



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de Botucatu



UNIVERSIDADE ESTADUAL PAULISTA “Júlio de Mesquita Filho”

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

LARISSA LUCENA PÉRICO

Investigação do papel dos hormônios sexuais na atividade cicatrizante do extrato hidroalcoólico das folhas de *Eugenia punicifolia* na doença ulcerosa péptica experimental.



Botucatu – SP

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ORIENTADORA: PROF. ADJA. CLÉLIA AKIKO HIRUMA LIMA

**COORIENTADORA: PROFA. DRA. LÚCIA REGINA MACHADO
DA ROCHA**

Tese apresentada ao Instituto de Biociências de Botucatu - UNESP, como requisito para a obtenção do título de Doutorado em Farmacologia e Biotecnologia

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Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Clélia Akiko Hiruma-Lima
Coorientador: Lúcia Regina Machado da Rocha
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Palavras-chave: Atividade cicatrizante; *Eugenia punicifolia* (Kunth) DC.; Hormônios sexuais; Myrtaceae; Úlceras pépticas.

BANCA EXAMINADORA

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Profa. Dra. Raquel de Cássia dos Santos (USF/Bragança Paulista – SP)

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Ao **Vinícius, Garboso** ou como gosto de chamá-lo **Garbs**, obrigada por ser meu braço direito e esquerdo. Obrigada por me ajudar e deixar tocar o laboratório junto com você. Obrigada por todos esses anos maravilhosos de confiança, amizade, amor, parceria, companheirismo, comidas, bebidas, choros e festas. Obrigada por ser o meu “pseudonamorado” nos últimos sete anos e por ter sido “casado” comigo por sete meses. Obrigada por cada puxão de orelha, broncas e conselhos tanto profissionais quanto pessoais. Obrigada por ter me dado a sua atenção e ter me ouvido falar das minhas angústias, medos, vitórias e derrotas. Você é o irmão que está comigo sete dias da semana, me apoiando em tudo e sim, neste exato momento eu estou chorando para conseguir escrever algo sobre você, porque apesar do meu jeito “dura”, o qual você levou um tempo para se acostumar, falando que eu era uma sargenta, no fundo você

sabe que eu tenho um coração mole, rsrs. Esta conquista não é somente minha, é nossa!!!

Muito obrigado as minhas amigas **Tainara, Nayara, Natália, Thaís, Fernanda e Carlinha** que sempre estiveram comigo me apoiando e me aconselhando. Elas me abraçaram nos dias de choro e também sorriram comigo nos momentos de alegria. Obrigada pela verdadeira amizade e companheirismo desde o momento em que cheguei em Botucatu, e que permanecerão pela vida.

A equipe do **Laboratório de Ensaaios Biológicos com Produtos Naturais** por toda ajuda e apoio prestados. **Rie** (Doku), **Gabriela** (Ximboca), **Bruna** (Nega), **Natalia** (Questão), **Marcelinho** e **Thiago** sem a colaboração de cada um de vocês este trabalho não seria concluído.

Agradeço a Raquel (**Pocotó**) por sua amizade, experiência, orientação e ensinamentos que tanto me fizeram crescer no mestrado e doutorado.

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Obrigado a todos do **Departamento de Fisiologia e Farmacologia**, alunos, servidores, técnicos e docentes que de alguma forma ajudaram na minha formação.

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A **FAPESP, Projeto Biota/FAPESP, CAPES e CNPQ** e pelo apoio financeiro.

OBRIGADA!

“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”.

Roger Von Oech

PRÓLOGO

O desenvolvimento do projeto de doutorado possibilitou a formação específica em Farmacologia e Biotecnologia, com estudos direcionados para a caracterização da atividade farmacológica de produtos naturais no tratamento da doença ulcerosa péptica. Durante a execução do projeto de doutorado, foram realizadas outras atividades, no intuito de enriquecer a minha formação profissional e científica.

Trabalhos apresentados na forma de painel em eventos científicos

2014:

- ➔ **Périco, L. L.**; Rodrigues, V. P.; Beserra, F. P.; Santos, R. C.; Heredia-Vieira, S. C.; Vilegas, W.; Bauab, T. M.; Varanda, E. A.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Antiulcerogenic, anti-inflammatory, antimicrobial and anti-mutagenic effects of hydroalcoholic extract from leaves of *Serjania marginata* Casar. (Sapindaceae).** Evento: XXIII Simpósio de Plantas Medicinais do Brasil - Da Etnofarmacologia à Biotecnologia no Desenvolvimento de Medicamentos à Base de Plantas Medicinais.
- ➔ Beserra, F. P.; **Périco, L. L.**; Rodrigues, V. P.; Saldanha, L. L.; Dokkedal, A. L.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Papel da isoforma óxido nítrico sintase induzível (iNOS) sobre o efeito antioxidante de *Cissus sicyoides* Linneu (Vitaceae) em modelos agudos de úlceras gástricas.** Evento: XXIII Simpósio de Plantas Medicinais do Brasil - Da Etnofarmacologia à Biotecnologia no Desenvolvimento de Medicamentos à Base de Plantas Medicinais.
- ➔ Rico, S. L. C.; **Périco, L. L.**; Beserra, F. P.; Rodrigues, V. P.; Cunha, J. C.; Nunes, D. S.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Avaliação da toxicidade e da atividade antiulcerogênica do óleo essencial de *Baccharis trimera* em roedores.** Evento: XI Encontro Mineiro de Biomedicina.
- ➔ Rodrigues, V. P.; Beserra, F. P.; **Périco, L. L.**; Dias, M. J.; Vilegas, W.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Avaliação do efeito do extrato hidroalcoólico das folhas de *Mimosa caesalpiniiifolia* benth (mimosoideae) sobre o trato gastrointestinal.** Evento: 17º Encontro Nacional de Biomedicina.
- ➔ Rico, S. L. C.; **Périco, L. L.**; Rodrigues, V. P.; Cunha, J. C.; Nunes, D. S.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Avaliação dos mecanismos de ação antiulcerogênico do óleo essencial de *Baccharis trimera* em roedores.** Evento: 17º Encontro Nacional de Biomedicina.
- ➔ Beserra, F. P.; Santos, R. C.; **Périco, L. L.**; Rico, S. L. C.; Kiguti, L. R. A.; Saldanha, L. L.; Rocha, L. R. M.; Dokkedal, A. L.; Hiruma-Lima, C. A. **Papel dos receptores muscarínicos, canais de cálcio e das prostaglandinas no efeito antiespasmódico e antidiarreico de *Cissus sicyoides* L. (Vitaceae).** Evento: 17º Encontro Nacional de Biomedicina.

2015:

- ➔ **Périco, L. L.**; Santos, R. C.; Rodrigues, V. P.; Ohara, R.; Vilegas, W.; Rocha, L.

- R. M.; Santos, C.; Hiruma-Lima, C. A. **Evaluation of toxicity and healing activity from the hydroalcoholic extract (leaves) of *Eugenia punicifolia* (Kunth) DC. in rodents.** Evento: IV International Symposium on Drug Discovery.
- ➔ Rodrigues, V. P.; Ohara, R.; Beserra, F. P.; **Périco, L. L.**; Dias, M. J.; Vilegas, W.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Anti-inflammatory effect of the hydroalcoholic extract of *Mimosa caesalpiniiifolia* Benth (Mimosidaceae) in animal model.** Evento: IV International Symposium on Drug Discovery.
 - ➔ Beserra, F. P.; Rodrigues, V. P.; **Périco, L. L.**; Santos, R. C.; Ohara, R.; Saldanha, L. L.; Dokkedal, A. L.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Role of prostaglandins in the antiulcerogenic and antidiarrheal effect of *Cissus sicyoides* Linneu (Vitaceae) in rodents.** Evento: IV International Symposium on Drug Discovery.
 - ➔ **Périco, L. L.** **Avaliação da atividade cicatrizante e dos mecanismos de ação do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (Kunth) DC frente a doença ulcerosa péptica experimental.** Evento: V SIMFAR - Simpósio de Farmacologia da UNESP.
 - ➔ Rodrigues, V. P.; **Périco, L. L.**; Rico, S. L. C.; Cunha, J. C.; Souza, L.P.; Vilegas, W.; Hiruma-Lima, C. A. **Avaliação da atividade cicatrizante gástrica do extrato hidroalcoólico das folhas de *Astronium urundeuva* (Allemão) Engl.** Evento: XIV Workshop da Pós-Graduação.

2016:

- ➔ **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Santos, R. C.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **Healing activity from the hydroalcoholic extract (leaves) of *Eugenia punicifolia* (Kunth) DC. (Myrtaceae) in gastric ulcer.** Evento: 6th International Congress of Aromatic and Medicinal Plants.
- ➔ Rodrigues, V. P.; **Périco, L. L.**; Ohara, R.; Rocha, C. Q.; Vilegas, W.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Anti-edematogenic activity of dichloromethane extract from the roots of *Arrabidaea brachypoda* (DC.) Bureal.** Evento: 6th International Congress of Aromatic and Medicinal Plants.
- ➔ Romano, K. S. F.; **Périco, L. L.**; Rodrigues, V. P.; Hiruma-Lima, C. A.; Gambero, A.; Santos, R. C. **Avaliação da atividade farmacológica do extrato das folhas de *Byrsonima intermedia* A. Juss. (Malpighiaceae) em modelo experimental de doença inflamatória intestinal em ratos.** Evento: XXII Encontro de Iniciação Científica, XV Encontro de Pós-Graduação, XI Encontro de Extensão Universitária e IX Seminários de Estudo do Homem Contemporâneo.
- ➔ **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Santos, R. C.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **Hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Kunth) DC. improvement injury induced by intestinal ischemia-reperfusion in rat.** Evento: VIII Simpósio Ibero-Americano de Plantas Medicinais e III Simpósio Ibero-Americano de Investigação em Câncer.
- ➔ Rodrigues, V. P.; **Périco, L. L.**; Ohara, R.; Vilegas, W.; Rocha, C. Q.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Antinociceptive activity of dichloromethane extract from the roots of *Arrabidaea brachypoda* (DC.) bureal.** Evento: VIII Simpósio Ibero-Americano de Plantas Medicinais e III Simpósio Ibero-

Americano de Investigação em Câncer.

- Ohara, R.; **Périco, L. L.**; Rodrigues, V. P.; Santos, R. C.; Vilegas, W.; Rocha, L. R. M.; Santos, L. C.; Hiruma-Lima, C. A. **Curative role of *Terminalia catappa* L. in a rat model of intestinal ischemia-reperfusion injury.** Evento: VIII Simpósio Ibero-Americano de Plantas Medicinais e III Simpósio Ibero-Americano de Investigação em Câncer.
- Ohara, R.; **Périco, L. L.**; Rodrigues, V. P.; Mazzolin, L. P.; Severi, J. A.; Hiruma-Lima, C. A. **Mechanisms involved in the gastroprotective effect of *Guapira noxia* (Netto) Lundell leaves.** Evento: VIII Simpósio Ibero-Americano de Plantas Medicinais e III Simpósio Ibero-Americano de Investigação em Câncer.

