enzyme (*horseradish peroxidase*, 8.5 U/mL). The reaction was followed by incubation for 10 minutes followed addition of 20 µL of sodium hydroxide (NaOH, 1 N) and the purple complex was quantified at 610 nm.

2.5.5 Horseradish peroxidase inhibition assay

The assay relies on the ability of peroxidase to use the H_2O_2 as hydrogen donor, to catalyze the oxidation of nonspecific electron donors such as guaiacol (Merrill, 1980). For the assay, 200 µL of samples or trolox (3.125-100 µg/mL), 1100 µL of 50 mM sodium phosphate buffer (pH 7.4, 37ºC), 200 µL of peroxidase (HRP) (7 nM), 200 µL of guaiacol (2 mM) and 300 µL of H_2O_2 (0.1 mM, to initiate the reaction) were added on cuvettes. The solutions were mixed and the reaction was followed for 40 s at 470 nm (Vellosa, 2008). The concentration of HRP (horseradish peroxidase) was determined from its molar extinction coefficient of absorbance at 403 nm. In this assay, the peroxidase activity is calculated by the initial rate of the kinetics reaction in the presence of H_2O_2 using guaiacol as substrate. The results were expressed as v_0 (s^{-1}; initial rate of guaiacol oxidation) and the percentage of inhibition (%) was calculated as v_0 reduction by samples or trolox, compared to the reaction without them.

2.5.6 Nitric oxide scavenging assay

This assay was based on the methodology presented by Marcocci, Maquire, Droy-Lefaix and Packer (1994) (with adaptations). The reaction occurred by the spontaneous release of nitric oxide (NO) by nitroprusside (NPS) in the presence of light. NO is transformed into nitrite by reacting to oxygen, being quantified by Griess reagent (1% w/v sulphanilamide, 0.1% w/v naphthylethylenediamine, 2.5% v/v ortho-phosphoric acid). The mixture consisted of 50 µL of the NPS solution (5 mM) in phosphate buffer (0.1 M, pH 7.0) prepared in the absence of light and 50 µL of samples or quercetin (concentrations of 25-800 µg/mL), incubated for 1 hour in room temperature with exposition to light. Griess reagent was added with incubation for 10 min without any light, the reading was made at 540 nm. The calibration curve of the sodium nitrite ($NaNO_2$) was performed to represent data in the concentration of formed nitrite (NO_2^-).

2.5.7 Superoxide anion scavenging assay

This assay was performed according to Suzumura, Yasuhara and Narita (1999). The superoxide anion ($O_2^{\bullet-}$) generated by the reaction between phenazine methosulphate (PMS) and β-Nicotinamide adenine dinucleotide (NADH), reduces nitrotetrazolium blue chloride (NBT) to the chromogen formazan, which the color

intensity is directly proportional to the concentration of radical. Antioxidant molecules react with $O_2^{\bullet-}$, inhibiting the formation of formazan. The reaction was performed with 30 µL of the sample or gallic acid, 7.5 µL of PMS (5 µM), 30 µL of NBT (45 µM), and 225 µL of sodium phosphate buffer (50 mM), pH 7.4. Two minutes later, 7.5 µL of NADH (0.125 mM) was added followed by incubation in the dark for 10 minutes and the reading performed at 560 nm.

2.6 Cell culture and viability

The viability on murine macrophages RAW 264.7 (ATCC TIB 71) and human gastric adenocarcinoma cells (AGS cells, ATCC CRL-1739) was performed using thiazolyl blue tetrazolium bromide (MTT-tetrazolium) method (Mosmann, 1983). Cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, incubated at 37°C/5%CO_2 until reached approximately 80-90% of confluence. After incubation, they were washed with PBS (pH 7.4). AGS cells were trypsinized (2 mL of 0.25% trypsin solution in serum-free DMEM) for 10 minutes and RAW cells were removed with *cell scraper* and then, cells were counted in Neubauer's chamber. For viability assessment, a concentration of $6x10^4$ cells/mL was adhered on a microplate, incubated for 24 h and pretreated with different concentrations of the samples (3.125-100 µg/mL) (or Cisplatin for AGS cells assay). After incubation for 24 h (RAW cells) or 48 h (AGS cells) the plates were prepared for MTT-tetrazolium (1 mg/mL) method and read at 540/620 nm. The assays were accompanied by cell growth control (without samples). A dose-effect curve was used to calculate the cytotoxic index (CI_{50} or CI_{90}) to determine the concentration capable to reduce the absorbance by 50% for AGS cells and 90% for RAW cells assay.

2.6.1 Inhibition of nitric oxide produced by macrophages

To determine the ability of the samples to inhibit the production of nitric oxide in RAW 264.7 macrophages, the cells ($1x10^6$ cells/mL) were pretreated with various concentrations (6.25-100 µg/mL) of samples or the standard N(ω)-nitro-L-arginine methyl ester (L-NAME), followed by stimulation with lipopolysaccharide of *E.* coli (LPS, 1 µg/mL) for 24 h. The supernatants were taken, and NO was quantified by Griess method. Cell viability was performed after experiments by MTT-tetrazolium method. Data were presented in nitrite concentration (µM) by the absorbance at 540 nm, using standard curve with sodium nitrite.

2.6.2 Inhibition of superoxide anion produced by macrophages

The inhibitory effect of samples on $O_2^{\bullet-}$ production in RAW 264.7 was determined by adding pretreated cells ($8x10^4$ cells/mL) with samples or apocynin (standard), stimulated with LPS (1 µg/mL) and incubated overnight. The supernatant was discarded to addition of NBT (1 mg/mL) and incubated again for 2 h. The cells were washed with methanol and after drying at 37ºC the formed formazan crystals were dissolved with potassium hydroxide (2 M) mixed for 5 minutes and developed by DMSO. Readings were performed at 620 nm and results followed up with control growth (Pinho, Sousa, Valentão & Andrade, 2011). The viability after the experiments was assisted by MTT-tetrazolium method.

2.6.3 Immunomodulatory activity

Immunomodulatory activity was determined in inhibition of cytokines TNF-α, IL-6 and induction of IL-10 produced by RAW 264.7. The cells ($1x10^6$ cells/mL for IL-6 and IL-10 and $2x10^5$ cells/mL for TNF-α assay) were pretreated with different samples concentrations (6.25 - 100 µg/mL), stimulated with LPS (1 µg/mL) (except for IL-10 assay) and incubated overnight at 37°C/5%CO_2. The supernatants were collected for detection and quantification of cytokines by the enzyme-linked immunosorbent assay (ELISA). The absorbance was measured at 450/570 nm, and the amount of the cytokines (pg/mL) was calculated from a standard curve prepared with purified cytokine.

2.7 Antibacterial activity against Helicobacter pylori

The anti-*H. pylori* activity was evaluated by determination of the Minimal Inhibitory Concentration (MIC), using the broth microdilution technique according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, M7-A6, 2003), and by Minimum Bactericidal Concentration (MBC). In this assay, *H. pylori* was cultured in a liquid medium (Brain Heart Infusion supplemented with 10% (v/v) of fetal bovine serum) at 37ºC/10%CO_2. The medium containing several concentrations of samples (32-1024 µg/mL) with the same volume of *H. pylori* suspension (approximately 10^6 UFC/mL) was added in microplate wells and the spectrophotometric readings were performed at 620 nm. After new incubation for 72 h at the same conditions, the plate was homogenized, and a new reading was performed at the same wavelength. Growth control (without samples), negative

control (culture medium with the samples and tested substances) and sterility control (BHI broth only) were also performed. MIC was graphically defined as the lowest concentration of antibacterial substance that promoted a decline of 90% in absorbance. Amoxicillin (5 mg/mL) and Metronidazole (10 mg/mL) were used as standard antibiotic. Data was expressed as percentage of *H. pylori* growth inhibition. MBC was defined as the lowest concentration of extract that inhibited colony formation on Columbia agar plates. In this test, the concentration that did not achieve growth was plated on Columbia agar plate containing 5% sheep blood (incubated at 37°C under an atmosphere containing 10% CO_2 for 72 h).

2.8 Urease inhibition

Urease inhibition was determined by the production of ammonia metabolized by urease through enzymatic catalysis. Briefly, 25 µL of urease (4 UI) and 25 µL of samples (32-1024 µg/mL) were added to a microplate and incubated for 2 h at room temperature. Furthermore, 25 µL of phenol red (0.02%) and 200 µL of 50 mM urea in 100 mM phosphate buffer (pH 6.8) were added. Absorbance readings were performed at 540 nm at time zero (T0) and read every 10 minutes until 40 min to evaluate the kinetics reaction. The result was expressed as percentage of urease activity inhibition. Boric acid was used as the positive control for urease inhibition (Tanaka, Kawase & Tani, 2004).

2.9 Morphological analysis of Helicobacter pylori

The *H. pylori* morphological analysis was performed after exposure to sub-inhibitory MIC (½ MIC) using a scanning electron microscope (SEM). The culture medium containing bacteria exposed to the samples was centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and 1 mL of Cacodylate buffer (0.1 M, pH 7.2) was added to the cell pellet, incubated in room temperature for 2 h and stored overnight in a refrigerator. New washings with Cacodylate buffer with subsequent centrifugations were performed. The cell pellet of the samples was post-fixed with Osmium tetroxide (OsO_4, 1%) and potassium ferrocyanide (1.25%) and incubated for 1 h in absence of light. After new washes, an aliquot of the cell pellet was placed at the center of a slide, dehydrated with ethanol, allowed to dry by critical CO_2 point, metalized, and subjected to reading under SEM with an accelerating voltage of 20 kV.

2.10 Statistical analysis

The assays were performed in triplicate and repeated at least three times, the results being represented by mean of triplicate ± standard deviation (SD). Statistical analysis was performed using one and two-way ANOVA with *post-hoc* test Bonferroni, considering significant results with $p < 0.05$. IC_{50} and CI_{50} or CI_{90} was calculated using non-linear regression. The statistical analysis was performed using GraphPad Prism 6.0.

3. Results

3.1 Phytochemical characterization of extract and fractions

The crude hydroalcoholic extract (SCE) yield was 5.76%, from 50 g of the seed, and after the partition, 22.1% of the ethyl acetate fraction (SEAP) and 14.6% of the hexane fraction (SHP), calculated from 2.88 g of SCE. Phytochemical characterization showed the presence of polyphenols, flavonoids, tannins, alkaloids and coumarins in all samples. SHP also presented positive reaction for triterpenes and SCE and SEAP for saponins and steroids.

The quantification results of phenolics, total tannins and flavonoids are shown in table 1. SEAP presented a higher amount of these compounds when compared with other samples, 366.79 ± 5.05 mgEA/g of phenolic content, 314.64 ± 2.53 mgEAG/g of total tannins and 28.09 ± 0.64 mgEQ/g of flavonoids.

Table 1: Determination of phenolic content, flavonoids and total tannins of crude extract and fractions from *Persea americana* Mill. seeds.

Samples	**Phenolic content (mgEGA/g)**	**Flavonoids (mgEQ/g)**	**Tannins (mgGA/g)**
SCE	58.75 ± 0.76	14.76 ± 0.35	36.13 ± 1.60
SEAP	366.79 ± 5.05	28.09 ± 0.64	314.64 ± 2.53
SHP	13.69 ± 0.50	12.86 ± 0.51	3.27 ± 4.12

Results expressed as mean (mgEGA/g or mgEQ/g - miligram equivalent gram of gallic acid or quercetin per gram of extract) ± SD.

3.2 Antioxidant activity

The antioxidant activity was evaluated by different chemical methods, presenting higher activity for SEAP. SEAP presented IC_{50} of 0.13 ± 0.01 for ABTS, 3.02 ± 0.17 for DPPH and IC_{50} of 9.39 ± 1.37 for HOCl (table 2). SEAP also had better activity in HRP enzyme inhibition with IC_{50} of 5.40 ± 0.17 and 95.71% ± 6.05 of enzyme inhibition at 100 µg/mL.

Table 2: Antioxidant activity of crude extract and fractions from *Persea americana* Mill. seeds.

Samples	**ABTS**	**DPPH**	**Hypochlorous acid**	**Hydrogen peroxide**	**HRP**
SCE	16.75 ± 2.21	44.48 ± 0.93	99.79 ± 0.05	58.80 ± 5.75	65.56 ± 10.83
SEAP	0.13 ± 0.01	3.02 ± 0.17	9.39 ± 1.37	13.58 ± 1.46	5.40 ± 0.17
SHP	12.50 ± 0.34	4.80 ± 0.29	44.87 ± 4.02	>100	28.22 ± 0.03
Gallic acid	0.04 ± 0.01	0.04 ± 0.01	-	-	-
Trolox	2.41 ± 0.02	2.32 ± 0.00	-	2.45 ± 0.60	3.16 ± 0.01
Quercetin	-	-	9.70 ± 0.87	-	-

Data represent mean IC_{50} (µg/mL) ± SD (n = 3). ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; HRP: *Horseradish peroxidase*; -: not rated. Gallic acid, trolox and quercetin were considered as standard controls.

3.3 Macrophages viability

The viability of macrophages RAW 264.7 treated with samples was evaluated to determine the concentrations with viability greater than 90%, which were used in immunomodulatory, in capture of NO and $O_2^{\bullet-}$ cell assays. As shown in Fig. 1, SCE and SAEP showed 100% of viability at all tested concentrations, but SHP was toxic at highest concentrations, 100 µg/mL and 50 µg/mL. Therefore, for SHP were chosen concentrations below 25 µg/mL (91.36% ± 7.23 viable cells).

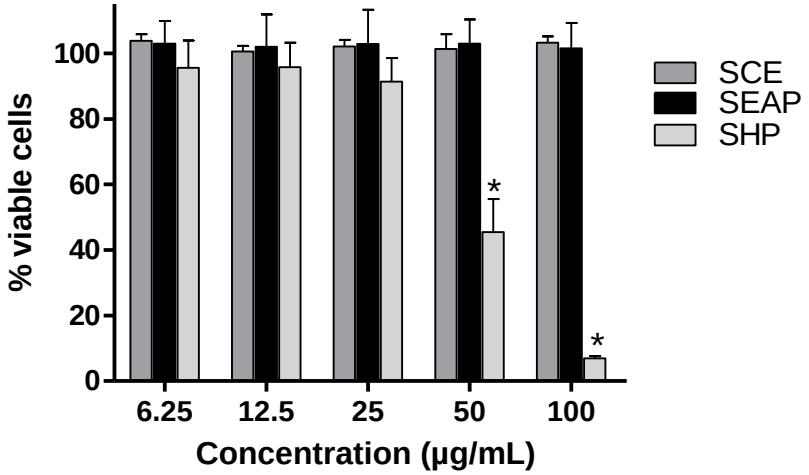


Fig. 1 - Effect of crude extract and fractions of seeds from *Persea americana* Mill. on cell viability of murine macrophages RAW 264.7. Results are expressed as percentage of viable cells. Data represent mean ± SD with *$p < 0.05$ considered significant when compared to cell growth without the samples.