2017:

- **Périco, Larissa Lucena**; Rodrigues, V. P.; Bueno, G.; Carvalho, N. C.; Ohara, R.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **Effects of female sex hormones on the healing of gastric ulcer in rats treated with *Eugenia punicifolia*.** Evento: XXVI Italo Latin American Congress of Ethnomedicine - SILAE and IX Congreso Colombiano de Cromatografía - IX COCOCRO.
- Emilio-Silva, M. T.; **Lucena Périco, Larissa**; Rocha, L. R. M.; Santos, R. C.; Hiruma-Lima, Clélia Akiko. **Effect of *Citrus aurantium* essential oil on castor oil-induced experimental diarrhea.** Evento: XXVI Italo Latin American Congress of Ethnomedicine - SILAE and IX Congreso Colombiano de Cromatografía - IX COCOCRO.
- de Cássia dos Santos, R.; **Lucena Périco, Larissa**; Peixoto Rodrigues, V.; Vilegas, W.; Hiruma-Lima, C. A. **Antinociceptive activity of methanolic extract from the leaves of *Byrsonima intermedia* A. Juss (Malpighiaceae).** Evento: XXVI Italo Latin American Congress of Ethnomedicine - SILAE and IX Congreso Colombiano de Cromatografía - IX COCOCRO.
- **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Santos, B. B.; Santos, R. C.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **From popular use to preclinical validation: a study of the healing activity of hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Myrtaceae) in rodents.** Evento: 49th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE).
- Rodrigues, V. P.; **Périco, L. L.**; Ohara, R.; Santos, R. C.; Vilegas, W.; Rocha, C. Q.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Antinociceptive and anti-inflammatory effect of the dichloromethane extract from the roots of *Arrabidaea brachypoda* (DC.) Bureau and its role on ASIC and TRPM8 receptors.** Evento: 49th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE).
- Ohara, R.; **Périco, L. L.**; Rodrigues, V. P.; Santos, B. B.; Santos, R. C.; Santos, L. C.; Vilegas, W.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Effects of the infusion of *Terminalia catappa* leaves on treatment of duodenal injury induced by ischemia-reperfusion in female rats.** Evento: 49th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE).
- **Périco, L. L.**; Rodrigues, V. P.; Santos, R. C.; Ohara, R.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **Hydroalcoholic extract obtained from *Eugenia punicifolia* leaves improvement injury induced by gastric ischemia-**

reperfusion in male and female rats. Evento: International Conference on Toxicology and Pharmacology.

- ➔ Santos, R. C.; Fagundes, F. L.; **Périco, L. L.**; Rodrigues, V. P.; Gotardo, E. M. F.; Gambero, A.; Hiruma-Lima, C. A. **Investigação da atividade farmacológica de extrato das folhas de *Arrabidaea brachypoda* (Bignoniaceae) em modelo animal de doença inflamatória intestinal em ratos.** Evento: 20º Encontro Nacional de Biomedicina.
- ➔ Romano, K. S. F.; Fagundes, F. L.; **Périco, L. L.**; Rodrigues, V. P.; Gotardo, E. M. F.; Gambero, A.; Hiruma-Lima, C. A.; Santos, R. C. **Investigação do potencial terapêutico do extrato das folhas de *Byrsonima intermedia* A. Juss (MALPIGHIACEAE) em modelo experimental de doença inflamatória intestinal com recidivas.** Evento: XXXII Reunião Anual da FeSBE.

Trabalhos apresentados na forma oral durante evento científico

2014:

- ➔ **Périco, L. L.** **Avaliação dos mecanismos de ação do extrato hidroalcoólico das folhas de *Serjania marginata* frente a modelos experimentais crônicos de úlcera péptica.** Evento: IV SIMFAR - Simpósio de Farmacologia da UNESP.

2015:

- ➔ **Périco, L. L.**; Santos, R. C.; Beserra, F. P.; Rodrigues, V. P.; Heredia-Vieira, S. C.; Vilegas, W.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Avaliação do efeito protetor do extrato hidroalcoólico das folhas de *Serjania marginata* Casar. (Sapindaceae) através de parâmetros bioquímicos da mucosa gástrica de ratos.** Evento: XIV Workshop da Pós-Graduação.

2016:

- ➔ Bueno, G.; **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Hiruma-Lima, C. A. **Avaliação da ação cicatrizante gástrica do óleo essencial de *Baccharis trimera* Less (Asteraceae).** Evento: XXVIII Congresso de Iniciação Científica da UNESP.
- ➔ Lima Júnior, M. A.; **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Hiruma-Lima, C. A.; Rocha, L. R. M. **Avaliação do efeito antidiarreico do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (Kunth) DC.** Evento: XXVIII Congresso de Iniciação Científica da UNESP.

2017:

- ➔ **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Vilegas, W.; Rocha, L. R. M.; Santos, C.; Hiruma-Lima, C. A. **Effects of sex hormones on the toxicity and healing of gastric ulcer in rats treated with *Eugenia punicifolia*, a medicinal plant from amazon region.** Evento: World Gastroenterological Conference.
- ➔ Lima Junior, M. A.; **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Hiruma-Lima,

C. A.; Rocha, L. R. M. **Avaliação da atividade gastroprotetora da infusão das folhas de *Terminalia catappa* L. em ratos.** Evento: XXIX Congresso de Iniciação Científica da UNESP.

➔ Nascimento, T. S.; **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Hiruma-Lima, C. A.; Rocha, L. R. M. **Avaliação do efeito antidiarreico da infusão de *Terminalia catappa* Linn em modelos animais.** Evento: XXIX Congresso de Iniciação Científica da UNESP.

➔ Carvalho, N. C.; **Périco, L. L.**; Rodrigues, V. P.; Ohara, R.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Efeito do extrato hidroalcoólico das folhas de *Eugenia punicifolia* na cicatrização das úlceras gástricas em ratas Wistar intactas e ovariectomizadas.** Evento: XXIX Congresso de Iniciação Científica da UNESP.

Participação em eventos científicos

2014:

- ➔ A Nature Conference: Chemical Probe-based Open Science: Uncovering new human and plant biology.
- ➔ I Simpósio de Ética no Uso de Animais.
- ➔ IV SIMFAR - Simpósio de Farmacologia da UNESP.
- ➔ XIII Workshop da Pós-Graduação.
- ➔ XXIII Simpósio de Plantas Medicinais do Brasil - Da Etnofarmacologia à Biotecnologia no Desenvolvimento de Medicamentos à Base de Plantas Medicinais.

2015:

- ➔ IV International Symposium on Drug Discovery.
- ➔ V SIMFAR - Simpósio de Farmacologia da UNESP.
- ➔ XIV Workshop da Pós-Graduação.

2016:

- ➔ 6th International Congress of Aromatic and Medicinal Plants.
- ➔ VIII Simpósio Ibero-Americano de Plantas Medicinais e III Simpósio Ibero-Americano de Investigação em Câncer.
- ➔ X Venha Conhecer o IB.

2017:

- ➔ XI Venha Conhecer o IB.
- ➔ 49th Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE).
- ➔ International Conference on Toxicology and Pharmacology.
- ➔ World Gastroenterological Conference.
- ➔ XV Encontro Anual da RNEC: Novos Talentos da Rede Pública.
- ➔ XXVI Italo Latin American Congress of Ethnomedicine - SILAE and IX Congreso Colombiano de Cromatografía - IX COCOCRO.

Palestras internacionais ministradas

- ➔ Effects of sex hormones on the toxicity and healing of gastric ulcer in rats treated with *Eugenia punicifolia*, a medicinal plant from amazon region. World Gastroenterological Conference – Toronto – Canadá. 2017.

Extensão Universitária

2015:

- ➔ Monitora do Difundindo e Popularizando a Ciência na Unesp - Reprodução de A a Z. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 48h.

2016:

- ➔ Monitora do Difundindo e Popularizando a Ciência na Unesp - Reprodução de A a Z. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 48h.

2017:

- ➔ Monitora do Difundindo e Popularizando a Ciência na Unesp - Reprodução de A a Z. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 48h.
- ➔ Expositora do Departamento de Fisiologia no projeto de extensão universitária "XI Venha conhecer o IB". Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 3h.

Cursos realizados

2014:

- ➔ Fundamentos e Aplicações de Células Tronco. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 8h.
- ➔ Métodos para Avaliação de Atividade Farmacológica. Universidade Federal de Goiás, UFG, Goiânia, Brasil. Carga horária: 3h.

2015:

- ➔ Boas Práticas de Pipetagem. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 3h.

- ➔ Thomson Reuters Integrity. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 4h.
- ➔ Redação Científica Internacional. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 8h.

2016:

- ➔ Como conseguir uma bolsa de estudo e financiamento para um projeto de pesquisa. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 4h.
- ➔ Aplicações de Citometria de Fluxo na Pesquisa Farmacológica de Plantas Medicinais. Universidade do Vale do Itajaí, UNIVALI, Itajaí, Brasil. Carga horária: 3h.
- ➔ Magpix Instrument Training. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 6h.

2017:

- ➔ Animals models in translational pharmacology. Sociedade Brasileira de Farmacologia e Terapêutica Experimental, SBFTE, Ribeirão Preto, Brasil. Carga: 3h.
- ➔ Western blot: princípios básicos e vantagens dos produtos Abcam. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 2h.
- ➔ Nova Tecnologia em Anticorpos Monoclonais Primários: vantagens, benefícios. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 2h.
- ➔ Como escrever um projeto de pesquisa. Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Brasil. Carga horária: 8h.

Prêmios e Títulos:

2014:

- ➔ Menção Honrosa no 17º Encontro Nacional de Biomedicina: Avaliação dos mecanismos de ação antiulcerogênico do óleo essencial de *Baccharis trimera* em roedores, UNESP – Universidade Estadual Paulista “Júlio de Mesquita Filho”.

2015:

- ➔ 2º Lugar na categoria “Oral-Pós-Graduação” no XIV Workshop da Pós-Graduação: “Avaliação do efeito protetor do extrato hidroalcoólico das folhas de *Serjania marginata* Casar. (Sapindaceae) através de parâmetros bioquímicos da mucosa gástrica de ratos”, Instituto de Biociências de Botucatu, UNESP – Universidade Estadual Paulista “Júlio de Mesquita Filho”.

2016:

- ➔ XXVIII Congresso de Iniciação Científica da UNESP: “Avaliação da ação cicatrizante gástrica do óleo essencial de *Baccharis trimera* Less (Asteraceae)”, Instituto de Biociências de Botucatu, UNESP – Universidade Estadual Paulista “Júlio de Mesquita Filho”.

2017:

- ➔ XXIX Congresso de Iniciação Científica da UNESP: “Avaliação do efeito antidiarreico do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (Kunth) DC em modelos animais”, Instituto de Biociências de Botucatu, UNESP – Universidade Estadual Paulista “Júlio de Mesquita Filho”.

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2014:

- ➔ Basting, R. T.; Nishijima, C. M.; Lopes, J. A.; Santos, R. C.; **Périco, L. L.**; Laufer, S.; Bauer, S.; Costa, M. F.; Santos, L. C.; Rocha, L. R. M.; Vilegas, W.; Santos, A. R. S.; Santos, C.; Hiruma-Lima, C. A. Antinociceptive, anti-inflammatory and antiulcer effects of hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Kunth) DC. in rodents. *Journal of Ethnopharmacology*. v.157, p.257 - 267, 2014.
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- ➔ Nishida, S. M.; Soares, A. C. S.; Soares, G. A.; Sakoda, J. N.; **Périco, L. L.**; Cavariani, M. M.; Rosa, M. E. D.; Ohara, R.; Rodrigues, V. P.; Rocha, L. R. M.; Hiruma-Lima, C. A. **Reprodução de A à Z Criando Material Didático: uma Interação Criativa entre Ensino Médio e Universidade**, 2017. Evento: XV Encontro Anual da RNEC: Novos Talentos da Rede Pública; Inst.promotora/financiadora: Rede Nacional Leopoldo de Meis de educação e ciência “Novos talentos da rede pública”

Participação em eventos, congressos, exposições, feiras e olimpíadas

- ➔ Apresentação de Poster no **X Venha conhecer o IB**, 2016. Expositora das Atividades do Departamento de Fisiologia.
- ➔ Apresentação de Poster no **XV Encontro Anual da RNEC: Novos Talentos da Rede Pública**, 2017. Reprodução de A à Z Criando Material Didático: uma Interação Criativa entre Ensino Médio e Universidade.

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Orientações e supervisões

2014:

- ➔ Vinícius Peixoto Rodrigues. **Avaliação da atividade cicatrizante gástrica do extrato hidroalcoólico das folhas de *Astronium urundeuva* (Allemão) Engl.** 2014. Curso (Biomedicina) - Universidade Estadual Paulista "Júlio de Mesquita Filho"

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- ➔ Journal of Medicinal Plant Research.
- ➔ BMC Complementary and Alternative Medicine.
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- ➔ Journal of Natural Products and Plant Research.

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- ➔ Farmacocinética
- ➔ Farmacodinâmica: Receptores e Interação Droga-
- ➔ Farmacologia do Sistema Nervoso Autônomo Simpático
- ➔ Fundamentos sobre Ética e Prática na Experimentação Animal
- ➔ Métodos de Estudo em Biologia Celular e Estrutural

RESUMO

As úlceras pépticas abrangem tanto as úlceras gástricas, quanto as úlceras duodenais e afetam cerca de 10% da população mundial, sendo 6,8% homens e 4,9% mulheres. A maior ocorrência de úlceras pépticas em homens do que em mulheres, provavelmente, está relacionada aos hormônios sexuais, pois existem relatos de que os hormônios femininos exercem um efeito protetor contra as ulcerações. A patogênese complexa e multifatorial desta doença ocorre devido a um desequilíbrio entre os fatores agressores [ácido clorídrico, pepsina, etanol, anti-inflamatórios não esteroidais (AINEs), estresse, *Helicobacter pylori*, entre outros] e os protetores (muco, bicarbonato, prostaglandinas e fluxo sanguíneo) da mucosa do estômago e/ou duodeno. Numa tentativa de proteger a mucosa gástrica do ácido clorídrico, melhorar a cicatrização da úlcera, e prevenir a sua recorrência, o controle farmacológico da secreção ácida tem sido uma das estratégias utilizadas ao longo dos anos. Dentre as opções disponíveis no mercado para o tratamento das úlceras pépticas existem os antagonistas de receptores H_2 , os inibidores da bomba de prótons e o uso de antibióticos para a erradicação da *H. pylori*. Entretanto, estes medicamentos podem promover não somente uma gama de efeitos colaterais, mas também uma menor eficácia no tratamento das úlceras, bem como o surgimento das recidivas logo após a interrupção do tratamento. Para tanto, se faz necessário as buscas de novas opções terapêuticas para tratamento adequado das úlceras pépticas a partir de produtos naturais. *Eugenia punicifolia* (Kunth) DC., uma espécie comumente encontrada na região Amazônica e no Cerrado, tem sido utilizada popularmente para o tratamento de feridas, infecções e inflamações. Em estudos realizados previamente em nosso laboratório, o extrato de *E. punicifolia* apresentou um efetivo efeito anti-inflamatório e gastroprotetor sobre a mucosa gástrica em modelos experimentais *in vivo* e *in vitro*. Porém, a presença de uma ação gastroprotetora de um extrato não assegura que o mesmo também possua ação cicatrizante. Portanto, o objetivo deste trabalho foi caracterizar os mecanismos envolvidos na ação cicatrizante do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (HEEP) ao combater os efeitos lesivos promovidos pela ação do etanol, ácido acético, AINEs e as injúrias oriundas de um processo de isquemia-reperfusão e analisar a diferença entre o efeito do extrato na cicatrização de machos e fêmeas (intactas e ovariectomizadas). Para tanto, foram desenvolvidos modelos experimentais *in vivo* no qual, inicialmente foi implantada a lesão gástrica ou duodenal nos ratos Wistar e posteriormente foi realizado o tratamento com HEEP em períodos que variaram de 1 a 14 dias de tratamento. Foram analisados a cicatrização gástrica e duodenal nos diferentes grupos experimentais e para isso utilizou-se parâmetros macroscópicos como a área de lesão, parâmetros bioquímicos antioxidantes (superóxido dismutase, glutatona reduzida, catalase, mieloperoxidase e peroxidação lipídica), pró e anti-inflamatórios (TNF- α , IL-1 β , IL-6, IFN- γ , IL-5 e IL-10), expressão proteica de fatores envolvidos no processo de cicatrização [fatores de crescimento endotelial vascular (VEGF) e epidermal (EGF), ciclooxigenase 1 e 2, marcadores pró-apoptóticos - caspase-3, caspase-8 e caspase-9 e antiapoptóticos, como a Bcl-2] e zimografia [metaloproteinases (MMP) 2 e 9]. A atividade de proliferação celular do HEEP em fibroblastos (células L929) também foi determinada por ensaios *in vitro*. Com a realização dos diferentes tratamentos foi possível avaliar os efeitos tóxicos através da evolução do peso corporal, letalidade, análises macroscópicas dos órgãos e alguns parâmetros bioquímicos no plasma. Adicionalmente, os efeitos do HEEP sobre a diarreia, a motilidade intestinal e o acúmulo de fluido intestinal *in vivo* assim como sua ação antimicrobiana *in vitro*, foram estudados. O HEEP foi capaz de cicatrizar a mucosa

gástrica de machos, fêmeas intactas (4 dias) e fêmeas ovariectomizadas (6 dias) no modelo de etanol 80% devido à redução da atividade da mieloperoxidase e aumento nos níveis de glutathione reduzida. Nas lesões gástricas induzidas por AINEs, os ratos machos tratados com HEEP (2 dias) apresentaram uma efetiva cicatrização da mucosa gástrica com redução nos níveis da citocina pró-inflamatória IL-5. O mesmo resultado não foi obtido com as fêmeas tratadas com HEEP durante o mesmo período. Nas lesões gástricas e duodenais promovidas pela isquemia e reperfusão, o tratamento de ratos machos e fêmeas (intactas e ovariectomizadas) com HEEP (6 dias) foi capaz de cicatrizar a mucosa apresentando aumento nas atividades de catalase e superóxido dismutase e redução da atividade de mieloperoxidase e dos níveis de glutathione reduzida. Os animais que foram submetidos a lesão gástrica induzida por ácido acético e tratados com o HEEP (14 dias) apresentaram uma efetiva ação cicatrizante. Os resultados da análise de western blotting demonstraram que o tratamento com HEEP por 14 dias no modelo de indução de lesão gástrica por ácido acético não alterou a expressão proteica (EGF, VEGF, COX-1 e 2, Bcl-2 e as caspases-8, 9 e 3), porém, a cicatrização da mucosa gástrica pelo tratamento com HEEP nesse modelo experimental foi devido ao aumento dos níveis de prostaglandina E₂, inibição da MMP-9 e manutenção constante da atividade da MMP-2 da mucosa gástrica. O efeito cicatrizante do HEEP apresenta um envolvimento com os hormônios sexuais femininos nos modelos de etanol 80%, isquemia-reperfusão e ácido acético. Podemos constatar que houve diferenças em relação ao tempo de tratamento, em que as fêmeas intactas apresentaram significativa ação cicatrizante durante um período menor de tratamento quando comparado com as fêmeas ovariectomizadas ou em relação ao grau de severidade da lesão, no qual as fêmeas intactas obtiveram uma área de lesão menor quando comparadas aos machos ou fêmeas ovariectomizadas. Além disso, houve diferença na expressão proteica de EGF, VEGF, Caspase-8 e Bcl-2, entre as fêmeas e os machos. Não foram observados efeitos tóxicos nos ratos machos e fêmeas com base na ausência de letalidade, alteração do peso corporal, análises macroscópicas dos órgãos e parâmetros bioquímicos do plasma pelo tratamento com HEEP. O efeito cicatrizante do HEEP também foi comprovado em estudos *in vitro* ao promover aumento da proliferação e cobertura de feridas riscadas em monocamadas de fibroblastos. Adicionalmente ao efeito antiulcerogênico, foi possível caracterizar que o extrato também possui ação antimicrobiana contra *Staphylococcus aureus* e foi capaz de reduzir o trânsito intestinal e prevenir ocorrência da diarreia induzida por agente catártico. Desta forma, podemos concluir que o HEEP pode contribuir efetivamente para a cicatrização da lesão gástrica e duodenal promovidas por uma grande diversidade de agentes ulcerogênicos e que os mecanismos envolvidos nesta ação se mostram diversificados e abrangentes de acordo com o grau de severidade da lesão e do tempo de tratamento do extrato. Nossos resultados demonstram que existe uma clara interferência do hormônio sexual feminino no processo de cicatrização das úlceras pépticas. Aliado a ação cicatrizante, o extrato também exerce uma ação antidiarreica e antimicrobiana, inclusive no combate a um dos principais indutores de gastroenterite no homem, a *S. aureus*.

Palavras-chave: *Eugenia punicifolia*; ação cicatrizante; hormônios sexuais femininos; úlceras pépticas.