3.4 Inhibition of superoxide anion and nitric oxide

Chemical and cellular assays in the capture of $O_2^{\bullet-}$ and NO were performed to evaluate the antioxidant effect of the samples. Inhibitory assays were performed on LPS-stimulated macrophages since none of the samples were able to induce $O_2^{\bullet-}$ or NO production.

SEAP at 100 µg/mL presented better results on these experiments: in $O_2^{\bullet-}$ assays, showed inhibition of 71.40% ± 8.57 of chemical (Fig. 2A) and 61.16% ± 3.56 of cellular $O_2^{\bullet-}$ production (Fig. 2B), similar to apocynin (61.48% ± 1.09) in the same concentration. In NO assays, SEAP presented inhibition of 51.30% ± 10.0 (6.92 µM ± 2.68) of chemical NO_2^- production compared to control (reaction without samples) (14.21 µM ± 2.27) (Fig. 3A). In the cell assay, SEAP inhibited 57.84% ± 9.79 of NO_2^- production (5.08 µM ± 1.18) compared to LPS (12.05 µM ± 2.39) (Fig. 3B).

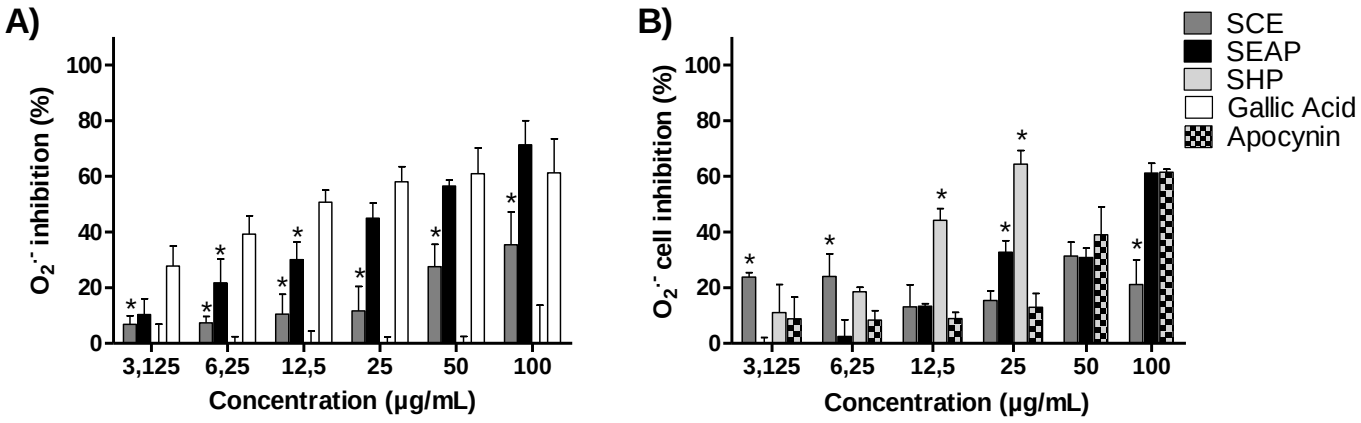


Fig. 2 - Effect of crude extract and fractions from *Persea americana* Mill. seeds against superoxide anion ($O_2^{\bullet-}$) in chemical (A) and cellular (B) assays. Results are expressed as percentage of inhibition. Columns represents mean ± SD (n = 3). *$p < 0.05$ *vs.* gallic acid (A) or apocynin (B).

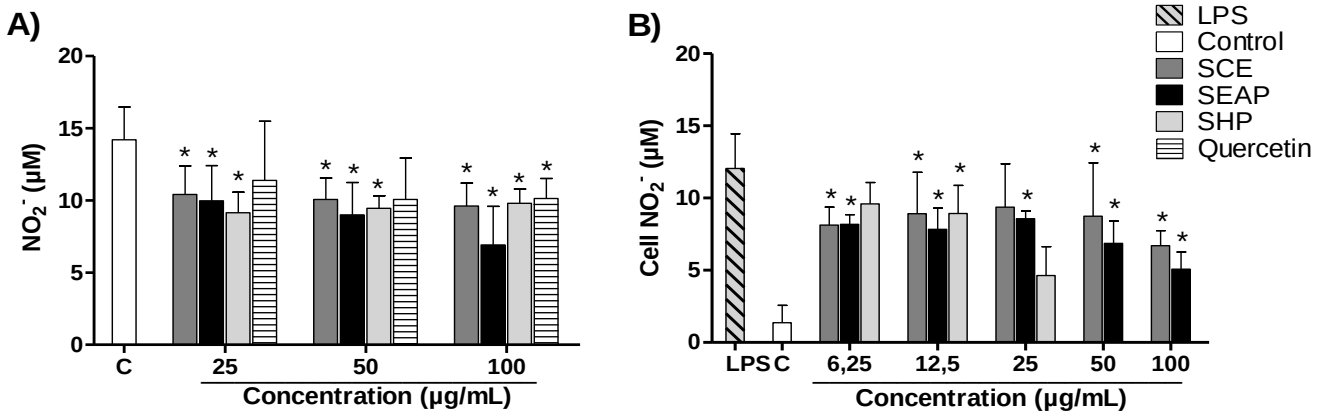


Fig. 3 - Effect of crude extract and fractions from *Persea americana* Mill. seeds against nitric oxide (NO) in chemical (A) and cellular (B) assays. Results are expressed as nitrite (NO^{2-}) concentration. Columns represents mean ± SD (n = 3). * $p < 0.05$ *vs.* control (A) and Lipopolysaccharide (LPS) (B). Reaction without samples served as control in A and cells incubated without LPS served as control in B.

3.5 Immunomodulatory activity

The immunomodulatory effect of extract and fractions was determined by TNF-α and IL-6 inhibition and by IL-10 induction assay. In the inhibition of IL-6 production, the results were significant for the following samples and concentrations: SEAP presented inhibition of 83.81% ± 9.40 and SCE of 73.77% ± 3.11 at 100 µg/mL (Fig. 4A). In the evaluation of the effect on TNF-α production, SEAP showed inhibition of 17.25% ± 2.24 at 100 µg/mL. SHP showed effect on lower concentrations: at 25 µg/mL, was observed inhibition of 30.66% ± 5.56 (Fig. 4B). In

IL-10 induction assay (Fig. 4C) samples did not produce effect, therefore results were not considered significant if compared to control (without LPS).

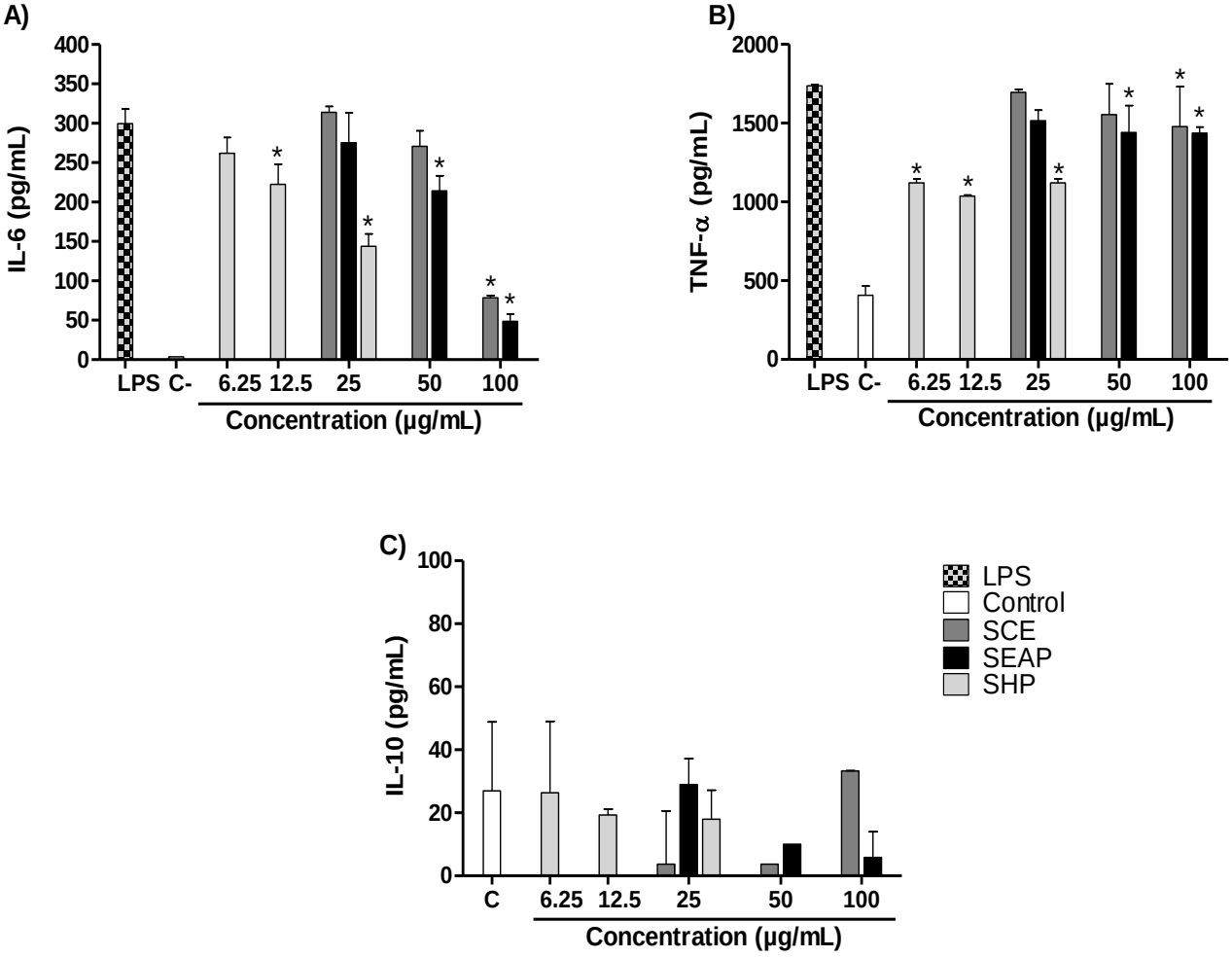


Fig. 4 - Immunomodulatory effect of crude extract and fractions from *Persea americana* Mill. seeds on macrophages RAW 264.7 stimulated with LPS. Data represent mean ± SD (n = 3). * Results were statistically significant ($p < 0.05$) to cytokine IL-6 and TNF-α assay compared with LPS control (A and B) and to IL-10 assay compared to control (without LPS).

3.6 Cytotoxicity in human gastric adenocarcinoma cells

The avocado seed fractions were able to inhibit AGS cells. SEAP presented CI_{50} of 55.43 ± 6.20 and SHP 11.80 ± 1.75, while for the standard Cisplatin was 7.70 ± 1.51. These data represent SEAP inhibition of 88.11% ± 4.78 at 100 µg/mL and SHP inhibition of 57.60% ± 0.35 at 25 µg/mL. Those concentrations presented viability greater than 90% on RAW cells (Fig. 5).

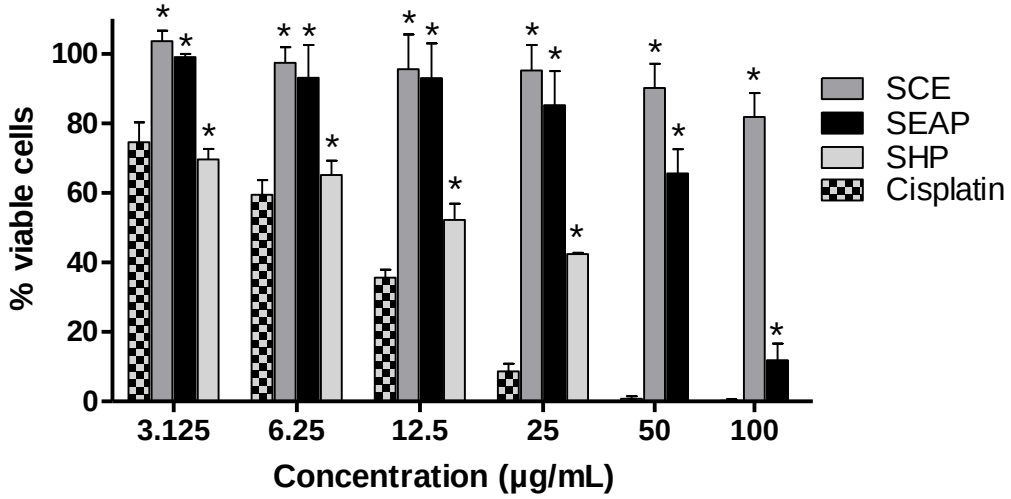


Fig. 5 - Evaluation of viability in gastric adenocarcinoma cell (AGS) by seeds from *Persea americana* Mill. and cisplatin. Results are expressed as percentage of viable cells ± SD with * $p < 0.05$ considered statistically significant when compared to cisplatin.

3.7 Antibacterial activity against Helicobacter pylori

Antibacterial activity of the samples was evaluated against two strains of *H. pylori*: ATCC 43504 and ATCC 43629. SEAP showed better activity for both strains, with MIC and MBC of 128 µg/mL for strain ATCC 43504 and MIC of 128 µg/mL and MBC of 256 µg/mL for strain ATCC 43629 (Fig. 6). Urease activity was evaluated but didn't present any significant results. At the highest tested concentration (1024 µg/mL), SCE inhibited 22.42% ± 0.8, SEAP inhibited 17.40% ± 8.6 and SHP inhibited 14.92% ± 2.1 of urease activity.

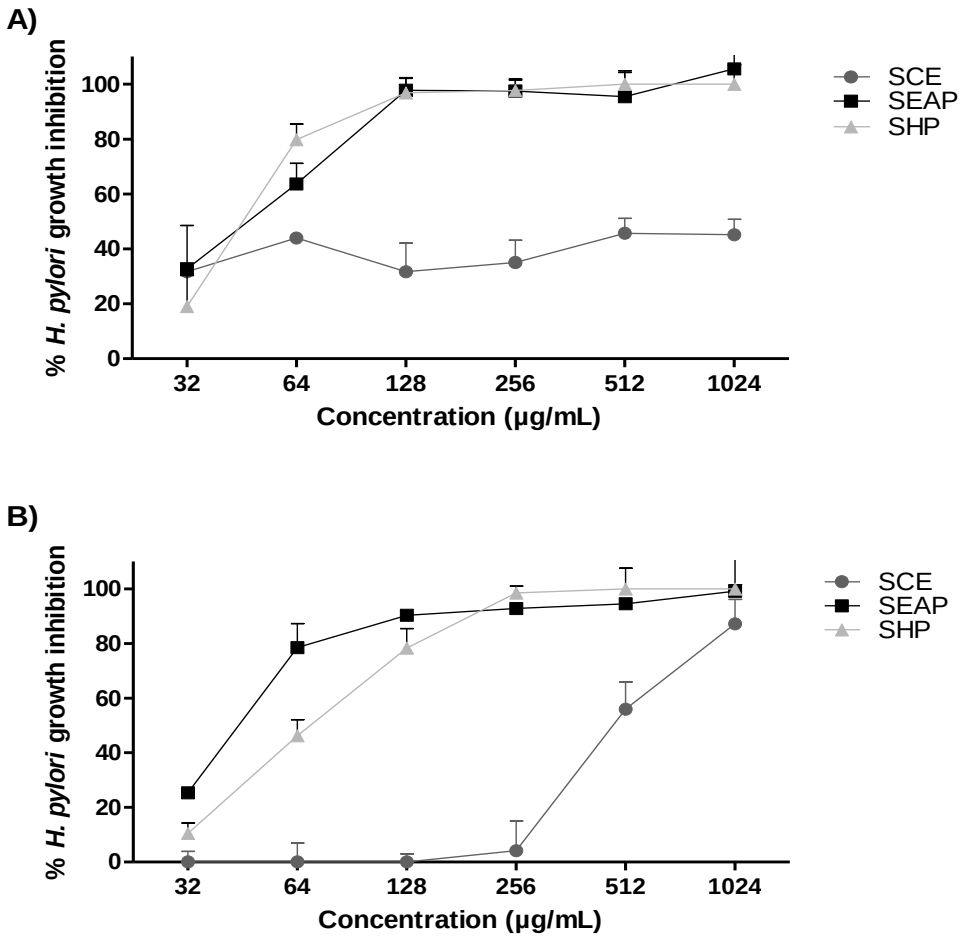


Fig. 6 - Effect on *Helicobacter pylori* growth after treatment with different concentrations of crude extract and fractions from *P. americana* Mill. seeds. A) *H. pylori* ATCC 43504; B) *H. pylori* ATCC 43629.

3.8 Morphological analysis of Helicobacter pylori

The morphological analysis of the *H. pylori* treated with fractions and amoxicillin in sub-MIC (½ MIC) concentrations was performed in scanning electron microscopy (SEM) (Fig. 7). Controls of both strains, ATCC 43504 (Fig. 7A) and ATCC 43629 (Fig. 7B) are in bacillary form, without morphological changes. Treatment with amoxicillin (0.125 µg/mL) in both strains presented slightly filamentous cells, *blebs* formation and other cell surface deformations (Fig. 7C and 7D), similar forms to SHP treatment, which also showed coccoid form in strain ATCC 43504 (Fig. 7E) and *blebs* with slight stretching of the cell wall for strain ATCC 43629 (Fig. 7F). On the other hand, the SEAP treatment showed a remarkable number of filamentous cells for both strains (Fig. 7G-H), with bacterial folding to convert to cocoide form in the strain ATCC 43629 (Fig. 7H). Figure 7I shows the predominance

of filamentous cells with SEAP treatment for strain 43504 and Figure 7J shows the morphological changes on SHP treatment for strain 43629.

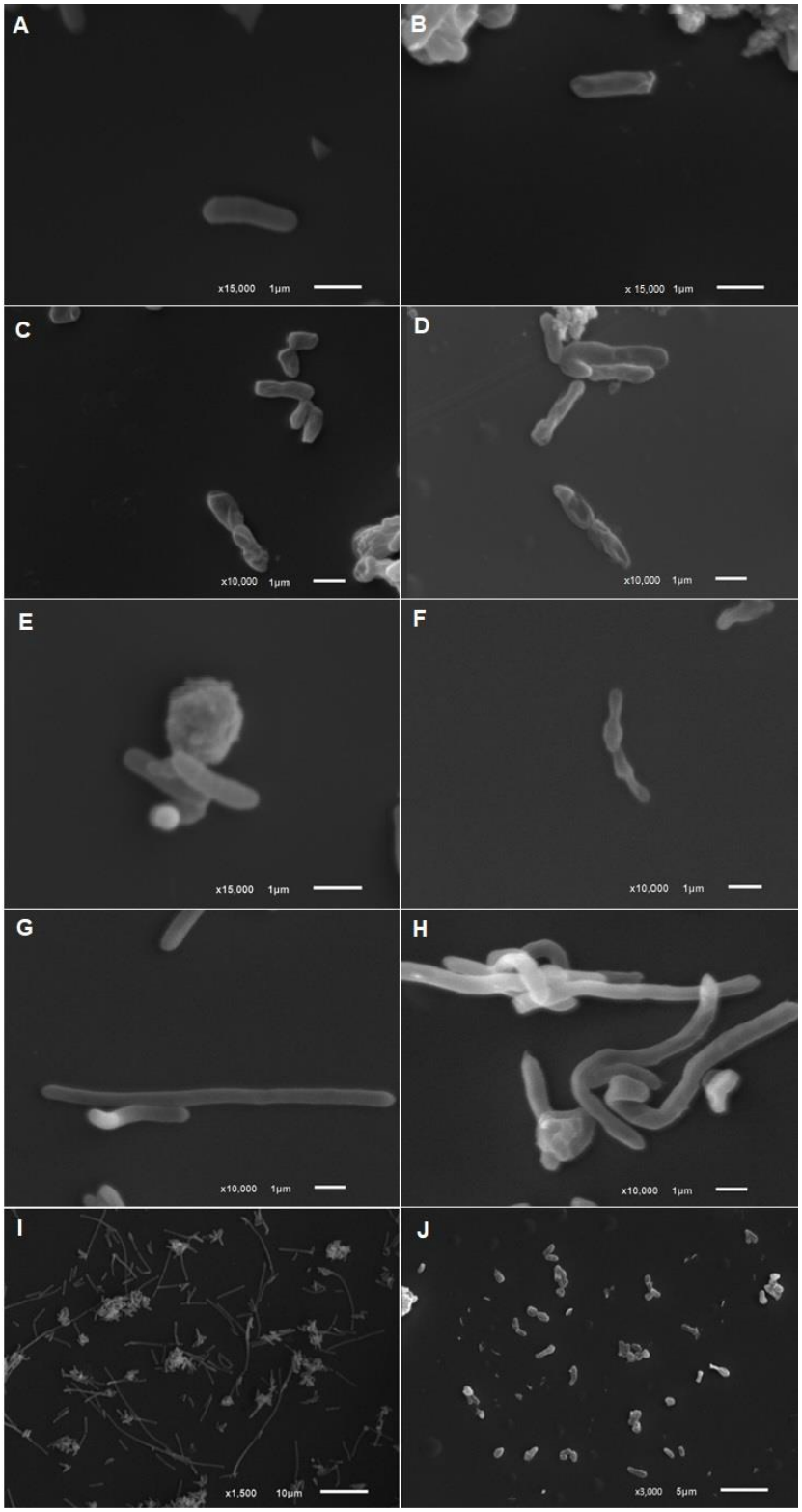


Fig. 7 - Scanning electronic microscopy of *H. pylori* bacteria exposed to different treatments. A) ATCC 43504 control; B) ATCC 43629 control; C) amoxicillin in ATCC 43504 and D) amoxicillin in ATCC 43629; E) SHP in ATCC 43504; F) SHP in ATCC 43629; G) SEAP in ATCC 43504; H) SEAP in ATCC 43629; I) SEAP in ATCC 43504 with magnification of 1500x; J) SHP in ATCC 43629 with magnification of 3000x.

4. Discussion

In our study, bioactive compounds from *Persea americana* Mill. seeds, showed to be a new alternative in the treatment of gastric diseases, since it is effective in a dose dependent manner against *H. pylori* strains, suppression of reactive species, immunomodulatory protection and antitumor activity, essential factors against the infection mechanism of this bacterium and to the gastric cancer development.

The content of phenolic compounds in avocado seeds were already reported, amounting to 88.2 mg/GAE/g in ethanolic extract (Soong & Barlow, 2004) and 51.6 mg/GAE/g from avocado seeds acetone/water/acetic acid extract (Wang, Bostic & Gu, 2010), being this last phenolic content superior than in the pulp, as already reported by other authors (Rodriguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011). However, SEAP presented higher phenolic content than other samples described in literature, being justified by the choosen solvent.

Less polar solvents, such as ethyl acetate, are suitable to extraction of lipophilic phenols, found in abundance in the avocado seed also selective for flavonoids extraction (Alothman, Bhat & Karim, 2009). Although more polar solvents are effective for extraction of polyphenols with antioxidant activity, less polar solvents are used when it is necessary to reduce the extract toxicity for cellular studies aiming human use, such as ethanol instead of methanol to obtain the crude extract (Saha, Debnath, Saha & Sarkar, 2011).

Other studies demonstrated that avocado seeds are rich in a complex mixture of polyphenolic compounds, including flavonoids and tannins. Among them, some have already been isolated and identified, ranging from the simple substances, like (+)-catechin and (-)-epicatechin, to highly polymeric substances which are the most abundant, such as proanthocyanidin and procyanidin A trimers, caffeoylquinic acid derivatives, besides flavonol monomers, epicatechin gallate and others hydroxycinnamic and hidroxibenzoic acids derivatives (Dabas, Shegog, Ziegler & Lambert, 2013; Kosińska et al., 2012; López-Cobo et al., 2016; Melgar et al., 2018).

Our results suggest that the presence of polyhydroxylated polyphenols can enhance the antioxidant activity of avocado seeds. These substances have different redox mechanisms, highlighting their ability to donate protons and receive electrons,

stabilizing by resonance or stable functional groups formation (Procházková, Boušová & Wilhelmová, 2011).

In this regard, SEAP displayed better results for all antioxidant assays compared to SCE and SHP, and also to other plants used in natural medicine for the treatment of gastric diseases, such as acetonitrile:chloroform extract from *Baccharis trimera* (IC_{50} 52 µg/mL against $O_2^{\bullet-}$, IC_{50} 15,50 µg/mL against HOCl and IC_{50} 66.70 µg/mL against H_2O_2) (Nunes et al., 2016) and methanolic extract from *Euphorbia umbellata* (IC_{50} 20.85 µg/mL, against peroxidase) (Minozzo et al., 2016).

For H_2O_2 inhibition assay two different assays was conducted to verify if the samples capture directly the radical or if they inhibit peroxidase enzyme. The fractions showed better peroxidase inhibitory effect instead supposed capture of radical, highlighting SEAP that obtained similar result when compared to trolox. These results are in agreement the study of Vellosa (2008), which observed that the inhibition by some flavonoids should not be due to the capture of H_2O_2 but by enzyme inhibition.

Patients infected with *H. pylori* also present high expression of inducible nitric oxide synthase (iNOS) which generates NO, an important pro-inflammatory mediator involved in exudation and leukocyte migration, able to react with $O_2^{\bullet-}$ to form peroxynitrite, a powerful and toxic oxidant that induces oxidative DNA damage. The expression of iNOS is closely related to the up-regulation of nuclear factor *kappa* B (NF-κB) pathway, activated in response to extracellular stimulus such as LPS. Besides leading to generation of reactive species, NF-κB is responsible for the regulation of the expression of a variety of genes that encode inflammatory cytokines, such as TNF-α and IL-6 (Butcher et al., 2017; Naito & Yoshikawa, 2002).

The cytokine TNF-α is mainly produced by monocytes, macrophages and lymphocytes, acting in the recruitment of pro-inflammatory cells and stimulating the production and release of cytokines, as IL-6. The IL-6 is chemotactic for neutrophils and mononuclear cells and starts the inflammatory cascade activation in the gastric mucosa, implicated in chronic active gastritis and gastric cancer development. Elevated levels of these cytokines and reduced IL-10 (anti-inflammatory) in *H. pylori* infections are strongly associated with a higher risk of developing chronic gastritis and cancer (Algood, & Cover, 2006; Sánchez-Zauco et al., 2017).

SEAP at 100 µg/mL was able to modulate $O_2^{\bullet-}$ and NO production both by chemical interaction and cellular pathway and also showed strongly inhibition of IL-6

production, even though did not have good results of TNF-α inhibition and IL-10 induction. In addition to direct inhibition of these pro-inflammatory mediators, it is possible that the phenolic compounds of SEAP regulate different pathways that produce these factors, which in excess, lead to chronic inflammation that results in cancer. Other studies have reported the ability of different procyanidins and flavonoids, which can also be found in avocado seed, to suppress iNOS expression (Au, Al-Talib, Au, Phan, & Frondoza, 2007), inhibit NADPH oxidase activity (3'-*O*-methyl epicatechin, procyanidin B2, (-)-epicatechin glucuronide and vanillic acid) (Steffen, Gruber, Chewe, & Sies, 2008), inhibit activation and regulation of the transcription factor NF-κB by different mechanisms (epigallocatechin gallate, epigallocatechin and procyanidin trimer) and also the constitutive activation of mitogen-activated protein kinase (MAPK proteins) (Terra et al., 2007, 2011).

The benefits of phenolic substances in avocado seeds go beyond their effectiveness against oxidative burst, since they also inhibit other inflammatory pathways such as immunomodulatory response, inhibiting pro-inflammatory mediators that increase inflammation and may lead to gastric cancer (Aravindaram & Yang, 2010; Du, Li, Zhang, Wang & Zhang, 2018; Lesjak et al., 2018).