ABSTRACT

Peptic ulcers include both gastric and duodenal ulcers and affect about 10% of the world population, being 6.8% men and 4.9% women. The higher occurrence of peptic ulcers in men than in women is probably related to sex hormones, as there are reports that female hormones exert a protective effect against ulcerations. The complex and multifactorial pathogenesis of this disease occurs due to an imbalance between the aggressive (hydrochloric acid, pepsin, ethanol, nonsteroidal anti-inflammatory drugs (NSAIDs), stress, *Helicobacter pylori*, among others) and protective factors (mucus, bicarbonate, prostaglandins and blood flow) of the stomach and duodenum mucosa. In an attempt to protect gastric mucosa from the hydrochloric acid, improve ulcer healing, and prevent recurrence, pharmacological control of acid secretion has been one of the strategies used over the years. Among the options available in the market for the treatment of peptic ulcers are H₂ receptor antagonists, proton pump inhibitors and the use of antibiotics for the eradication of *H. pylori*. However, these drugs may promote not only a range of side effects, but also a lower efficacy in the treatment of ulcers, as well as the occurrence of relapses soon after discontinuation of treatment. Therefore, it is necessary to search for new therapeutic options for appropriate treatment of peptic ulcers from natural products. *Eugenia punicifolia* (Kunth) DC., a specie commonly found in the Amazon region and in the Cerrado, has been popularly used for the treatment of wounds, infections and inflammation. In previous studies in our laboratory, the extract of *E. punicifolia* showed an effective anti-inflammatory and gastroprotective effect on the gastric mucosa in experimental models *in vivo* and *in vitro*. However, the presence of a gastroprotective action of an extract does not ensure that it also has healing action. Therefore, the objective of this work was to characterize the mechanisms involved in the healing action of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) by combating the harmful effects promoted by ethanol, acetic acid, NSAIDs and injuries originating from a process of ischemia-reperfusion and analyzing the difference between the healing effect of the extract on male and female (intact and ovariectomized). For this, experimental models were developed *in vivo* in which the gastric or duodenal lesion was initially implanted in the Wistar rats and later the treatment with HEEP was performed in periods ranging from 1 to 14 days of treatment. The gastric and duodenal healing in the different experimental groups was analyzed using macroscopic parameters such as lesion area, antioxidant and biochemical parameters (superoxide dismutase, reduced glutathione, catalase, myeloperoxidase and lipid peroxidation), pro and anti-inflammatory agents (TNF, IL-6, IFN- γ , IL-5 and IL-10), protein expression of factors involved in the healing process [vascular endothelial (VEGF) and epidermal (EGF) growth factor, cyclooxygenase 1 and 2, pro-apoptotic markers - caspase-3, caspase-8 and caspase-9 and antiapoptotic markers such as Bcl-2] and zymography [MMPs 2 and 9]. With the accomplishment of the different treatments it was possible to evaluate the toxic effects through the evolution of the body weight, lethality, macroscopic analysis of the organs and some biochemical parameters in the plasma. In addition, the effects of HEEP on diarrhea, intestinal motility and intestinal fluid accumulation *in vivo* as well as its antimicrobial action *in vitro* were studied. HEEP was able to heal the gastric mucosa of males, intact females (4 days) and ovariectomized females (6 days) in the ethanol 80% model due to reduce of myeloperoxidase activity and increased levels of reduced glutathione. In the gastric lesions induced by NSAIDs, male rats treated with HEEP (2 days) showed an effective healing of the gastric mucosa with reduction in the levels of the pro-inflammatory cytokine IL-5. The same result was not obtained with the females treated with HEEP during the same period. In the gastric and duodenal lesions promoted by ischemia and

reperfusion, the treatment of male and female (intact and ovariectomized) rats with HEEP (6 days) was able to heal the mucosa presenting an increase in the catalase and superoxide dismutase activities and reduction of myeloperoxidase activity and levels of reduced glutathione. Animals that underwent gastric injury induced by acetic acid and treated with HEEP (14 days) presented an effective curative action. The results of western blotting analysis demonstrated that 14-day HEEP treatment in the acetic acid gastric injury induction model did not alter protein expression (EGF, VEGF, COX-1 and 2, Bcl-2 and caspase-8, 9 and 3); however, healing of the gastric mucosa by treatment with HEEP in this experimental model was due to increased levels of prostaglandin E₂, inhibition of MMP-9 and constant maintenance of MMP-2 activity of the gastric mucosa. The healing effect of HEEP shows an involvement with female sex hormones in the ethanol 80%, ischemia-reperfusion and acetic acid models. We observed that there were differences in treatment time, in which the intact females had a significant healing effect during a shorter treatment period when compared to the ovariectomized females or in relation to the degree of lesion severity, in which the intact females obtained a lower lesion area compared to males or ovariectomized females. In addition, there was a difference in the protein expression of EGF, VEGF, Caspase-8 and Bcl-2 between females and males. No toxic effects were observed in male and female rats based on the absence of lethality, body weight change, macroscopic organ analysis and biochemical parameters of plasma by treatment with HEEP. The healing effect of HEEP has also been demonstrated with *in vitro* studies in promoting increased proliferation and coverage of scratched wounds in fibroblast monolayers. In addition to antiulcerogenic effect, it was possible to characterize that the extract also has antimicrobial activity against *Staphylococcus aureus* and was able to reduce the intestinal transit and prevent occurrence of diarrhea induced by the cathartic agent. In this way, we can conclude that HEEP can effectively contribute to healing of the gastric and duodenal lesions promoted by a great diversity of ulcerogenic agents and that the mechanisms involved in this action are diversified and comprehensive according to the degree of severity of the lesion and the time of treatment of the extract. Our results demonstrate that there is a clear interference of the female sexual hormone in the healing process of peptic ulcers. Combined with healing action, the extract also exerts an anti-diarrheal and antimicrobial action, including in combating one of the major inducers of gastroenteritis in humans, *S. aureus*.

Keywords: *Eugenia punicifolia*; healing action; female sex hormones; peptic ulcers.

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LISTA DE ABREVIATURAS E SÍMBOLOS

AINES ou NSAIDs – Anti-inflamatórios não esteroidais ou Non Steroidal Anti-inflammatories

ALT - Alanine amino transferase

ARH2 - Antagonists of type 2 histamine receptor

AST - Aspartate aminotransferase

BSA - Bovine serum albumin

CARB - Carbenoxolone

CAT – Catalase

CCK – Colesciticinina

CEUA - Comitê de Ética sobre o Uso de Animais ou Ethics Committee on the Use of Animals

CGRP – Peptídeo relacionado ao gene da calcitonina ou calcitonin gene related peptide

COX – 1 – Ciclooxygenase 1

COX – 2 – Ciclooxygenase 2

COX – Ciclooxygenase

DMEM - Dulbecco's Modified Eagle Medium

DTNB - 5,5'-Dithiobis (2-nitrobenzoic acid)

E. coli – *Escherichia coli*

E. punicifolia – *Eugenia punicifolia*

ECM - Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EROs ou ROS – Espécie reativa de oxigênio ou Reactive Oxygen Specie

FBS - Fetal bovine serum

GPx – Glutathione peroxidase

GSH – Glutathione reduzida ou reduced glutathione

H. pylori – *Helicobacter pylori*

H₂O₂ – Peróxido de hidrogênio ou hydrogen peroxide

HCO₃⁻ - Bicarbonato

HEEP – Extrato hidroalcoólico das folhas de *Eugenia punicifolia* ou Hydroalcoholic extract from the leaves of *Eugenia punicifolia*

HTAB – Hexadecyl trimethyl ammonium bromide

I/R ou I-R ou IR – Isquemia e Reperusão ou Ischemia-Reperfusion

IBP ou PPI – Inibidores da bomba de prótons ou Proton Pump Inhibitor

IFN- γ – Interferon gamma

IL-10 – Interleukin 10

IL-1 β – Interleukin 1 β

IL-5 – Interleukin 5

IL-6 – Interleukin 6

KCl - Potassium chloride

KH₂PO₄ - Potassium dihydrogen phosphate

LPO - Lipid peroxidation

LZ – Lansoprazole

MDA – Malondialdeído ou Malondialdehyde

MHA - Mueller Hinton Agar

MHB - Mueller Hinton broth

MIC – Minimal inhibitory concentration

MMP - Matrix metalloproteinases

MPO – Mieloperoxidase ou Myeloperoxidase

NADPH - β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate

NaOH - Sodium hydroxide

NBT - Nitrotetrazolium Blue chloride

NO – Óxido nítrico ou nitric oxide

NOS – Óxido nítrico sintase

O₂⁻ - Ânion superóxido ou free radical superoxide

O₂ - Molecular oxygen

OH⁻ - Hydroxyl

OMS ou WHO – Organização Mundial da Saúde ou World Health Organization

OVZ – Ovariectomized female rats

PBS – Phosphate buffered solution

PGE₂ – Prostaglandina do tipo E2

PGI₂ – Prostaciclina ou prostacyclin

PGs – Prostaglandinas

PND – Postnatal day

QOUH - Quality of ulcer healing

S. aureus – *Staphylococcus aureus*

SDS - Sodium dodecyl sulfate

SOD – Superóxido dismutase ou superoxide dismutase

TBA - 2-thiobarbituric acid

TCA - Trichloroacetic acid

TFFs – Família de fatores trefoil

TFN- α – Tumor necrosis factor alpha

TGI – Trato gastrointestinal

VEGF – Vascular endothelial growth factor

γ -GT - γ -glutamyl transpeptidase

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APRESENTAÇÃO

A maneira de exposição dos capítulos foi feita com o objetivo de encaminhar os artigos para a publicação em revistas científicas, o que facilita e agiliza o processo de publicação dos resultados.

O **capítulo 1** contém uma abordagem ampla sobre as úlceras pépticas, a homeostasia da mucosa gástrica, os fatores protetores e agressores da mucosa, bem como o tratamento e problemática atual sobre a terapêutica. Este capítulo também contém informações sobre o objeto de estudo (o extrato hidroalcoólico das folhas de *Eugenia punicifolia*), etnofarmacologia e os estudos fitoquímicos. Encontra-se também descrito no capítulo os objetivos (geral e específicos) do trabalho.

No **capítulo 2** encontra-se o artigo: **“Can the gastric healing effect of a medicinal plant be the same in males and females? The curative action of *Eugenia punicifolia* (Kunth) DC in NSAID and ethanol experimental models”**. Este estudo mostra os resultados do efeito cicatrizante da *Eugenia punicifolia* nos modelos de indução de lesão gástrica com etanol 80% e AINEs, assim como a elucidação de seus mecanismos de ação e a influência dos hormônios sexuais nesta resposta.