The fractions have also shown to be efficient against gastric adenocarcinoma cells at concentrations which macrophage viability was maintained. Even though the lowest value of CI_{50} was for SHP, we emphasize that concentrations above 50 µg/mL produces low viability on macrophages. Therefore, SEAP may be considered more viable for human use as antitumor, since it did not affect viability at higher concentrations and has demonstrated better antioxidant and immunomodulatory activity. In a similar way, Ding et al. (2007) reported the antitumor activity of several compounds isolated from avocado (polyphenols, steroids, triterpenoid and tannins) by different mechanisms such as selective induction of cell cycle arrest, growth inhibition and induction of apoptosis in pre-cancer and cancer cell lines. Besides acting inhibiting tumor growth, it is important to highlight that natural products could be used as adjuvant cancer therapies due to its antioxidant activity, which prevents DNA attack by reactive oxygen species, blocking the initial genetic modification step of carcinogenesis preventing the development of primary tumors (Aravindaram & Yang, 2010).

The effect of the seeds from *P. americana* against *H. pylori* was evaluated, and our results suggests that it can be an alternative to its treatment. Phenolic

derivates, such as catechin, catechin-derived procyanidins, quercetin and gallic acid have shown potent anti-*H. pylori* properties for different mechanisms. The active substances in mokdanpi extract, gallic acid and catechin, showed protective effects against *H. pylori* and gastric cancer (Jung, Bae & Jeong, 2013). Pastene et al. (2010) reported that an apple peel polyphenol-rich extract (APPE, 24% procyanidins) exerted a dual anti-*H. pylori* effect, inhibiting the bacteria adherence process in the gastric mucosa and the activity of vacuolating cytotoxin A (VacA). In addition, according to Rempe, Burris, Lenaghan and Stewart (2017), quercetin can inhibit *H. pylori* by cell membrane disruption, DNA intercalation, DNA gyrase inhibition, Type III secretion inactivation, dehydratase inhibition (HpFabZ) and protein kinase inhibition.

Electron microscopy evidenced that *H. pylori* treated with amoxicillin showed the majority of cells with blebs and coccoid (sporulated) forms. These changes are atributted to the cell wall damage during the conversion of helical to cocoide form, as a result of covalent interaction of this β-lactam with different PBPs (Penicillin Binding Proteins), a set of enzymes involved in the synthesis of the peptidoglycan layer of the bacterial cell wall, more specifically PBP 1 (66-kDa) in coccoid form and PBPs 3 (60-kDa) and 2 (63-kDa) in helical form (Deloney & Schiller, 1999; Damasceno, Rodrigues, Gonçalves & Kitagawa, 2017). Similar changes were found in the treatment with SHP in both strains, suggesting that this fraction may act similarly to amoxicillin on PBPs, destabilizing the cell structure and destroying the membrane as seen with membrane blebbing and conversion to cocoide form. On the other hand, filamentation was the dominant altered morphology observed in the treatment with SEAP, also observed for the antimicrobial aztreonam (Deloney & Schiller, 1999; Dús et al., 2013). Its mechanism of action can be directly associated only with the interaction with PBP2, essential in the helical form and related in septum formation, making it difficult to separate cells during cell division.

The *H. pylori* infectious process is very complex and its treatment requires intervention in different ways to prevent it from intensifying and leading to cancer, which makes it difficult to find effective medicines. In summary, all these results show the effectiveness of seeds from *Persea americana* Mill. in combating and preventing mechanisms related to the gastric inflammatory process, based on its anti-*Helicobacter pylori* property and antitumoral gastric activity with the absence of toxicological effects. Associated with these effects, avocado seeds contain powerful antioxidants that help the immunomodulatory protection and can be an alternative in

the treatment of gastric diseases. However, further studies of these extracts are required to properly identify the mechanisms in which seeds of avocado exert your antiinflammatory and antitumoral effects.

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Conflicts of interests: The authors declare no conflicts of interests.

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Capítulo 2

Artigo 2: Avocado seeds (*Persea americana* Mill.) prevents indomethacin-induced gastric ulcer in mice

Original Article

Avocado seeds (*Persea americana* Mill.) prevents indomethacin-induced gastric ulcer in mice

Brena Ramos Athaydes[a], Gisele Maziero Alves[b], Arícia Leone Evangelista Monteiro de Assis[c], João Victor Dutra Gomes[e], Ricardo Pereira Rodrigues[a], Bianca Prandi Campagnaro[b], Thiago de Melo Costa Pereira[b,d], Breno Valentim Nogueira[c], Dâmaris Silveira[e], Ricardo Machado Kuster[f], Rodrigo Rezende Kitagawa[a], Rita de Cássia Ribeiro Gonçalves[a]*

[a]Graduate Program in Pharmaceutical Sciences, Department of Pharmaceutical Sciences, Federal University of Espirito Santo, Vitoria, ES, Brazil.

[b]Graduate Program in Pharmaceutical Sciences, Vila Velha University (UVV), Vila Velha, ES, Brazil.

[c]Laboratory of Cellular Ultrastructure Carlos Alberto Redins (LUCCAR), Department of Morphology, Federal University of Espirito Santo, Vitoria, ES, Brazil.

[d]Federal Institute of Education, Science and Technology (IFES), Vila Velha, ES, Brazil.

[e]Health Science College, University of Brasilia (UnB), Brasília, DF, Brazil.

[f]Graduate Program in Chemistry, Department of Chemistry, Federal University of Espirito Santo, Vitoria, ES, Brazil.

*Corresponding author: Department of Pharmaceutical Sciences, Federal University of Espirito Santo, Av. Marechal Campos, 1468, CEP 29043-900, Maruipe/ES, Brazil;
E-mail address: rita.goncalves@ufes.br

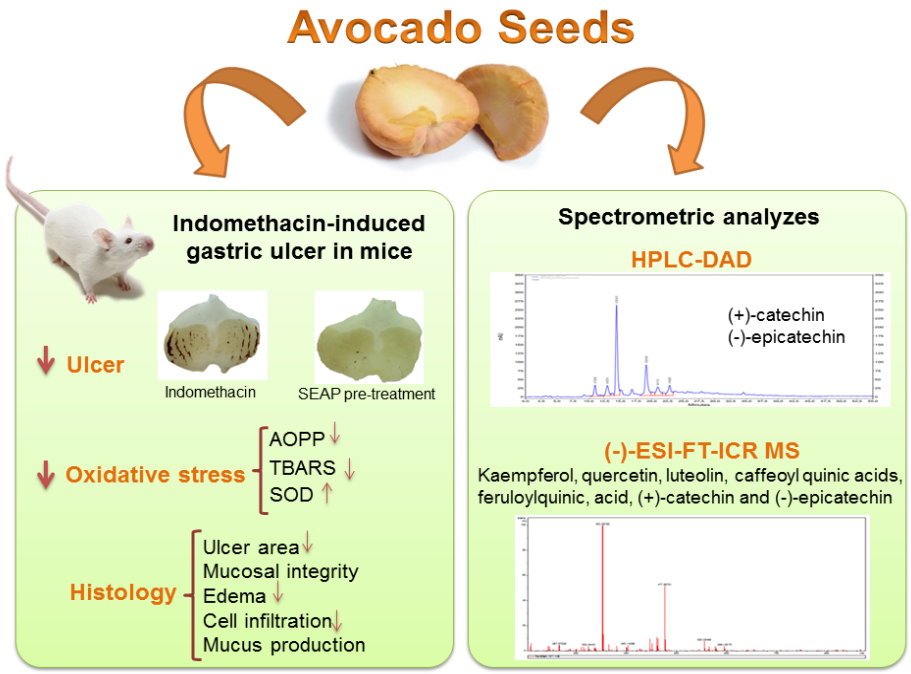
Abstract

The long-term use of anti-inflammatory is the most common cause of gastric ulcer disease, one of the major gastrointestinal disorders affecting people worldwide. *Persea americana* Mill. (avocado) seed is a by-product generally discarded as waste, but can be used to treat gastric disorder due to its anti-inflammatory, antioxidant and antimicrobial activities. The aim of the present study was to evaluate the potential protective effects of the ethyl acetate fraction of avocado seeds (SEAP) extracts against indomethacin-induced gastric ulcer in mice. It was found that SEAP in all tested doses (10, 35 and 75 mg/kg) were effective in mitigating oxidative stress through a decrease on the oxidized products levels and increasing SOD activity, also preventing the rise in ulcer and lesions areas and histological changes induced by indomethacin. Chemical analysis using (-)-ESI-FT-ICR MS revealed the presence of (-)-epicatechin and (+)-catechin, confirmed by HPLC-DAD, and other important phenolic compounds in avocado seeds, such as caffeoylquinic acid, flavonoids, phenylpropanoids and tannins, substances that promote inhibition of pathways involved in gastric ulcer formation. Thus, avocado seeds extract may be a suitable natural source for the prevention and treatment of gastric ulcer.
Keywords: Antioxidants, Anti-ulcer Agents, Avocado Seeds, Indomethacin, *Persea americana*, Polyphenols, Stomach ulcer.

Highlights:

- Avocado seeds prevent gastric ulcers and mucosal injury induced by indomethacin.
- SEAP prevents oxidative stress by increasing SOD levels and decreasing MDA and AOPP.
- The antioxidant activity is due to its high polyphenols content.

Graphical Abstract:



1. Introduction

Gastric ulcer is a common digestive disorder caused by an imbalance between aggressive factors (gastric hydrochloric acid, pepsin, reactive free radicals and oxidants) and defensive mechanisms (mucus barrier, bicarbonate, mucosal blood flow and others) present in the gastric mucosa. Oxidative stress, alcohol intake, *Helicobacter pylori* infection and the chronic use of medicines such as non-steroidal anti-inflammatory drugs (NSAIDs) are relevant etiological factors for the development of stomach ulcers (Farzaei, Abdollahi, & Rahimi, 2015; Yuan, Padol, & Hunt, 2006).

NSAIDs are one of the most frequently used drugs in clinical medicine worldwide, comprising approximately 30 million people using it and this number is increasing due to the widespread existence of pain, inflammation and pyrexia, which require their frequent administration (Drini, 2017; Harirforoosh, Asghar, & Jamali, 2013). Indomethacin is one of the most widely used NSAIDs worldwide. Unfortunately, since the COX enzyme is involved in maintenance of integrity of gastric mucosa, mucus secretion and increment of mucosal blood flow, the use of NSAIDs may develop gastric ulcer, culminating with leukocyte endothelium interaction, neutrophils infiltration and oxidative stress (Drini, 2017; Suleyman, Albayrak, Bilici, Cadirci, & Halici, 2010; Utsumi et al., 2006), characterized by the

excessive production of reactive oxygen species (ROS) whereas the antioxidant parameters are reduced. ROS lead to a higher level of DNA and protein oxidation and lipid peroxidation, that have a destroying effect on the integrity of biological tissues, mediating gastric injury, as well as the inflammatory process (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014; Lee, Cheng, Lee, & Chu, 2017; Suleyman et al., 2010).

Although currently available medicines against gastric ulcers are effective, most of these drugs exhibit several side effects, and depending on the cause, the therapy can be long and expensive (Yuan et al., 2006). A large number of plants and their secondary metabolites with gastroprotective effect can be found in literature, being a valuable alternative to treat gastric ulcer (Awaad, El-Meligy, & Soliman, 2013). Due to its antioxidant effects, they are responsible for decreasing lipid peroxidation, protein and DNA damage, assisting in the prevention of inflammation that leads to gastric ulcers (Bi, Man, & Man, 2014).

Persea americana Mill. (Lauraceae), commonly known as avocado, is a native plant from Mexico and Central America and can be found in almost all tropical countries. Avocado is mainly consumed as a fresh fruit because of its well-established benefits. Most of the chemical and bioactivity studies are focused on the pulp and little is known about the avocado seed, which can also be of great interest due to its anti-inflammatory (by decreasing the generation of pro-inflammatory mediators IL-6 and PGE_2), anticancer, antimicrobial, antihypertensive and antioxidant effects, activities described by other authors (Dabas, Shegog, Ziegler, & Lambert, 2013a), but no information is currently available about the gastroprotective effects of *P. americana* seeds extract. Several chemical characterizations evidenced a large amount of polyphenols, such as catechins, procyanidins and others tannins, flavonols, triterpenes, lipids and fatty acids in avocado seeds (Dabas et al., 2013; Kosińska et al., 2012).

Combining pharmacological and analytical studies improves the understanding of the use of medicinal plants and their possible therapeutic and adverse effects. Therefore, the aim of the present study was to investigate the polyphenols contents and the gastroprotective effectiveness of seeds from *Persea americana* Mill. ethyl acetate partition (SEAP) in the indomethacin-induced acute ulcer model. We determined the effect of SEAP on protein oxidation and lipid peroxidation levels, as well as on superoxide dismutase (SOD) activity, the potential of epithelial injury

prevention and mucus production, which are important parameters to identify the oxidative damages in the stomach tissue.

2. Materials and Methods

2.1 Botanical material and extract preparation

Seeds of *P. americana* Mill. was collected in Cariacica, Espirito Santo, Brazil, (20º22'43.2"S; 40°22'14.6"W) in March 2016, identified by Dr. Luciana Dias Thomaz, Department of Botany, Federal University of Espirito Santo, where the voucher specimen was deposited (VIES 38282). The seeds were grated and dried at 40ºC for 5 days. The hydroalcoholic extract was performed by turbolysis at 10% w/v with 70% ethylic alcohol. The resultant solution was filtered, submitted to evaporation at 50ºC under reduced pressure in a rotary evaporator until complete elimination of ethanol, fractionated with ethyl acetate and dried at room temperature.

2.2 Chemical compounds analysis

2.2.1 HPLC-DAD

The ethyl acetate seeds extract of *Persea americana* Mill. was solubilized in methanol (3 mg/mL), and 10 µL of this sample was analyzed using LaChrom Elite HPLC system (Hitachi®, Tokyo, Japan) liquid chromatograph equipped with auto-sampler L2200, L2130 pump, L2300 column oven was set at 25°C and a L2455 diodo array detector (DAD) (Hitachi®, Tokyo, Japan). The separation of SEAP was performed by reverse phase C-18 column (5 µm, 150 mm x 4.6 mm), in combination with an appropriate guard column (4.0 mm x 4.0 mm; 5 µm of particle size) (Merck®, Germany). The analysis was performed at a wavelength fixed at 280 nm.

The eluents used were aqueous phosphoric acid (1%) (solvent A) and acetonitrile (solvent B). The gradient employed was 90% A and 10% B for 0 min, 70% A and 30% B for 40 min, 50% A and 50% B for 50 min, 90% A and 10% B for 51 min, and 90% A and 10% B for 55 min at a flow rate of 0.6 mL/min. Data acquisition was performed using ExChrom Elite software (version 3.3.2 SP1) (Scientific Software Inc.). The compounds present in the extract were compared according to their UV–Vis spectra (230-400 nm) and retention times with commercial standards (Leite et al., 2014).