O **capítulo 3** contém o artigo: **“Antioxidant and anti-inflammatory potentials of *Eugenia punicifolia* in healing gastric and duodenal injury induced by ischemia-reperfusion in male and female rats”**. Neste artigo temos os resultados do efeito cicatrizante do extrato hidroalcoólico das folhas de *Eugenia punicifolia* em modelos de indução de lesão gástrica e duodenal por isquemia e reperfusão. Caracterizamos os possíveis mecanismos de ação envolvidos nesta cicatrização, através de técnicas bioquímicas, bem como o efeito dos hormônios sexuais.

No **capítulo 4** temos o artigo: **“Effects of *Eugenia punicifolia* in gastric ulcer healing and differences between sexes”**. Neste estudo temos o efeito cicatrizante do extrato de *Eugenia punicifolia* no modelo de indução de lesão gástrica por ácido acético tanto em machos quanto em fêmeas intactas e ovariectomizadas e avaliamos a qualidade da cicatrização por técnicas de biologia molecular. Além disso, analisamos a toxicidade subaguda do HEEP nos animais.

O **capítulo 5** é constituído de resultados adicionais que obtivemos durante o desenvolvimento desta tese. Neste capítulo nós temos o artigo intitulado: **“Antidiarrheal and antimicrobial activities of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Kunth) DC *in vivo* and *in vitro*”**. Neste trabalho nós obtivemos dados sobre as propriedades antidiarreicas e antimicrobianas exercidas pelo extrato de *Eugenia punicifolia*, dados estes que complementam as potencialidades exercidas por esta planta.

CAPÍTULO 1

1. INTRODUÇÃO

Úlceras pépticas

As úlceras pépticas abrangem tanto as úlceras gástricas, quanto as úlceras duodenais (MALFERTHEINER et al., 2009) e afetam cerca de 5-10% da população mundial, sendo 6,8% homens e 4,9% mulheres (KO et al., 2016; LANAS; CHAN, 2017). As lesões descritas como ulcerosas variam de acordo com a profundidade das erosões causadas que podem ser desde danos epiteliais superficiais até lesões mais profundas que penetram na mucosa e causam perfuração (TURNER, 2010; STEWART; ACKROYD, 2011).

No estômago, normalmente, as úlceras estão localizadas na menor curvatura, porém, podem ocorrer a partir do piloro até a cárdia. A localização típica da úlcera duodenal é no bulbo, no qual os conteúdos gástricos entram em contato com o intestino delgado (MALFERTHEINER et al., 2009) (Fig. 1).

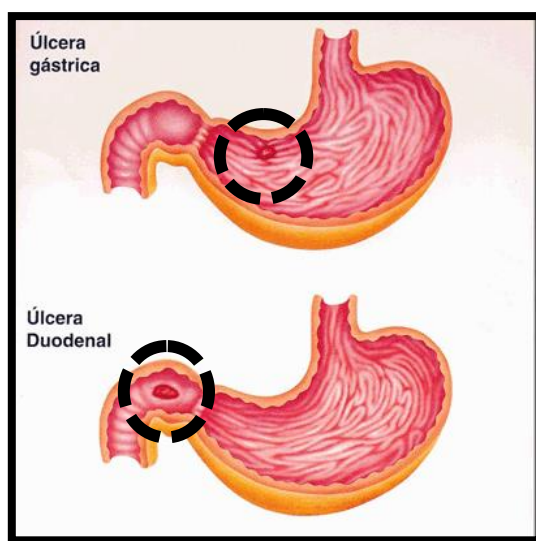


Figura 1: Imagem ilustrativa de úlceras gástricas e duodenais. (Disponível em http://www.gastrosite.com.br/s_estomago.php - Acessado em 09/01/2018).

A incidência das úlceras pépticas (0,1 – 0,3% ao ano) varia de acordo com a idade, sexo, localização geográfica e está associada a complicações graves, incluindo sangramentos, perfurações, obstrução gastrointestinal e malignidade. Assim, esta condição clínica representa um problema de saúde mundial devido à sua alta morbidade

e mortalidade (BROWN; WILSON, 1999; DIMALINE; VARRO, 2007; LANAS; CHAN, 2017).

O sangramento e a perfuração são as principais complicações da úlcera péptica e elas se manifestam como melena (sangue nas fezes) ou hematêmeses (vômito com sangue), entretanto pode não ocorrer sintomas de alerta em quase metade dos pacientes (LANAS; CHAN, 2017). As internações hospitalares decorrentes do sangramento das úlceras pépticas diminuíram de forma constante em todo o mundo, mas a taxa de letalidade permanece estável em 5-10% (LANAS et al., 2011; MALMI et al., 2014). A perfuração tipicamente apresenta um início repentino de dor intensa na parte superior do abdômen e a mortalidade pode atingir até 20% dependendo da idade e da comorbidade (LANAS; CHAN, 2017).

Os principais sintomas da úlcera péptica são dor epigástrica e sintomas de dispepsia, como plenitude, distensão abdominal, saciedade precoce e náuseas, embora muitas vezes as úlceras crônicas podem ser assintomáticas (MALFERTHEINER et al., 2009).

Os pacientes com úlceras duodenais tipicamente sentem fome ou têm dores abdominais noturna. Em contraste, aqueles com úlceras gástricas apresentam dores abdominais pós-prandial, náuseas, vômitos e perda de peso. Os idosos são frequentemente assintomáticos ou têm apenas sintomas leves (LANAS; CHAN, 2017). Os pacientes com úlceras pépticas frequentemente apresentam recorrências e a prevenção desta recorrência é o objetivo mais importante, a longo prazo, para diminuir a morbidade e a mortalidade (LANAS; CHAN, 2017). Os custos com as doenças ulcerosas pépticas por pacientes são estimados em 163 à 866 dólares por ano (BARKUN; LEONTIADIS, 2010).

O desenvolvimento das úlceras pépticas tem sido associado ao surgimento da urbanização com aumento da incidência em indivíduos nascidos a partir do século 19, devido principalmente a mudança no estilo de vida (SUSSER; STEIN, 1962; SONNENBERG, 2006).

A endoscopia é utilizada como padrão ouro para o diagnóstico da doença ulcerosa péptica. Além da exclusão da doença maligna, a detecção da infecção por *Helicobacter*

pylori com exames histológicos ou testes de detecção da urease rápida são essenciais para um subsequente plano de tratamento (LANAS; CHAN, 2017).

A descoberta da *H. pylori*, primeiramente isolada e cultivada pelos pesquisadores australianos Warren e Marshall em 1982 (WARREN; MARSHALL, 1983), revolucionou o mundo da gastroenterologia, com um benefício inestimável para milhões de pessoas em todo o mundo. Além disso, o conceito de que os microorganismos não sobrevivem no ambiente gástrico tornou-se desatualizado, após uma longa história de relatos não reconhecidos sobre a presença de bactérias espirais no estômago (PELLICANO et al., 2011). Como o estômago humano é um lugar hostil para a maioria das bactérias, a fim de sobreviver ao ambiente gástrico, a *H. pylori* desenvolveu um mecanismo de resistência ao ácido que, juntamente com os fatores de colonização, ajudam a bactéria a superar a barreira mucosa. Em particular, através da enzima urease, o microorganismo cria ao seu redor uma nuvem de produtos químicos neutralizantes que oferece proteção contra o ácido (ROSSO et al., 2015).

Lanas e Chan, (2017) relataram que a erradicação da infecção por *H. pylori* por si só, é suficiente para cicatrizar as úlceras pépticas e prevenir o sangramento recorrente. No entanto, a obtenção de um tratamento bem-sucedido para combater a infecção por *H. pylori* é um desafio global devido à crescente prevalência e resistência dessa bactéria gram negativa aos antibióticos.

Como descrito anteriormente, as úlceras pépticas ocorrem com mais frequência em homens do que em mulheres, entretanto estas diferenças são menos acentuadas após 45 anos de idade, provavelmente porque a incidência das úlceras aumenta em mulheres menopausadas. Alguns trabalhos relatam que as diferenças na incidência da doença entre os sexos estão relacionadas aos hormônios sexuais e que os hormônios femininos exercem um efeito protetor nas ulcerações (EL-TABLAWEY et al., 2012). Curiosamente, mulheres grávidas ou mulheres que utilizam pílulas contraceptivas (estrogênio/progesterona) apresentam menor frequência de úlceras duodenais (VESSEY et al., 1992). Além disso, as úlceras duodenais seguem um curso mais grave em mulheres menopausadas do que em mulheres pré-menopausa (REDCHITS; PETROV, 1995). Assim, similarmente a outros órgãos, o estrógeno pode ter um efeito protetor no trato gastrointestinal (SMITH et al., 2008). Em 2012, El-Tablawy e colaboradores demonstraram que o estrógeno diminui a secreção ácida gástrica e reduz a formação de

úlceras pépticas, efeitos esses que podem indicar uma nova possibilidade terapêutica para a terapia de reposição hormonal, a proteção do TGI (trato gastrointestinal). Dong e Kaunitz (2006) relataram que o aumento na incidência das úlceras pépticas em mulheres está relacionado com a sua participação cada vez mais frequente no mercado de trabalho e em atividades sociais. No entanto, embora se tenha argumentos sobre a ação benéfica do estrógeno em relação as úlceras pépticas, os estudos são desatualizados, escassos e muitas vezes contraditórios (SANGMA et al., 2014).

Embora os estudos indiquem que a etiologia da úlcera ainda seja desconhecida, é aceito que as úlceras pépticas, tanto em homens quanto em mulheres, são decorrentes de processos multifatoriais e se desenvolvem quando os fatores agressores (agentes endógenos, exógenos e/ou infecciosos) superam os mecanismos de defesa da mucosa (TULASSAY; HERSZÉNYI, 2010; GOMES SILVA; DE SOUSA, 2011).

Homeostasia da mucosa gástrica

As úlceras pépticas resultam de um desequilíbrio entre agentes agressores exógenos, como o estresse, consumo de álcool, tabagismo, ingestão prolongada de anti-inflamatórios não esteroidais (AINEs), infecção por *Helicobacter pylori*, etc., e os mecanismos de proteção endógenos, tais como a barreira mucosa, secreção de muco, efluxo de bicarbonato, aumento dos níveis antioxidantes endógenos enzimáticos e não enzimáticos, regeneração celular, prostaglandinas, óxido nítrico, compostos sulfidrílicos dentre outros (AMARAL et al., 2013; JÚNIOR et al., 2014; WALLACE, 2008; WANG; WANG, 2011).

Em resposta aos fatores agressores da mucosa há os fatores protetores, dentre os quais estão: o componente pré-epitelial (muco, bicarbonato e barreira fosfolipídica) que é a primeira linha de defesa e o componente epitelial [uma camada contínua de células epiteliais superficiais conectadas por junções oclusivas e responsáveis pela geração de bicarbonato, muco, fosfolipídios, prostaglandinas (PGs), e proteínas de choque térmico] que constitui a segunda linha de defesa da mucosa (LAINE et al., 2008). Outros mecanismos de defesa incluem a contínua renovação celular [acompanhada de fatores de crescimento e PG do tipo E₂ (PGE₂)], a manutenção do fluxo sanguíneo através dos microvasos na mucosa, a geração de óxido nítrico (NO), PGs e inervação sensorial

(LAINE et al., 2008; DIMALINE; VARRO, 2007; HENRIKSNÄS et al., 2006; NAYEB-HASHEMI; KAUNITZ, 2009) (Fig.2).

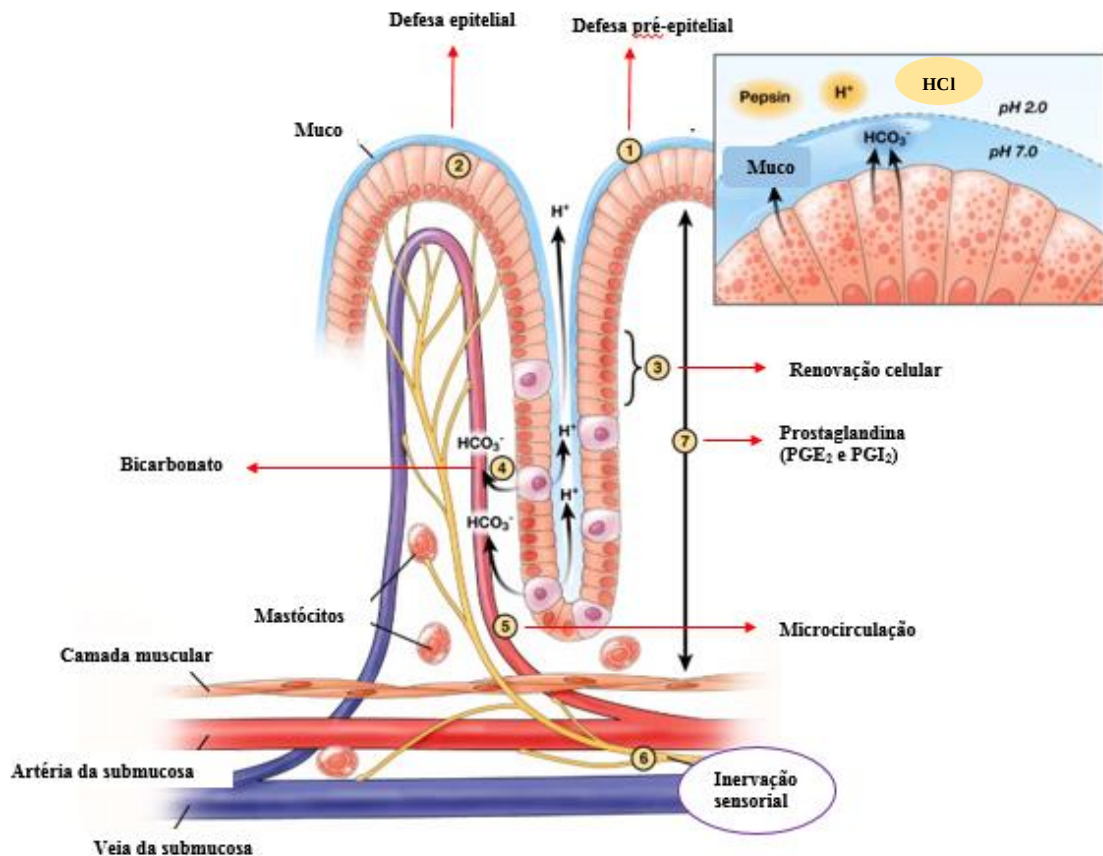


Figura 2: Ilustração dos fatores protetores da mucosa (Adaptado de TARNAWSKI et al., 2013).

Fatores protetores da mucosa

A exposição regular do estômago às enzimas ácidas produzidas endogenamente requer a presença de uma "barreira" mucosa gástrica eficiente. A primeira evidência experimental para a barreira de muco-bicarbonato foi relatada há cerca de três décadas (ALLEN, 1978; ALLEN; GARNER, 1980). Essa barreira constitui a primeira linha de defesa da mucosa (barreira pré-epitelial) e é formada por gel mucoso, bicarbonato (HCO_3^-) e fosfolípidos, que recobrem a superfície mucosa (LICHTENBERGER, 1999; ALLEN; FLEMSTRÖM, 2005).

O muco gástrico consiste em um gel viscoso, elástico, aderente e transparente secretado pelas células epiteliais superficiais. Ele é formado por aproximadamente 95% de água e 5% de mucinas que cobrem a mucosa gastrointestinal por inteiro, e sua superfície luminal é revestida com uma película de fosfolípides surfactantes com fortes propriedades hidrofóbicas. O HCO_3^- é secretado pelas células epiteliais superficiais e o seu papel é neutralizar a difusão de ácido, formando uma camada com o gel de muco aderente (barreira de muco-bicarbonato) que mantém um pH quase neutro ($\sim 7,0$) na interface da superfície da mucosa (HILLS et al., 1983; LICHTENBERGER, 1999; REPETTO; LLESUY, 2002; TULASSAY; HERSZÉNYI, 2010).

A barreira de muco-bicarbonato é a única barreira pré-epitelial entre o epitélio e o lúmen. Quando essa barreira está sobrecarregada e se decompõe nas diferentes condições da doença, a próxima série de mecanismos de proteção entram em ação, incluindo o reparo epitelial, manutenção e distribuição do fluxo sanguíneo da mucosa (TULASSAY; HERSZÉNYI, 2010).

A próxima linha de proteção da mucosa é formada por uma camada continua de células epiteliais superficiais (barreira epitelial), que secretam muco e HCO_3^- , geram PGs, proteínas de choque térmico, peptídeos da família de fatores trefol (TFFs), e as catelicidinas. Esta barreira epitelial tem a finalidade de separar o lúmen digestivo dos compartimentos internos do organismo. A sua principal função é manter uma troca seletiva de diferentes substâncias (secreções, nutrientes, etc.) entre esses dois compartimentos e assegurar a proteção do organismo contra a penetração de microrganismos e outros antígenos exógenos, essencialmente contidos nas comidas. Neste contexto, dois elementos cruciais da barreira epitelial digestiva garantem estas funções: as células epiteliais e as junções intercelulares (junções oclusivas). Ambas estruturas fornecem duas vias para transporte transepitelial: as rotas transcelulares e paracelulares, respectivamente (MATYSIAK-BUDNIK et al., 2003; LAINE et al., 2008; TULASSAY; HERSZÉNYI, 2010). Devido à presença de fosfolípidos nas superfícies das células epiteliais, essas células são hidrofóbicas e, portanto, repelem agentes prejudiciais ao ácido e à água (LICHTENBERGER et al., 1983).

As proteínas de choque térmico geradas pelas células epiteliais gástricas são essenciais na manutenção da homeostasia celular, crescimento celular normal e para sobrevivência durante vários estresses celulares, como o aumento da temperatura, estresse oxidativo e

agentes citotóxicos, prevenindo a desnaturação proteica e protegendo as células contra lesões (GOMES SILVA; DE SOUSA, 2011).

A família de fatores trefoil (TFFs) compreende um grupo de pequenos peptídeos (6.5-12 kDa) secretados abundantemente pelo epitélio superficial, e que desempenham um importante papel na integridade da mucosa (TAUPIN; PODOLSKY, 2003). Eles regulam a reepitelização por estimular a migração celular e exercem ação protetora da mucosa contra uma ampla gama de produtos químicos, tóxicos e medicamentos (LAINE et al., 2008), bem como inibem a apoptose, a inflamação e aumentam a função da barreira de muco (TAUPIN; PODOLSKY, 2003; HERNÁNDEZ et al., 2009; TULASSAY; HERSZÉNYI, 2010).

As PGs são sintetizadas pelas células epiteliais da mucosa gástrica a partir do metabolismo do ácido araquidônico através da ação da enzima ciclooxigenase (COX). A capacidade das PGs de atenuar ou até mesmo prevenir completamente o dano na mucosa causada por substâncias agressoras como o álcool, bile concentrada ou soluções hiperosmolares tem sido denominada de "citoproteção" (BRZOZOWSKI et al., 2005). Particularmente as PGE₂ e prostaciclina (PGI₂) apresentam efeitos "citoprotetores" no epitélio gastrointestinal e, portanto, podem ser cruciais para a manutenção da integridade gástrica (GOMES SILVA; DE SOUSA, 2011). De fato, está bem estabelecido que a inibição da síntese de PG resulta na redução do fluxo sanguíneo da mucosa gástrica e conseqüentemente no dano dessa mucosa (ABDEL-SALAM et al., 2001).

Além disso, outros mediadores como o NO, peptídeo relacionado ao gene da calcitonina (CGRP), bem como alguns hormônios incluindo a gastrina, a colecistocinina (CCK), grelina, leptina e o peptídeo liberador de gastrina também estão relacionados com a proteção da mucosa gástrica contra danos induzidos por substâncias agressoras (GOMES SILVA; DE SOUSA, 2011).

Outro mecanismo de defesa da mucosa é a modulação da microcirculação gástrica que exerce um papel essencial na manutenção da integridade gástrica, especialmente por fornecer oxigênio e nutrientes e remover substâncias tóxicas. Ao nível da muscular da mucosa, a maioria das artérias gástricas se ramificam em capilares, que entram na lâmina própria e se direcionam para as células epiteliais glandulares gástricas. Na base

das células epiteliais de superfície, os capilares convergem para vênulas (LAINE et al., 2008; TULASSAY; HERSZÉNYI, 2010).

As células endoteliais também são capazes de gerar potentes agentes vasodilatadores como o NO e a PGI₂. O NO é produzido a partir da L-arginina em uma reação catalisada pela enzima óxido nítrico sintase (NOS) (ANDREW; MAYER, 1999; FÖRSTERMANN; SESSA, 2012). É uma molécula importante de sinalização biológica que influencia a circulação regulando o tônus vascular do músculo liso e modulando a pressão arterial sistêmica exercendo, portanto, efeitos positivos na defesa da mucosa no sistema gastrointestinal (BERG et al., 2004). Tanto o NO quanto o PGI₂ se opõem à ação prejudicial de vasoconstritores nas mucosas como tromboxano A₂, leucotrieno C₄ e endotelina. Consequentemente, estes agentes mantêm a viabilidade das células endoteliais e previnem a adesão de plaquetas e leucócitos às células endoteliais microvasculares, impedindo o comprometimento da microcirculação e, assim, protegendo a mucosa gástrica contra lesões (LAINE et al., 2008). Além de manter o fluxo sanguíneo gástrico, o NO protege o trato gastrointestinal inibindo a secreção de ácido gástrico das células parietais, estimulando o muco e a secreção de bicarbonato e promovendo a angiogênese *in vivo* e *in vitro* (BROWN et al., 1993; MA; WALLACE, 2000).