2.2.2 Identification of substances by ultra-high resolution and accuracy mass spectrometry (-)ESI FT-ICR MS

The ethyl acetate seeds extract of *Persea americana* Mill. was also analyzed in a mass spectrometer (Model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany), which was set to operate in negative ion mode, ESI(-), over a mass range of m/z 200–1300. The parameters of the ESI(-) source were as follows, nebulizer gas pressure of 0.5–1.0 bar, capillary voltage of + 3–3.5 kV, and transfer capillary temperature of 250°C. The mass spectrum was processed using the Compass Data Analysis software package (Bruker Daltonics, Bremen, Germany). A resolving power, $m/\Delta m_{50\%} \approx$ 500,000, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height of m/z ≈ 400 and a mass accuracy of <1 ppm, provided unambiguous molecular formula assignments for singly charged molecular ions (Freitas et al., 2013). Elemental compositions of the compounds were determined by measuring the m/z values. The unsaturation level of each molecule could be deduced directly from its double bond equivalent (DBE), following the equation DBE = c - h/2 + n/2 + 1, where c, h, and n are the numbers of carbon, hydrogen, and nitrogen atoms, respectively.

2.3 Experimental animals

Male Swiss albino mice (8 weeks, 25-35 g) provided by the “Laboratório de Acompanhamento Experimental” of Vila Velha University (UVV) were used to evaluate the gastroprotective activity. The animals were maintained under standard laboratory conditions of 12 h light/dark cycle and controlled temperature (~23°C), with free access to food and water. Fasting of food (18 h) was used prior to the experiments since standard drugs or extract was administered exclusively orally (by gavage). Moreover, the animals were kept in cages with raised floors to prevent coprophagia. The number of animals and intensity of ulcerogenic agents were the minimum necessary to demonstrate consistent results. All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory animals as recommended by the National Institutes of Health (NIH 85-23), and the study protocols were approved by the Institutional Animal Care Committee (Protocol #433 /2017 – CEUA UVV).

2.4 Indomethacin-induced gastric lesions and experimental groups

The experiment was performed according to the method of Djahanguiri (1969) and Pereira et al. (2017) with slight modifications. Mice were randomly divided into six groups of five animals each to receive vehicle (distillated water, control), indomethacin 40 mg/kg, lansoprazole 30 mg/kg (Boyacioglu et al., 2016) or SEAP (10, 35, 75 mg/kg) by oral gavage. Thirty minutes later, the mice of lansoprazole and SEAP groups received indomethacin by gavage (40 mg/kg, ulcerogenic agent). After six hours, animals were anesthetized and euthanized with sodium thiopental overdose (100 mg/kg, i.p), then thoracotomy was performed. Blood was collected by cardiac puncture in the right ventricle and transferred to tubes flushed with heparin. Blood samples were centrifuged at 3500 rpm for 15 minutes, the plasma separated and freezing in -80°C for biochemical analysis. The stomachs were rapidly removed, opened along the greater curvature and gently rinsed with 0.9% saline solution for assessment of ulcerative lesions as described below.

2.5 Macroscopic analysis and calculation of ulcerative lesions areas

Once opened, the stomachs were placed between two glass slides with graph paper and lightly pressed for macroscopic analysis. Stomach images were captured and analyzed by the software ImageJ® version 1.50b. The lesion index was expressed in percentage, according to the formula:

Ulcerative lesions (%) = (ulcerative area [mm^2])/(total area [mm^2]) × 100

Data are presented in variation of the lesion in relation to the control group, which corresponds to factor 1.0 (Szelenyi & Thiemer, 1978). After macroscopic analysis, the right side of the stomach was used to prepare the homogenate and the left side preserved in 10% formaldehyde buffer solution for histological analysis. The stomach tissues were homogenized in 0.5 mL of ice-cold phosphate buffer (0.1 M, pH 7.4) with a Turrax homogenizer (UltraStirrer, ULTRA80) and centrifuged at 3500 rpm, 4°C for 10 min. The supernatants were removed and stored at -80ºC for biochemical analysis.

2.6 Histological analysis and mucin content determination

The stomach tissues (*n* = 5 from each group) were diaphanized in xylol baths and the material was embedded in paraffin. The resulting tissue blocks were cut into histological sections (2.5 µm) and placed on microscope slides. Two slides were

prepared from each sample containing three consecutive cuts, one stained with hematoxylin/eosin (HE) and the other with HE and Periodic Acid-Schiff (PAS) for staining mucin-like glycoproteins in stomach, analyzed with an optical microscope Olympus AX70 using 20x and 40x objectives with Zeiss camera image acquisition system (AxioCam ERc5S model, Oberkochen, Germany).

The histological images were captured, saved and analyzed by an unbiased examiner without previous information about the groups. All the sections were classified according to four scores (score 0 – without ulcer or tissue changes; score 1 – superficial tissue changes; score 2 – about half tissue with architectural/cellular changes; score 3 - advanced changes across tissue thickness (Minozzo et al., 2016). The data were plotted as ulcerative lesions (%) and calculated by fold-variation in relation to control group. To assess the level of gastric mucin, 10 different fields of gastric lesions per animal were randomly used to calculate the average percentage of stained area and calculated with the software ImageJ® with 40x objective.

2.7 Determination of Advanced Oxidation Proteins Products (AOPP)

AOPP analysis were performed according to Witko-Sarsat et al. (1998), with modifications. The plasma and stomach homogenates were diluted 1:10 for the experiment. Briefly, 40 µL of sample and 160 µL of phosphate-buffer (0.1 M, pH 7.4) or chloramine-T standard solutions (0 to 100 µM), 10 µL of potassium iodide (1.16 mol/L, KI) and 20 µL of glacial acetic acid were added in each well of 96-well microplate and stirred for six minutes. The reading was performed in ELISA iMark® Absorbance Reader (BioRad, Washington, USA) at 340 nm against a blank containing 200 µL of phosphate-buffer, 10 µL of KI and 20 µL of acetic acid. The AOPP content was determined based on the standard chloramine-T linear curve with correlation coefficient greater than 0.95. The results were expressed as µmol/mg protein, previously quantified by the Bradford method (Bradford, 1976).

2.8 Determination of lipid peroxidation (TBARS)

The thiobarbituric acid reactive substances was determined on the basis of the method by Buege and Aust (1978) to analyze the degree of lipid peroxidation, as indicated by the amount of malondialdehyde (MDA) generated and spectrophotometrically detected through the formation of a chromogen at 532 nm. In duplicate, 100 µL of plasma or stomach homogenate was mixed with 20 µL of 10%

SDS in microtubes and 250 µL of the color reagent (thiobarbituric acid 0.037% + trichloroacetic acid 15% + hydrochloric acid 0.25 M). The mixture heated at 95ºC for 15 minutes in dark state and cooled for 5 minutes. After this period, the microtubes were centrifuged at 3500 rpm at 4°C, and the supernatant was placed in a 96-well microplate and read at 540 nm using a microplate reader iMark® Absorbance Reader (BioRad, Washington, USA) against the blank (mixture without sample and the color reagent). The standard curve was performed from TBARS Assay kit (Cayman Chemical, Michigan, USA) and MDA levels were expressed in µM.

2.9 Superoxide dismutase activity (SOD)

The superoxide dismutase activity was evaluated by the SOD Determination Kit (Sigma-Aldrich®). This kit uses a superoxide, xanthine and xanthine oxidase anion generation system and evaluates the ability of the test solution to inhibit the superoxide anion reaction with WST (2-(4 iodophenyl)-3-(4-nitrophenyl)-2H-5-tetrazolium). The reaction forms the formazan compound, with color intensity read at 450 nm after incubation for 20 min at 37°C. The results were expressed as amount of SOD (UI/mL) from the standard curve.

2.10 Statistical analysis

The values were expressed as mean ± SEM. Differences observed between the doses were achieved by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test and values $p < 0.05$ were considered significant. Statistical analyses were carried out using the software GraphPad Prism version 5.0.

3. Results and Discussion

The present study shows the antiulcerogenic activity of the ethyl acetate partition from *Persea americana* Mill. extract, a rich source of phenolic compounds, in acute gastric lesions induced by indomethacin, as well as its influence on gastric acid secretion parameters, such as mucin, and in markers related to oxidative damage, such as protein oxidation, lipid peroxidation and superoxide dismutase.

Farzaei et al. (2015) and Awaad et al. (2013) described the importance of polyphenols as bioactive molecules with potential to be applied in the management of

peptic ulcer with protective actions that prevent the ulcer development process by promoting cytoprotection, re-ephitelialization and suppressing oxidative damage due to its antioxidant properties, since they reverse damage caused by the imbalance between redox defense system.

We have observed a high level of polyphenolic compounds (Figure 1) in SEAP. Table 1 shows the pseudo-molecular ions $[M-H]^-$, molecular formula, measured m/z values, DBE, and mass error of several molecules in SEAP. They were generated as negative ions due to the major presence of phenolic compounds in avocado seeds. To each peak are associated an exact molecular formula and some important values such as error, double bound equivalent and signal intensity. A low value for the error represents greater accuracy of the molecular formula. Equivalence in double bonds (DBE) shows the amount of cycles and unsaturations present in the molecule. The intensity of the signal represents how acidic a molecule is compared to another one. In this context, at similar concentrations in the extract, carboxylic acids compared to phenolic compounds will have more intense peaks since they are stronger acids.

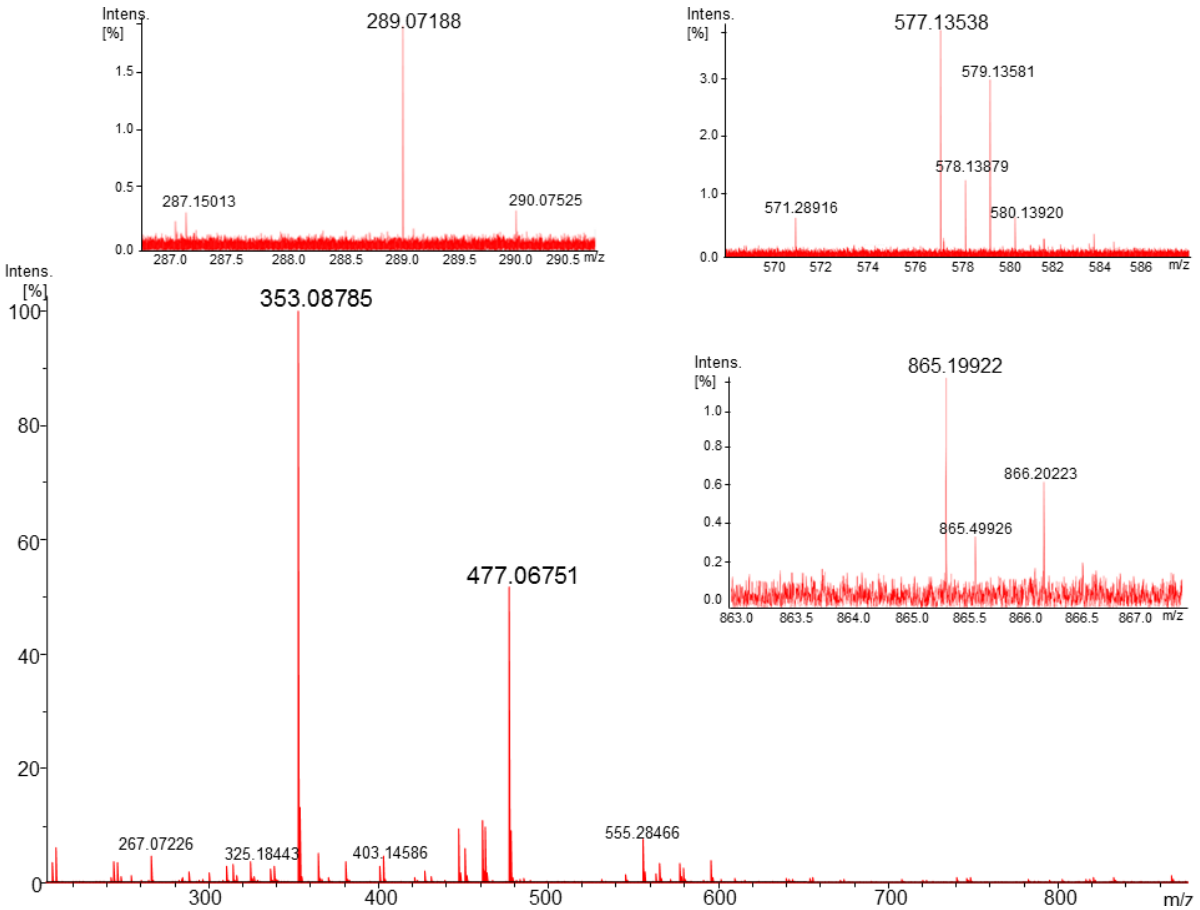


Figure 1 - (-)-ESI FT-ICR MS spectra of *Persea americana* Mill. seeds extract.

Table 1: Accurate mass data for proposed substances or class of substances for *Persea americana* Mill. seeds extract using (-)-ESI-FT-ICR MS.

$[M-H]^-$	Molecular Formula	Error (ppm)	DBE	Signal Intensity (%)	Proposed Substance or Class of Substance
209.06677	$C_7H_{13}O_7$	- 0.46	1.5	3.57	Carbohydrate
211.08242	$C_7H_{15}O_7$	- 0.44	0.5	6.16	Inositol
255.23307	$C_{16}H_{31}O_2$	- 0.45	1.5	1.35	Palmitic acid
279.23309	$C_{18}H_{31}O_2$	- 0.47	3.5	0.33	Linoleic acid
281.24866	$C_{18}H_{33}O_2$	- 0.21	2.5	0.25	Oleic acid
283.26439	$C_{18}H_{35}O_2$	- 0.49	1.5	0.34	Stearic acid
285.04062	$C_{15}H_9O_6$	- 0.54	11.5	1.03	Luteolin/ kaempferol
289.07188	$C_{15}H_{13}O_6$	- 0.41	9.5	1.96	Catechin/epi-catechin
301.03551	$C_{15}H_9O_7$	- 0.44	11.5	1.81	Quercetin
327.21784	$C_{18}H_{31}O_5$	- 0.44	3.5	1.01	Oxylipin
329.23343	$C_{18}H_{33}O_5$	- 0.25	2.5	0.53	Oxylipin
337.09305	$C_{16}H_{17}O_8$	- 0.47	8.5	2.42	*p*-hydroxycoumaroyl quinic acid
353.08785	$C_{16}H_{17}O_9$	- 0.12	8.5	100	Caffeoylquinic acid
364.99745	$C_{15}H_9O_9S$	- 0.47	11.5	5.22	Luteolin sulfate/kaempferol sulfate
367.10357	$C_{17}H_{19}O_9$	- 0.32	8.5	0.71	Feruloyl quinic acid
380.99233	$C_{15}H_9O_{10}S$	- 0.36	11.5	3.75	Quercetin sulfate
401.13022	$C_{14}H_{25}O_{13}$	- 0.40	2.5	2.95	Disaccharide
403.14586	$C_{14}H_{27}O_{13}$	- 0.37	1.5	4.53	Disaccharide
431.09855	$C_{21}H_{19}O_{10}$	- 0.42	12.5	1.09	Kaempferol rhamnoside or isomer
447.09344	$C_{21}H_{19}O_{11}$	- 0.36	12.5	9.54	Kempferol hexoside, luteolin hexoside, quercetin rhamnoside or isomers
451.10365	$C_{24}H_{19}O_9$	- 0.42	15.5	5.97	Catechin/epicatechin + Ph-C_3
461.0727	$C_{21}H_{17}O_{12}$	- 0.33	13.5	10.92	Kaempferol hexuronic acid or isomer
463.08836	$C_{21}H_{19}O_{12}$	- 0.34	12.5	9.79	Quercetin hexoside or isomer
477.06751	$C_{21}H_{17}O_{13}$	- 0.10	13.5	51.76	Quercetin hexuronic acid or isomer
577.13538	$C_{30}H_{25}O_{12}$	- 0.39	18.5	3.42	Catechin/epicatechin dimers (condensed tannin)
579.13581	$C_{26}H_{27}O_{15}$	- 0.45	13.5	2.68	Kaempferol disaccharide (hexose-pentose) or isomer
595.1307	$C_{26}H_{27}O_{15}$	- 0.41	13.5	3.80	quercetin disaccharide (hexose-pentose) or isomer
865.19922	$C_{45}H_{37}O_{18}$	-0.79	27.5	1.05	Catechin/epicatechin trimers (condensed tannin)

(-)-ESI-FT-ICR MS spectrum of SEAP shows the presence of peaks representing several classes of secondary metabolites such as flavonoids, phenylpropanoids and tannins. Carbohydrates and fatty acids were also present but as primary metabolites. Flavonoids represent the major group in terms of distribution of molecules, with several compounds, as shown in table 1. Kaempferol, quercetin, luteolin and their derivatives like glycosides and sulfates were found. Quercetin hexuronic acid/isomer was found at $[M-H]^-$ = 477.06751. The most intense peak of the spectrum $[M-H]^-$ = 353.08785 indicated the presence of caffeoyl quinic acids, such as chlorogenic acid and its isomers, important phenylpropanoids found in several medicinal plants and already found in avocado seeds (Dabas, Shegog, Ziegler, & Lambert, 2013b; Figueroa, Borrás-Linares, Lozano-Sánchez, & Segura-Carretero, 2018; López-Cobo et al., 2016; Melgar et al., 2018). Other phenylpropanoids, but with low signal intensity, were feruloylquinic acid isomers at $[M-H]^-$ = 367.10357. Precursors of condensed tannins were found at $[M-H]^-$ = 289.07188, (+)-catechin and (-)-epicatechin. These flavan-3-ols were confirmed by HPLC-DAD when the extract was compared to authentic standards. Retention times for the compounds were 14.4 min and 19.1 min, respectively (Figure 2). Dimers and trimers of these flavan-3-ols (condensed tannins) were detected by (-)-ESI-FT-ICR MS at $[M-H]^-$ = 577.13538 and 865.19922, respectively. The presence of these flavonoids in avocado seed, by the same analysis, has previously been described by other authors (Figueroa et al., 2018; Kosińska et al., 2012a; López-Cobo et al., 2016). These substances are found in other plants used in traditional medicine as antiulcer, as well as for the treatment of lesions caused by indomethacin (Awaad et al., 2013; Somensi et al., 2017).

Indomethacin is the NSAID of choice for this type of experiment due to its ulcerogenic potential, higher than the other NSAIDs. Its mechanism of action is by a non-selective inhibition of COX enzyme involved in the production of prostaglandins which are found to produce a gastroprotective effect not only via decreasing acid secretion, but also by increasing the gastric mucus level. Moreover, indomethacin increases oxidant parameters while decreasing antioxidant parameters and elicited both local and vascular mechanisms that promote extensively damage in the gastric mucosa (Suleyman et al., 2010). Therefore, this model is also well-accepted as oxidative stress-induced stomach disease, since the ulcers might be mediate by oxidative stress.

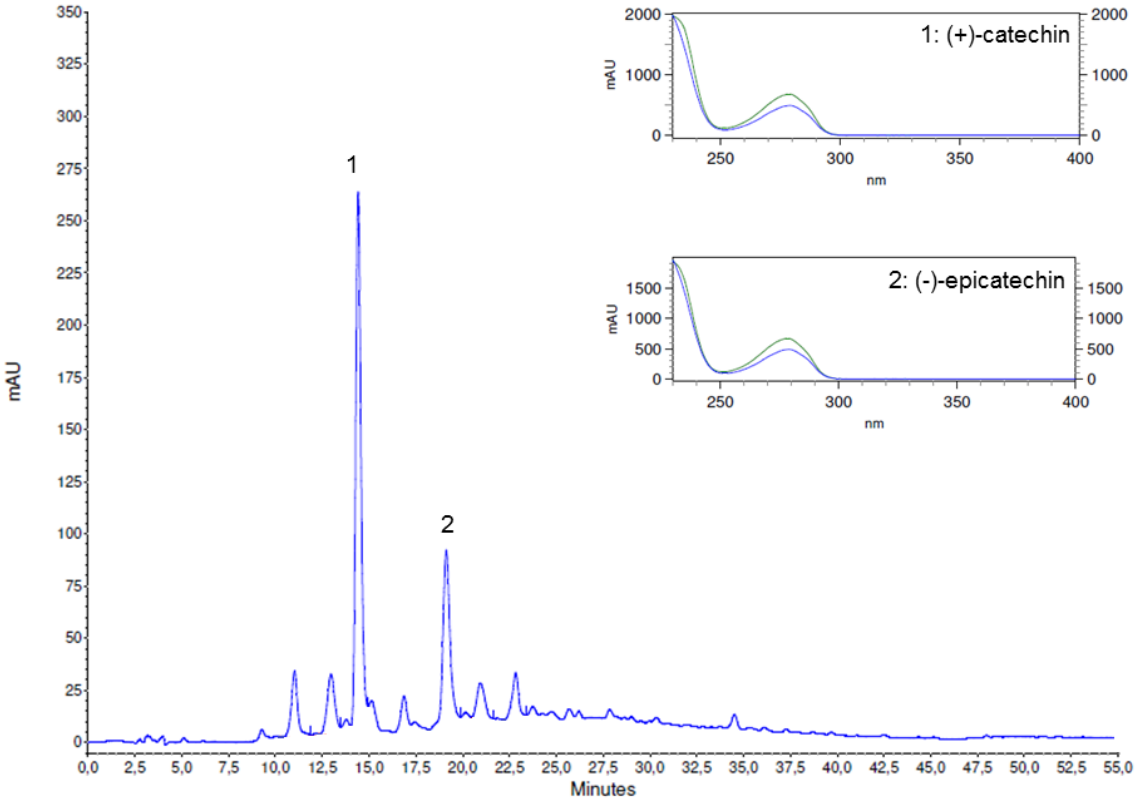


Figure 2 – HPLC-DAD chromatogram of *Persea americana* Mill. seeds extract.

Macroscopic analysis showed that pre-treatment with SEAP significantly reduced the total indomethacin-induced gastric lesion area in a dose-dependent manner, as observed in figure 3. The treatment with indomethacin induced multiple macroscopic lesions, with irregular sizes and shapes in the gastric mucosa of mice (18.73 ± 2.52 UI, ulcer indices). As expected, no lesions were detected in the control group mucosa. Interestingly, the animals treated with SEAP 10 (2.89 ± 1.75 UI), 35 (2.41 ± 1.24 UI) and 75 (1.51 ± 0.72 UI) mg/kg obtained superior results than the lansoprazole group (3.53 ± 1.50 UI), a classic proton pump inhibitor.

Histological analysis confirmed that pre-treatment with SEAP prevented indomethacin-induced histological damage in the superficial layers of the gastric mucosa with congestion by HE staining (Figure 4). In the control group, we observed the gastric epithelium with organized glandular structure and normal mucosa and submucosa, considered as score 0 (Figure 4A). The administration of indomethacin induced several evidences of gastric damage, such as disruption of the surface epithelium and significantly high necrotic lesions, which were associated with destruction of glandular architecture beyond loss or disorganization of the cellular

epithelium and inflammatory alterations, representing lesions with score 3 (Figure 4B). The presence of inflammatory sites results in mucosal edema, extensive infiltration by polymorphonuclear cells in the upper part of the submucosal layer and part of mucosa, release of oxygen metabolites and cell membrane peroxidation, which is consistent with previous reports (Blandizzi et al., 2005; Boeing et al., 2016; El-Ashmawy, Khedr, El-Bahrawy, & Selim, 2016; Pereira et al., 2017; Utsumi et al., 2006; Yadav et al., 2012).

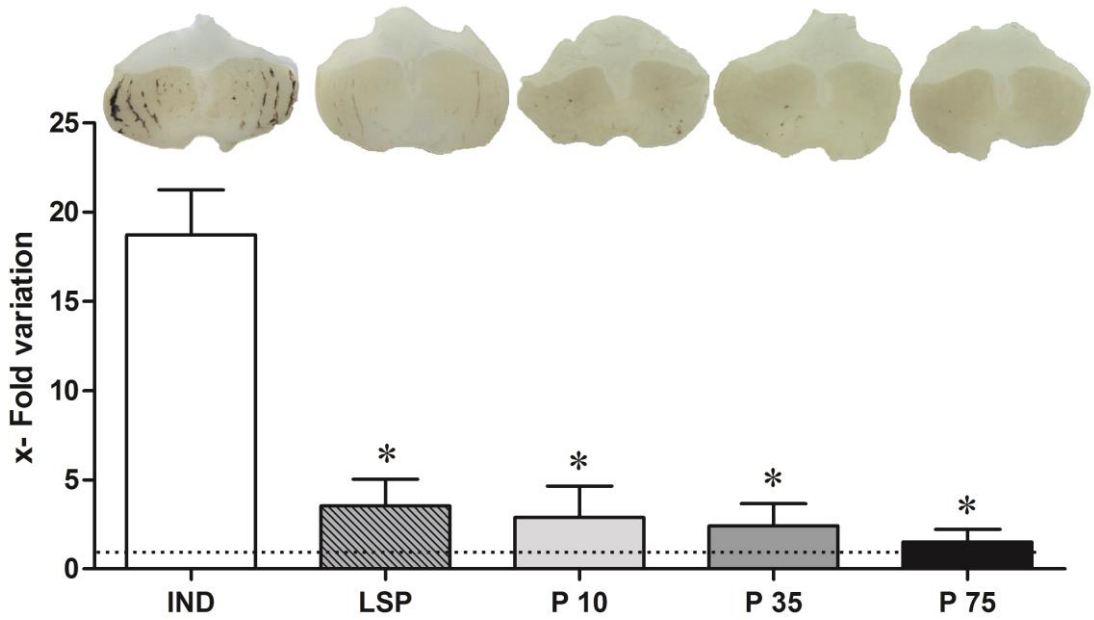


Figure 3 - Effect of seeds from *Persea americana* Mill. in gastric ulcers induced by indomethacin. At the top, macroscopic representative images of each group. The lesion scores are expressed as mean ± SEM of ulcer area (mm²). The bars represent the fold variation relative to control group (without treatment). Dashed line means the control group as a value of 1.0. IND: Indomethacin; LSP: Lansoprazole. * $p < 0.05$ compared to indomethacin group. $n = 5$ per group.

Although we confirmed that the pre-treatment with lansoprazole (score 1) protects the stomach against lesions induced by indomethacin, we observed shallow mucosal lesions on the gastric mucosa (Figure 4C). Interestingly, the pre-treatment with SEAP 35 (score 1) and 75 (score 1) mg/kg also maintained the integrity of the mucosa with a reduction in mucosal edema and leucocyte infiltration, as shown by the reduction or absence of the ulcer area in treated mice (Figure 4D-F). It also revealed a mild disruption of the surface epithelium in the lowest tested concentration (SEAP 10 mg/kg, score 1).

The production of mucus is an indicator of local gastric mucosal defense, which can be analyzed by Periodic Acid-Schiff stain. The mucus that is secreted onto

the surface of much of the stomach is composed mostly by mucin, a macromolecular glycoprotein that accelerates epithelial recovery and forms a mucoid layer that promotes tissue repair. The histochemical staining for mucin-like glycoproteins is shown on the right side of Figure 4.

Histological analysis showed that SEAP 10 mg/kg (Figure 4D) and 75 mg/kg (Figure 4F) significantly prevented this damage, with increased mucus production by the stomach mucosal cells in order to 2.36 ± 0.45 and 2.38 ± 0.55 times compared to the control, respectively (Figure 4G). However, lansoprazole was more effective in this protection mechanism, with an increase of 3.67 ± 0.33 times in relation to control. To better evaluate the therapeutic response of SEAP, further investigations should be carried out with new experiments with chronic treatment or in higher concentrations. For example, the crude ethanolic extract of *Vernonia condensata*, in a concentration of 300 mg/kg, increased the production of mucin in 119%, but in a chronic model (Boeing et al., 2016). The high tannin content present in SEAP can be related to this protection mechanism, since the interactions between tannins and biological macromolecules cause their precipitation over the mucosa, resulting in a mucoprotective barrier, an impenetrable layer to harmful agents since the created complex could act as a lipid peroxidation inhibitor (Jakobek, 2015).