A defesa da mucosa gástrica também é regulada, pelo menos em parte, pelo sistema nervoso e fatores hormonais (STROFF et al., 1995; PESKAR, 2001; MÓZSIK et al., 2001). A mucosa gástrica e os vasos submucosos são inervados por neurônios sensoriais aferentes primários e nervos que formam um plexo denso na base da mucosa. Os neurônios aferentes constituem um sistema de emergência que é solicitado quando a mucosa gástrica está em perigo por agentes nocivos. Assim, a ativação desses nervos na presença de ácido gástrico promove a liberação de neurotransmissores, como a substância P e o CGRP, que relaxam o músculo liso que circunda as arteríolas, resultando em elevação do fluxo sanguíneo da mucosa, aumento do muco e do pH intracelular na superfície no estômago. Esta ação de proteção da mucosa ocorre muito provavelmente através da vasodilatação de vasos submucosos mediados pela geração de NO. Neste sentido, a interferência com qualquer aspecto das inervações sensoriais prejudica a resposta hiperêmica e, portanto, diminui a resistência da mucosa gástrica à

lesão (TANAKA et al., 1997; HOLZER, 2007; LAINE et al., 2008; TULASSAY; HERSZÉNYI, 2010).

Fatores agressores da mucosa

Apesar de sua natureza robusta e multifacetada, muitos fatores diretamente relacionados ao desempenho da defesa da mucosa podem alterar a barreira epitelial e estimular a formação de lesões mucosas, sendo as mais importantes a secreção ácida, bactérias e seus produtos, AINEs, álcool, espécies reativas de oxigênio (EROs), bem como diferentes compostos químicos (WALLACE, 1992; PESKAR; MARICIC, 1998; TULASSAY; HERSZÉNYI, 2010).

Um importante agente indutor de úlceras pépticas é a bactéria espiralada *H. pylori*. Ela é uma bactéria gram-negativa, micro-aerófila, de crescimento lento, geralmente adquirida durante a infância, cujo habitat é a superfície luminal do epitélio gástrico (PELLICANO et al., 2016). A prevalência da infecção varia de acordo com fatores socioeconômicos e idade, contudo, pelo menos 50% da população mundial são portadores do microrganismo, com uma prevalência muito maior nos países em desenvolvimento do que nos países desenvolvidos (TONKIC et al., 2012).

A infecção por *H. pylori* é atualmente aceita como uma das causas mais importante de gastrite e úlcera péptica em seres humanos. A fisiopatologia da úlcera péptica envolve: 1) danos na mucosa mediada pelos fatores de virulência da bactéria (os principais fatores são as citotoxinas, urease, fosfolipases e adesinas); 2) resposta inflamatória na mucosa tanto para dano tecidual quanto aos antígenos de *H. pylori*; 3) distúrbios na secreção de gastrina, somatostatina e de ácidos; e 4) desenvolvimento de metaplasia gástrica duodenal (PELLICANO et al., 2016).

Antes da descoberta da *H. pylori*, as úlceras eram conhecidas pela sua recorrência, e durante anos a prática padrão era manter os pacientes em tratamento com medicamentos supressores de ácido (MALFERTHEINER et al., 2009). A terapia de primeira linha padrão utilizada para o tratamento da bactéria, consiste em um inibidor da bomba de prótons (IBP) e dois antibióticos, como claritromicina e amoxicilina ou metronidazol administrados por 7-14 dias (denominada terapia tripla) ou com bismuto/tetraciclina (terapia quadrupla) (CHEY et al., 2007; FOCK et al., 2009; FALLONE et al., 2016;

MALFERTHEINER et al., 2017). Com a crescente prevalência de resistência dessa bactéria aos antibióticos, no entanto, a eficácia da erradicação da *H. pylori* com este regime caiu de mais de 90% há duas décadas até menos de 70% em muitos países (FALLONE et al., 2016; MALFERTHEINER et al., 2017; LANAS; CHAN, 2017).

Assim, devido à diminuição da eficácia do tratamento de primeira linha e ao aumento dos relatos de resistência aos antibióticos em todo o mundo, novas abordagens e direções são necessárias para gerenciar essa problemática infecção, por isso, esforços são dirigidos para o desenvolvimento e fornecimento de novas estratégias anti-*H. pylori* (TALEBI; ABADI, 2017).

Outro fator importante na atualidade e diretamente relacionado com a lesão gástrica é a utilização cada vez maior de AINEs. À medida que a prevalência da infecção por *H. pylori* diminuiu decorrente da introdução da antibioticoterapia, a incidência das úlceras gástricas por AINEs aumentou, principalmente com o uso cada vez mais frequente desse medicamento na terapia antitrombótica de doenças cardiovasculares (GOMES SILVA; DE SOUSA, 2011).

Os AINEs aumentam o risco de desenvolver complicações da úlcera péptica em quatro vezes (LANAS; CHAN, 2017). Mais de 90% das prescrições para AINEs são feitas para pacientes com idade superior a 65 anos. Os AINEs são comumente usados para tratar dor, febre, inflamação (CHEN et al., 2018) e também para o tratamento de doenças cardiovasculares (TAKEUCHI et al., 2014). A aspirina, um AINE, tem sido utilizada como terapia de prevenção para infarto agudo do miocárdio ou acidente vascular cerebral ou isquêmico e angina (SQUIZZATO et al., 2017), porém, o principal problema com o uso desses medicamentos é que eles induzem lesões da mucosa gástrica previsíveis, incluindo complicações no trato gastrointestinal superior e inferior (LAINE et al., 2008; SOSTRES et al., 2010).

O principal mecanismo através do qual os AINEs causam úlceras e complicações gastrointestinais é através da inibição da COX, uma enzima chave na biossíntese de PGs. Existem duas isoformas bem identificadas de COX, a COX-1 e COX-2 (LAINE et al., 2008; SOSTRES et al., 2010). A isoforma COX-1 é expressa na maioria dos tecidos, produzindo PGs que desempenham um papel protetor essencial no estômago estimulando a síntese e secreção de muco e bicarbonato, aumentando o fluxo sanguíneo

e promovendo a proliferação epitelial. Assim, a síntese de PG mediada pela COX-1 é principalmente responsável pela manutenção da integridade da mucosa gástrica. Por outro lado, a COX-2 tem pouca ou nenhuma expressão na maioria dos tecidos, mas é rapidamente induzida após resposta a estímulos inflamatórios. Portanto, esta isoforma é o principal alvo para medicamentos anti-inflamatórios (GOMES SILVA; DE SOUSA, 2011).

Nesse contexto, os inibidores não-seletivos de AINE tradicionais (inibem tanto a COX-1 como a COX-2), como a indometacina ou a aspirina, causam danos ao estômago com uma diminuição acentuada do conteúdo de PGE₂ na mucosa gástrica (GOMES SILVA; DE SOUSA, 2011). Este efeito ocorre através da inibição da isoforma COX-1, criando um ambiente gástrico mais suscetível ao ataque tóxico por fatores endógenos e exógenos (VANE; BOTTING, 1995). Além disso, a inibição da COX-1 bloqueia a produção de tromboxano, o que aumenta o sangramento quando existe um local ativo de sangramento gastrointestinal (LANAS; SCHEIMAN, 2007; SOSTRES et al., 2010). Esta afirmação é apoiada pelo fato de que os inibidores seletivos de COX-2 (coxibes), que não inibem a COX-1 em doses terapêuticas, não afetam a produção de PGs na mucosa e não produzem dano gástrico em modelos experimentais (LAINE et al., 2008).

No entanto, é reconhecido que as PGs derivadas da COX-2 são geradas na margem da úlcera e desempenham um papel importante na cicatrização da úlcera através do desencadeamento da proliferação celular, promoção da angiogênese e restauração da integridade mucosa (KONTUREK et al., 2005; SOSTRES et al., 2010). Enquanto os inibidores seletivos da COX-2 são eficazes na redução da dor e do dano gastrointestinal, eles tem sido associados a um risco aumentado de eventos cardiovasculares adversos (WALKER; BIASUCCI, 2018). Essas observações indicam que, em contraste com o conceito inicial, a COX-2 desempenha um papel importante na defesa da mucosa gástrica (GOMES SILVA; DE SOUSA, 2011).

De fato, a indometacina e outros AINEs, que inibem ambas as isoformas da enzima COX, produzem danos mais severos no tecido gástrico, como sangramento gastrointestinal, do que fármacos mais seletivos (DELANEY et al., 2007). Portanto, a indometacina tornou-se um dos medicamentos de primeira escolha para induzir as úlceras gástricas experimentais (SIGTHORSSON et al., 2000; SÜLEYMAN et al., 2004).

Assim, devido à prevalência e severidade das complicações gastrointestinais relacionadas aos AINEs, os esforços têm sido empenhados para a prevenção da lesão da mucosa induzida por esses medicamentos. Nesse sentido, uma vez que a terapia com AINEs e até mesmo com coxibes retarda a cicatrização de úlceras pépticas ativas, a melhor maneira de prevenir a lesão na mucosa é evitar o uso de AINEs, substituí-la por um agente menos tóxico para a mucosa gastroduodenal ou fazer a utilização de fármacos antiulcerogênicos que fortaleçam a barreira mucosa e evitem a lesão por AINEs (SOSTRES et al., 2010; FIORUCCI; SANTUCCI, 2011).

Além dos AINEs, um dos agentes exógenos mais irritantes para a mucosa é o etanol. Em todo o mundo, o etanol tem sido usado há séculos em ambientes sociais, médicos, culturais e religiosos. Atualmente, é considerado uma droga de abuso, relacionados a uma ampla gama de danos físicos, mentais e sociais, e responsável por 3,3 milhões de mortes e 5,1% das doenças globais são atribuídas ao seu consumo (WHO, 2014). Entre os vários sistemas de órgãos que medeiam os efeitos do etanol no corpo humano, o TGI desempenha um papel particularmente importante. Ele pode interagir diretamente com a mucosa gástrica ou pode atuar através de um mecanismo mais geral que afeta a liberação de hormônios e a regulação das funções nervosas envolvidas na secreção ácida (BODE; BODE, 1997; CHARI et al., 1993).