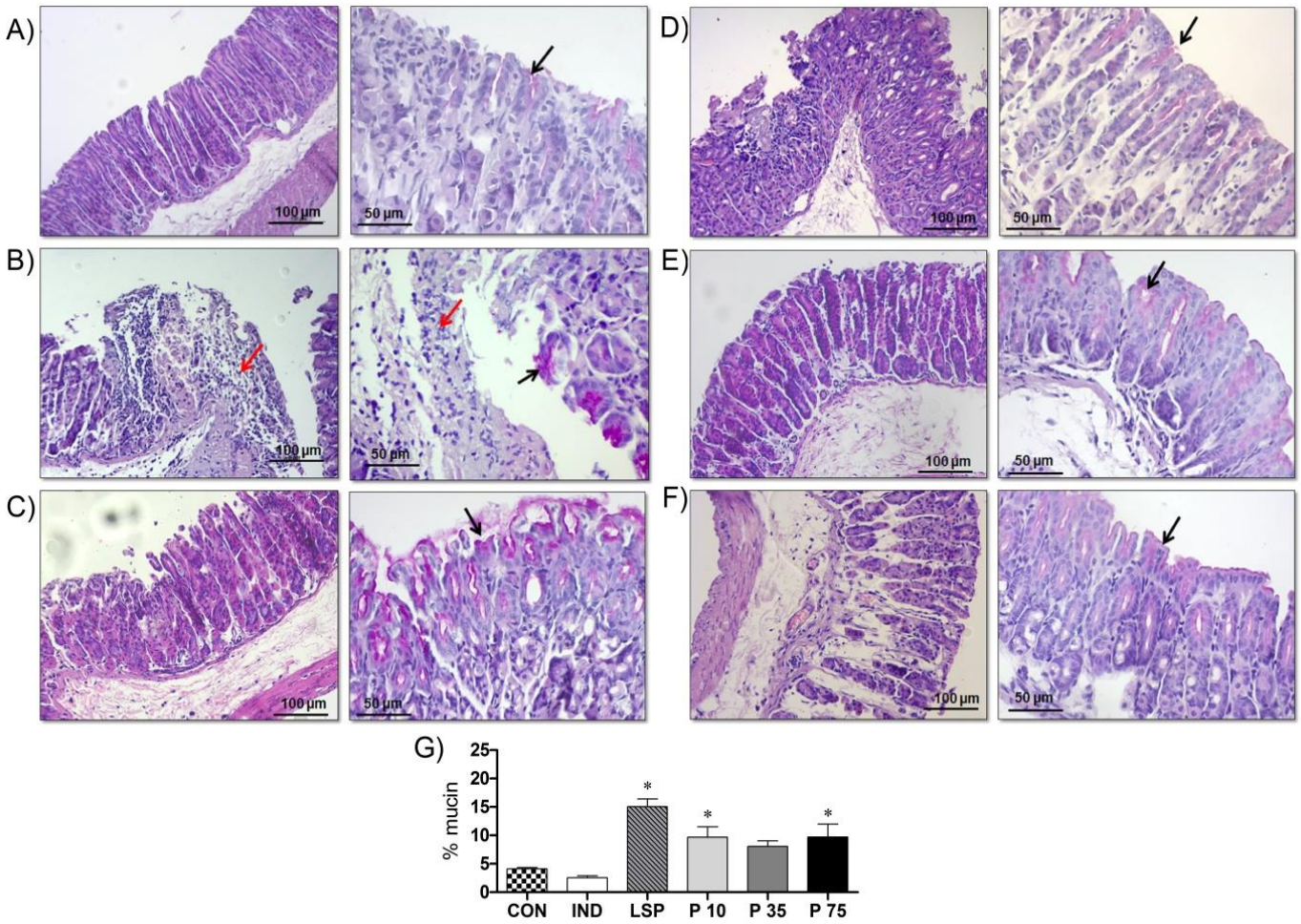


Figure 4 - Effects of *Persea americana* Mill. seeds on the histological evaluation of indomethacin-induced gastric mucosa damage in mice. Sections stained with hematoxylin and eosin (HE) on left side and sections stained with Periodic Acid-Schiff (PAS) on the right side. A) Control; B) Indomethacin - several polymorphonuclear cells can be evidenced (red arrow); C) Lansoprazole; D) SEAP at 10 mg/kg; E) 35 mg/kg and F) 75 mg/kg; G) Mucin stained with PAS quantified by ImageJ® program. Black arrow: mucin. The results were expressed as mean ± SEM, $n = 5$ per group. * $p < 0.05$ compared to control group. Objective of 20x (scale bar: 100 µm) on left side and 40x (scale bar: 50 µm) on right side.

Utsumi et al. (2006) showed that the generation of ROS at the mucus layer on the interface or in the intracellular compartment of epithelial cells contributes to the development of indomethacin-induced ulcer. Oxidative damage mediates gastric mucosal injuries, such as ulceration, erosion and hemorrhage: hemodynamic changes caused by reactive species that, if untreated, may lead to gastric cancer. This pathogenic process is mediated by oxidized lipids and proteins, potentiated by the decreased activity of anti-oxidant factors, such as superoxide dismutase enzyme (Pérez, Taléns-Visconti, Rius-Pérez, Finamor, & Sastre, 2017; Suleyman et al., 2010).

In this way, we evaluated the biochemical parameters of inflammation on plasma and stomach homogenate through the determination of advanced products of protein oxidation and lipid peroxidation levels (by MDA marker) and quantification of the antioxidant enzyme SOD. All results are shown in Figure 5. Indomethacin (40 mg/kg) significantly increased mucosal and plasma oxidized protein levels (12.70 ± 5.68 µmol/mg protein). Pre-treatment with SEAP significantly reduced the accumulation of AOPP induced by indomethacin in plasma in a dose-dependent manner, presenting better results when compared to lansoprazole. But for the stomach homogenate, SEAP have significant results only at the concentration of 75 mg/kg (19.07 ± 8.53 µmol/mg protein), as shown in Figure 5A.

The production of membrane lipoperoxides was measured by MDA concentration, which is the final product of lipid peroxidation. MDA concentration was significantly reduced by SEAP 35 mg/kg, both in plasma and gastric tissue, with reduction of 90% and 61%, respectively, in relation to indomethacin group (Figure 5B). This result might be due to the reduction of oxidative gastric injury caused by oxygen radicals (Sreeja et al., 2018). According to Blandizzi et al. (2005), one of the antioxidant effects from lansoprazole (antiulcer reference) is to prevent MDA production in the mucosa, but in our study SEAP at 35 mg/kg obtained better results in reducing this marker.

One strategy of cell membrane protection is increase the activity of intracellular enzymatic antioxidants, such as SOD, since this enzyme catalyzes the dismutation of the superoxide radical (Bhattacharyya et al., 2014). The results are shown in Figure 5C. The plasma SOD levels were effectively increased in the SEAP 75 mg/kg group with a 4.25-fold increase (23.20 ± 12.73 UI/mL) compared to the indomethacin group (5.45 ± 2.44 UI/mL). Likewise, the treatment of indomethacin-induced ulcers with lycopene, the main antioxidant compound present in tomatoes (Boyacioglu et al., 2016) and a methanol extract of leaves from *Sphenodesme involucrata* (Sreeja et al., 2018) were effective to decrease the levels of ulcer index and lipid peroxide, and also increase antioxidant enzymes, such as SOD.

Antioxidants mechanisms of SEAP may be due to substances derived from catechin, the main family of phenolic compounds and the major compound found in avocado seeds (Melgar et al., 2018). Its gastric protective actions may be associated with the nitric oxide release reduction and may regulate the gastric acid secretion by the inhibition of gastric H+, K+ -ATPase enzyme (Baggio et al., 2007). The increase

of gastric mucus and its antioxidant activity (Hamaishi, Kojima, & Ito, 2006; Lee et al., 2017) contributes directly to the reduction of the oxidative stress, as well reducing paracrine/endocrine parameters involved in the gastroprotective effect, such as somatostatin, gastrin, and histamine (Sato, Matsui, & Arakawa, 2002). Likewise, polyphenols such as condensed tannins and flavonoid heterosides derivatives from quercetin and kaempferol presents in hydroalcoholic extract from bark of *Persea major* (Lauraceae) at 300 mg/kg prevented indomethacin induced gastric lesions in animal model by increasing mucin contents and reducing the oxidative and inflammatory parameters at the ulcer site as reported by Somensi et al. (2017), findings that corroborate with our results.

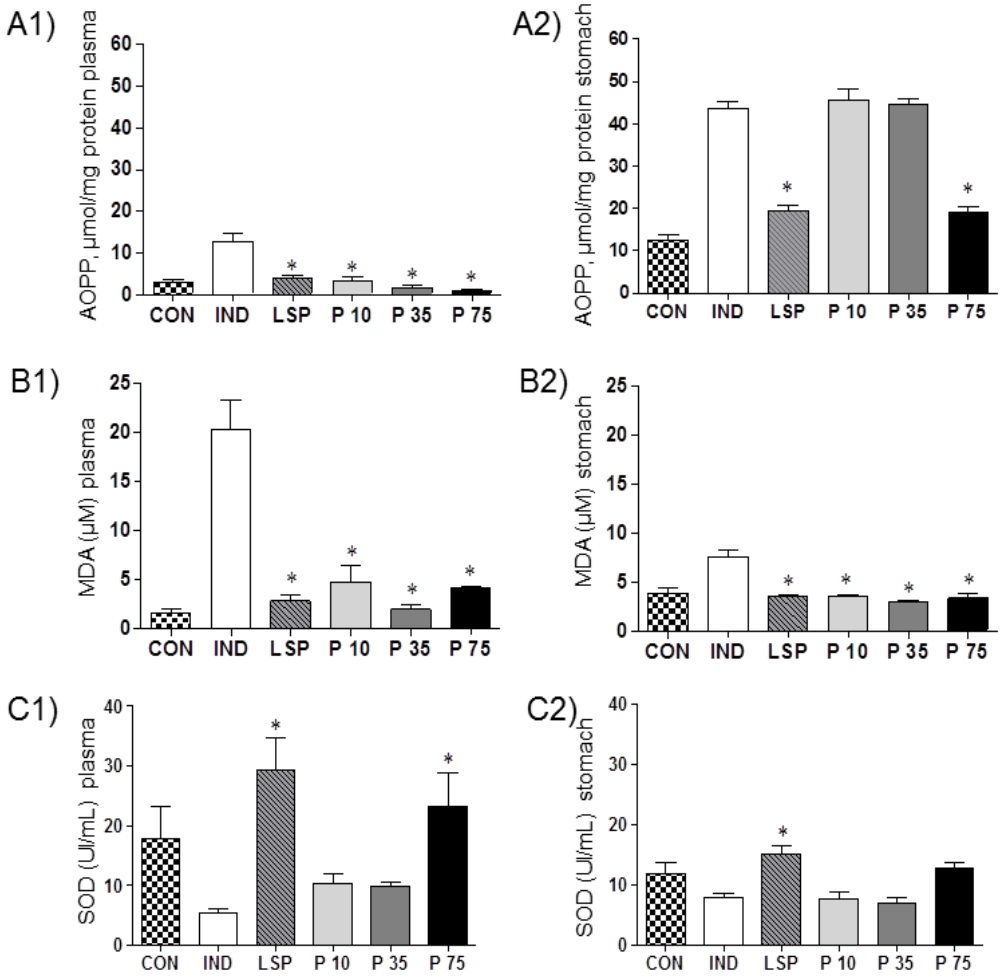


Figure 5 - Effects of *Persea americana* Mill. seeds on Advanced Oxidation Protein Products (AOPP) (A), lipid peroxidation products expressed as concentration of malondialdehyde (MDA) (B) and superoxide dismutase (SOD) (C), in plasma (1) and stomach tissue (2) from mice with acute gastric ulcers lesions induced by indomethacin. Each value represents the mean ± SEM of groups. CON: Control; IND: Indomethacin; LSP: Lansoprazole. * $p < 0,05$ compared to indomethacin group. $n = 5$ per group.

4. Conclusions

In summary, the gastroprotective activity of SEAP could be by the single or synergistic action of polyphenolic components. The mode of action in the gastroprotective activity of SEAP was in part, mediated by the increase of endogenous antioxidant enzymes (SOD) activity and decrease of oxidant factors or by its anti-inflammatory property, inhibiting the infiltration of inflammatory mediators like neutrophils and leucocyte and reducing edema in the gastric tissue, with influence on the increase of mucus production. The result described here highlights the use of avocado seeds as a valuable source to be used in the prevention of gastric mucosal injury induced by indomethacin. However, further studies need to be conducted on order to better understand the correlation between these phenolic compounds and its bioactivities for preventing gastric ulcers.

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Conflicts of interests

The authors declare no conflicts of interests.

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III. CONCLUSÕES GERAIS

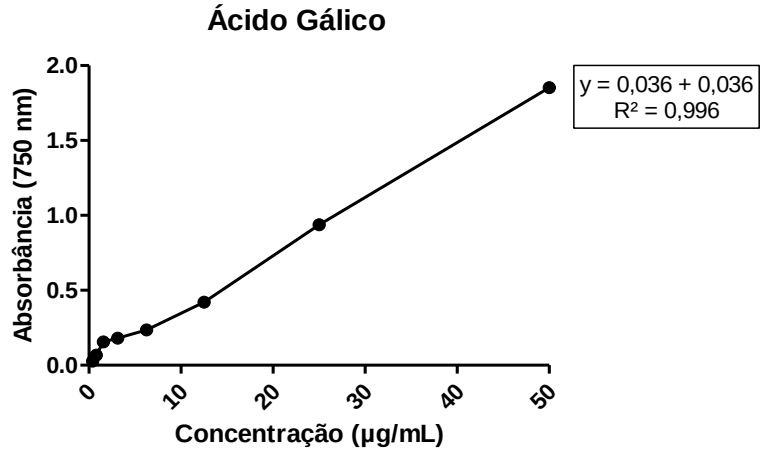
Nossos resultados mostram que a semente de *Persea americana* Mill., um resíduo do abacate que geralmente é descartado, possui efeitos gastroprotetores atuando por diferentes mecanismos. O extrato bruto e frações mostraram potencial antioxidante frente aos radicais artificiais ABTS•+ e DPPH•, frente ao ácido hipocloroso e inibiram a atividade da peroxidase, mas apenas a SEAP mostrou atividade inibitória do radical ânion superóxido, além de obter os melhores resultados antioxidantes.

A viabilidade celular em macrófagos murinos RAW 264.7 foi superior a 90% nas concentrações de 100 µg/mL no SCE e SEAP e na concentração de 25 µg/mL na SHP. Nessas concentrações, as frações foram capazes de inibir o ânion superóxido e óxido nítrico, além de reduzirem os níveis de TNF-α e IL-6 produzidos por macrófagos em resposta ao estímulo do LPS e também reduziram a viabilidade de células de adenocarcinoma gástrico humano. Além disso, as frações apresentaram melhores respostas inibitórias no crescimento de duas cepas de *Helicobacter pylori* (ATCC 43504 e 43629), sendo o principal mecanismo de ação da SHP a formação de bolhas e septação da parede celular e o aparecimento de formas cocoides, e na SEAP houve predomínio de filamentação bacteriana. Nenhuma das frações apresentou significativa atividade inibitória da enzima urease.

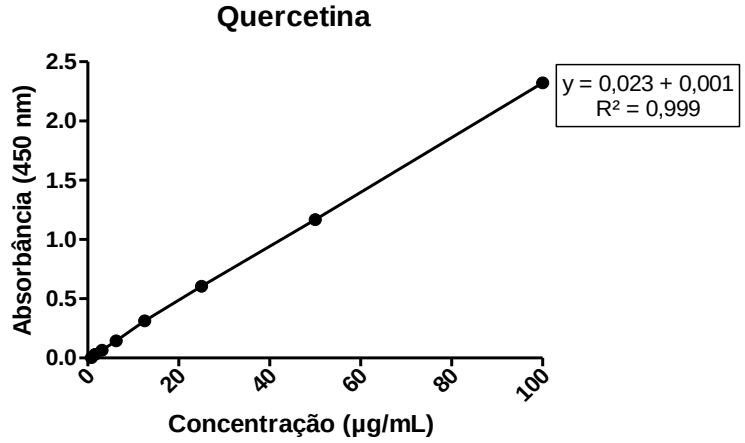
Com base nos resultados alcançados, as propriedades gastroprotetoras da SEAP dependem do seu alto conteúdo polifenólico, representado por flavonoides, fenilpropanoides e taninos. Esses compostos promovem efeitos protetores contra úlcera induzida por indometacina em modelo animal, dados confirmados por análise histológica, através da redução da profundidade e gravidade das lesões, com redução da infiltração de leucócitos e do edema submucoso de maneira dose-dependente. Além disso, SEAP evitou o estresse oxidativo provocado por indometacina através da redução dos parâmetros bioquímicos oxidantes (MDA e AOPP) e aumento da atividade da enzima antioxidante SOD. Esses dados podem abrir novas investigações do potencial gastroprotetor das sementes de *Persea americana* Mill. para possibilitar seu uso terapêutico.

APÊNDICES

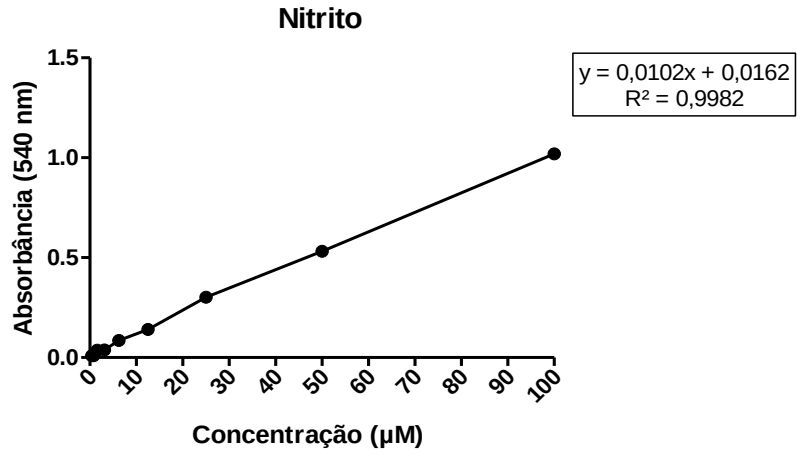
Curvas de calibração



Apêndice A: Curva de calibração do ácido gálico

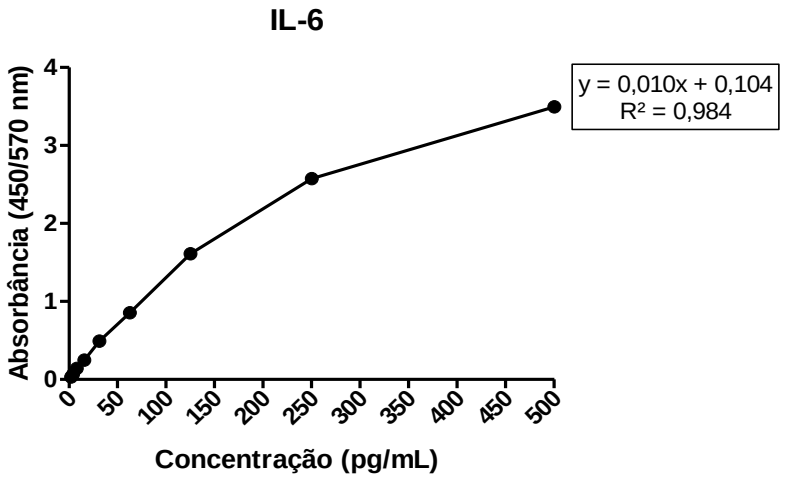


Apêndice B: Curva de calibração da quercetina

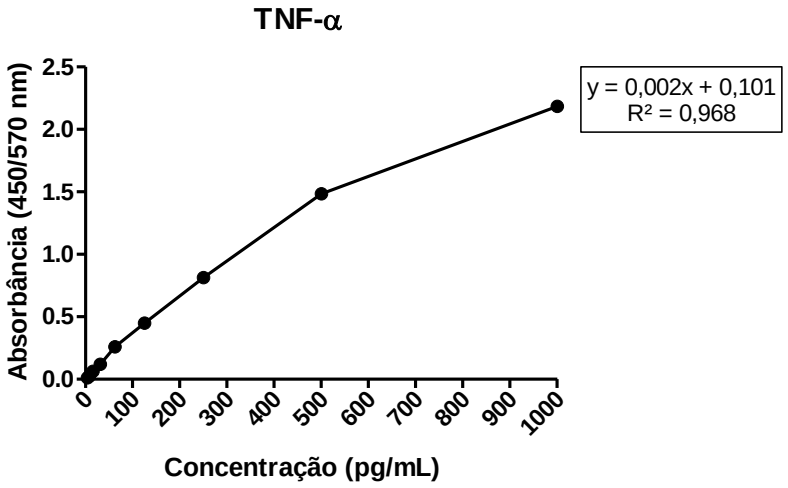


Apêndice C: Curva de calibração de nitrito ($NaNO_2$)

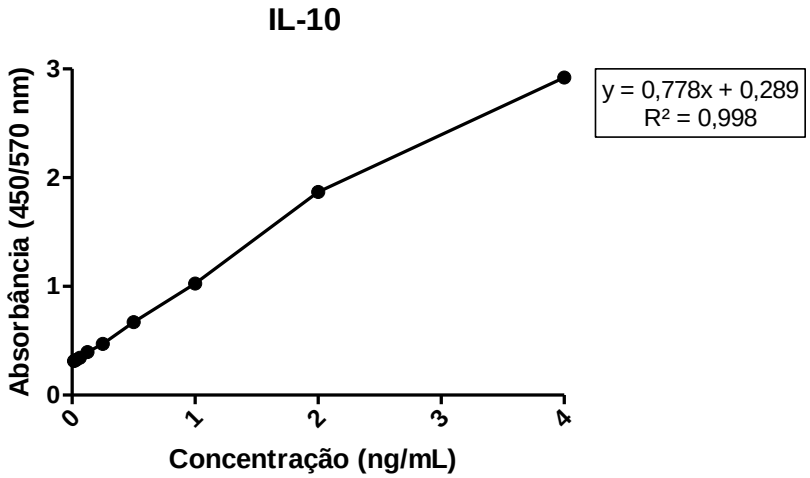
Curvas dos padrões das interleucinas



Apêndice D: Curva padrão da interleucina IL-6

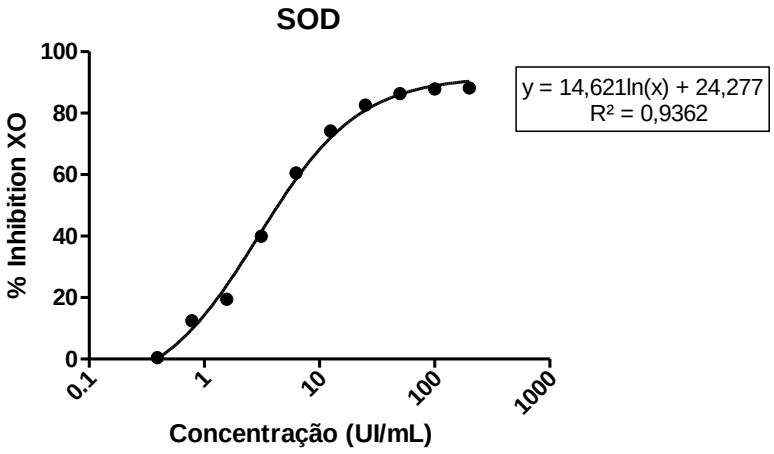


Apêndice E: Curva padrão da citocina TNF-α

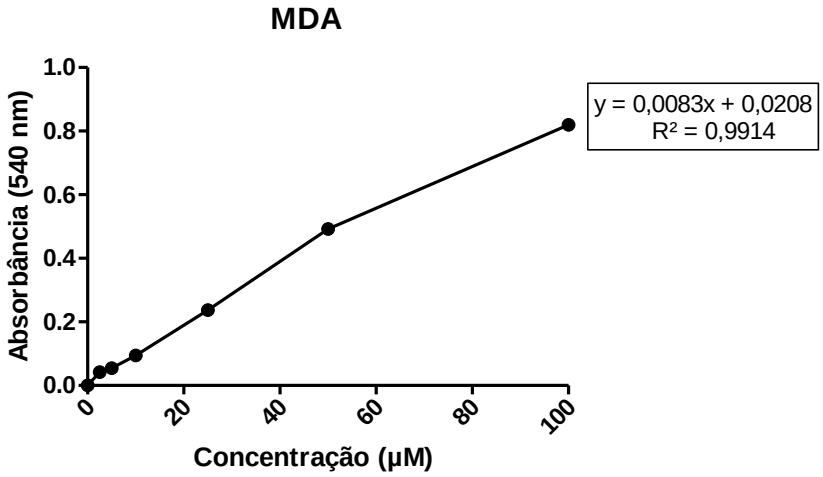


Apêndice F: Curva padrão da interleucina IL-10

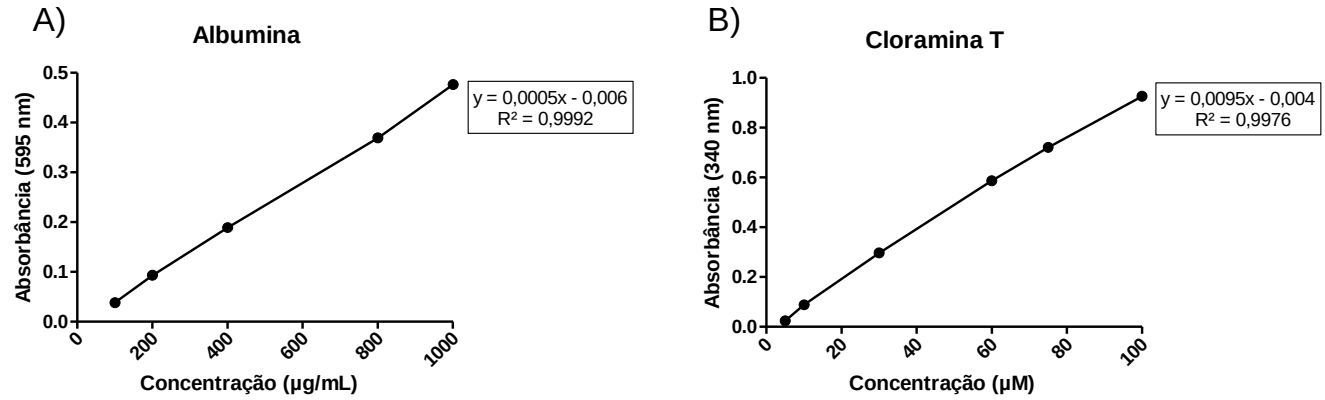
Curvas dos padrões dos ensaios bioquímicos



Apêndice G: Curva padrão de SOD



Apêndice H: Curva padrão de Malondealdeído (MDA)



Apêndice I: Curvas dos padrões para o ensaio da AOPP. A) Curva padrão de albumina para ensaio de Bradford; B) Curva padrão de Cloramina T para o ensaio da AOPP

ANEXO

UNIVERSIDADE
VILA VELHA
ESPÍRITO SANTO

Universidade Vila Velha

Comissão de Ética, Bioética e Bem Estar Animal (CEUA-UVV)

PARECER DO RELATOR

Parecer Nº433/2017

Pesquisador (a) Responsável: **Prof. Dr. Thiago de Melo Costa Pereira**

Tipo de Pesquisa: **Avaliação do efeito de frações de *Persea americana* (Mill.) em camundongos submetidos à lesões gástricas**

Instituição onde será desenvolvido: Laboratório do NUPECFARMA

Situação: **APROVADO**

Ao analisar o projeto de pesquisa: **"Avaliação do efeito de frações de *Persea americana* (Mill.) em camundongos submetidos à lesões gástricas"**, tendo como pesquisador(a) responsável **Prof. Dr. Thiago de Melo Costa Pereira**, que irá utilizar a(s) seguinte (s) espécie (s) animal (s) **90 (noventa) camundongos machos, provenientes do Biotério da Universidade Vila Velha**. Esta CEUA-UVV considera que o projeto se encontra adequado e satisfatoriamente de acordo com as exigências das Resoluções que regem esta Comissão e ao CONCEA.

Assim, mediante a importância social e científica que o projeto apresenta, a sua aplicabilidade e conformidade com os requisitos éticos, sou de parecer favorável à realização do projeto classificando-o como **APROVADO**, pois o mesmo **atende** aos Requisitos Fundamentais da Normas de Conduta para a Utilização de Animais no Ensino, Pesquisa e Extensão na Universidade Vila Velha.

Vila Velha , **04 de julho de 2017.**

Prof. João Luiz Rossi Junior

Relator da CEUA-UVV.

Universidade Vila Velha

Rua Comissário José Dantas de Melo, 21, Boa Vista, Vila Velha-ES, CEP.: 29102-770
E-mail barbara.loureiro@uvv.br

Anexo A: Parecer do Comitê de Ética (CEUA-UVV)