O etanol penetra rapidamente na mucosa gástrica, causando danos na membrana, esfoliação as células e provocando erosões teciduais (SIDAHMED et al., 2013). A lesão por etanol afeta predominantemente a porção glandular do estômago (SIDAHMED et al., 2013) e pode ser atribuída a alguns mecanismos tais como: a formação de radicais livres derivados do oxigênio (KWIECIENÍ et al., 2002); decréscimo nas concentrações de grupamentos sulfidrílicos no conteúdo da mucosa gástrica (GARDÈS-ALBERT et al., 1993); ruptura do muco endógeno e aumento da secreção de ácido gástrico, bem como pelos danos ocorridos na mucosa gástrica em decorrência das lesões hemorrágicas instaladas (RAJASEKARAN et al., 2012); apoptose celular (HOSHINO et al., 2002); indução de peroxidação lipídica e diminuição dos níveis de glutathione reduzida (DAS et al., 2012). O etanol também induz lesão no endotélio vascular da mucosa, causando distúrbios na microcirculação e promovendo a isquemia, resultando com isso em um desequilíbrio com grande produção de radicais livres no local (PAN et al., 2008).

O estresse oxidativo e a depleção de antioxidantes foram considerados como passos cruciais na geração dos danos causados na mucosa induzida pelo etanol e, portanto, foram amplamente investigados em vários estudos (HIROKAWA et al., 1998; ARAFA; SAYED-AHMED, 2003).

A aplicação de etanol pela via oral tem sido utilizada como um método experimental reprodutível para induzir lesões da mucosa gástrica em animais (SZABO et al., 1981). Os efeitos da administração aguda na mucosa gástrica de ratos ou camundongos são dependentes da dose e os danos surgem depois de 30 minutos após a ingestão do agente lesivo e atinge um pico em cerca de 60 minutos após a ingestão (KNOLL et al., 1998).

Outro fator agressor importante para a mucosa gastroduodenal é o processo de isquemia-reperfusão (I/R). A isquemia é um evento onde o suprimento sanguíneo em determinada área é interrompido, podendo resultar em morte tecidual. Os danos causados por uma interrupção do suprimento de sangue para um órgão ou tecido seguido pela reintrodução de sangue na área afetada são chamados de I/R. Esse processo é conhecido por induzir lesões na mucosa gastroduodenal predominantemente devido à formação excessiva de EROs, adesão dos neutrófilos às células endoteliais e disfunção microvascular (UEDA et al., 1989; BRZOZOWSKI et al., 2000). A reperfusão de tecidos isquêmicos, paradoxalmente, agrava o processo de lesão e leva à liberação adicional de EROs e mediadores pró-inflamatórios e à atração de células inflamatórias infiltrando os tecidos (CHAMOUN et al., 2000; PIPER et al., 2003). No intestino, lesões por I/R são conhecidas por serem associadas à morbidade e mortalidade durante o choque hemorrágico (CORCOS; NUZZO, 2013; FISHMAN et al., 2014), correção de aneurisma da aorta abdominal, doença isquêmica intestinal, enterocolite necrosante (RIAZ et al., 2002; DIMMITT et al., 2003) e neonatal (NANKERVIS, et al., 2008), isquemia mesentérica aguda (YASUHARA, 2005) vólvulo (SCHWARTZ et al., 2012), trauma (CORCOS; NUZZO, 2013), doenças cardiopulmonares (SASTRY et al., 2014), rejeição de transplante intestinal (MALLICK et al., 2004) além de colite isquêmica e vasculite (CORCOS; NUZZO, 2013). Estima-se que a taxa de sobrevivência para pacientes humanos e veterinários com isquemia intestinal é inferior a 50% (TROMPETER et al., 2002; BLIKSLAGER, 2003; YASUHARA, 2005; GREGORY et al., 2011) uma vez que a hipóxia do tecido, inflamação e infiltração de células resultam em perda da barreira da mucosa, que é composta de uma única camada de células epiteliais

colunares, ligadas entre si por junções interepiteliais, que coletivamente impedem a translocação de bactérias e toxinas associadas na circulação sistêmica (PODOLSKY, 1999). Desde que a isquemia intestinal é raramente evitável, a maioria das pesquisas no campo concentram-se no avanço de técnicas de detecção precoce da isquemia e desenvolvimento de alvos terapêuticos que contribuam para minimizar os danos pós-isquemia (período de reperfusão) atribuídas às EROs, associados com a ativação de enzimas da mucosa produtora de oxidantes, liberação de fatores quimiotáticos e subsequentemente, infiltração de neutrófilos (GONZALEZ; MOESER; BLIKSLAGER, 2015). Portanto, intervenções terapêuticas para proteger contra a lesão de reperfusão têm como alvo a inibição tanto da lesão oxidante, quanto da ativação dos neutrófilos (RIAZ et al., 2002; SHAFIK, 2013). Radicais livres de oxigênio são considerados mediadores fundamentais no surgimento da lesão da mucosa nesse modelo (KITANO et al., 1997; NAKAMOTO et al., 1997). Dentre esses radicais, a hidroxila, inicia uma reação em cadeia de radicais livres conhecidos como peroxidação lipídica com danos na membrana celular (UEDA et al., 1989). Algumas enzimas antioxidantes como a superóxido dismutase (SOD), catalase (CAT) atuam na proteção da mucosa contra a lesão por I/R por inibir a produção de EROs e desintoxicação do ânion superóxido ($O_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2) (OGINO et al., 1988; STEIN et al., 1990).

Radicais livres e defesa antioxidante

Radical livre pode ser definido como qualquer espécie molecular que contenha um elétron desemparelhado em um orbital atômico. A presença de um elétron desemparelhado resulta em certas propriedades comuns que são compartilhadas pela maioria dos radicais. Muitos radicais são instáveis e altamente reativos. Eles podem doar um elétron ou aceitar um elétron de outras moléculas, portanto, comportando-se como oxidantes ou redutores (CHEESEMAN; SLATER, 1993). Os radicais livres que contêm oxigênio mais importantes em muitos estados de doença são o radical hidroxila, o $O_2^{\bullet-}$, o H_2O_2 , o oxigênio singleto, o hipoclorito, o radical de óxido nítrico e o radical peroxinitrito (YOUNG; WOODSIDE, 2001).

Os radicais livres atacam macromoléculas importantes que levam ao dano celular e à ruptura homeostática. Os alvos dos radicais livres incluem todos os tipos de moléculas no corpo, entre elas, os lipídios, ácidos nucleicos e proteínas (LOBO et al., 2010).

O estresse oxidativo, decorre de um desequilíbrio entre a excessiva produção de radicais livres e a redução nas defesas antioxidantes. Esse desequilíbrio gera danos contra uma ampla gama de espécies moleculares incluindo lipídios, proteínas e ácidos nucleicos (MCCORD, 2000). Pode ocorrer em tecidos submetidos a traumas, infecções, toxinas, lesões por calor e exercícios excessivos. Esses tecidos lesionados produzem um aumento de enzimas geradoras de radicais (por exemplo, xantina oxidase, lipogenase, ciclooxygenase) ativação de fagócitos, liberação de ferro livre, íons de cobre ou uma ruptura das cadeias de transporte de elétrons da fosforilação oxidativa, produzindo excesso de EROs (LOBO et al., 2010).

A peroxidação lipídica é um processo que envolve subprodutos que podem reagir diretamente com outras biomoléculas, resultando no aumento das lesões bioquímicas. A peroxidação lipídica ocorre em ácidos graxos poli-insaturados localizados nas membranas celulares e prossegue com a reação em cadeia. O radical hidroxila inicia a formação de EROs e assim, a peroxidação lipídica é propagada. Devido à peroxidação lipídica, são formados vários compostos, por exemplo, alcanos, malondialdeídos e isoprostanos. Estes compostos são utilizados como marcadores no ensaio de peroxidação lipídica e são indicadores de muitas doenças tais como as doenças neurodegenerativas, lesão por I/R e diabetes (LOVELL et al., 1995; LOBO et al., 2010).

Para a defesa contra ataques de radicais livres, as células desenvolveram diferentes sistemas antioxidantes para manter a homeostase celular (CNUBBEN et al., 2001). O organismo possui sistemas antioxidantes enzimáticos e não-enzimáticos. Os antioxidantes enzimáticos são as CAT, Glutathione Peroxidase (GPx) e SOD (SINDHU et al., 2004). O sistema antioxidante não enzimático, por sua vez, é formado por substâncias como flavonoides da dieta, vitamina E, polifenóis, albumina, carotenoides, ácido úrico, vitaminas e a glutathione reduzida (GSH) (DEVASAGAYAM et al., 2004).

A participação da atividade das enzimas antioxidantes (como SOD, CAT e GPx) tem sido investigada na patogênese da úlcera gástrica (NAITO et al., 2014). A formação local de $O_2^{\bullet}$ pode levar à ativação da SOD, que catalisa a sua dismutação para H_2O_2 . Consequentemente a inativação do H_2O_2 em H_2O se dá pela atuação das enzimas CAT ou GPx (KWIECIEŃ et al., 2002) (Fig. 3).

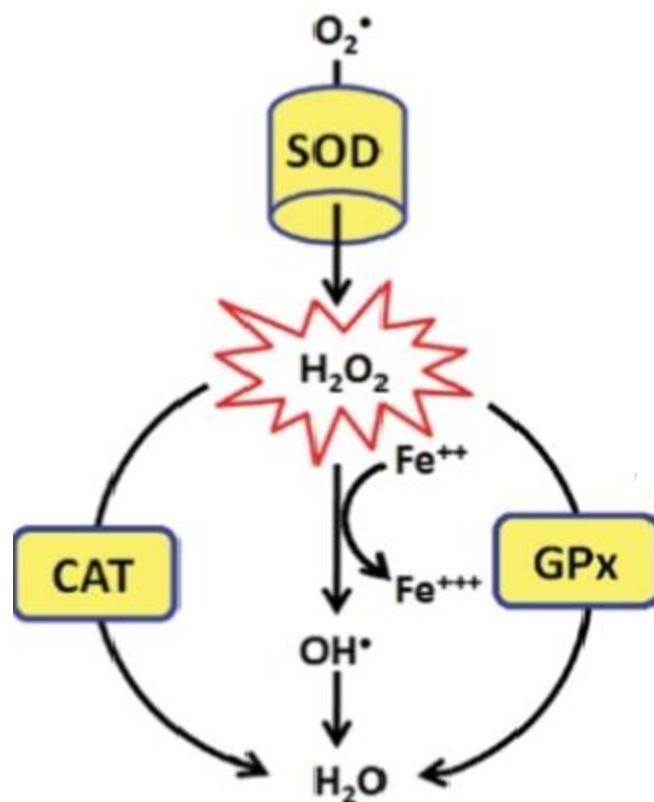


Figura 3. Esquema de atuação de algumas enzimas antioxidantes (Disponível em: http://openi.nlm.nih.gov/detailedresult.php?img=2835884_omcl0301_0002_fig005&req=4. Acessado em 14/01/2018).

A GSH é um tripeptídeo contendo cisteína encontrado na maioria das formas da vida aeróbica (MEISTER; ANDERSON, 1983). A GSH tem propriedades antioxidantes uma vez que o grupo tiol, na sua porção de cisteína, é um agente redutor e pode ser oxidado de forma reversível e reduzido (LOBO et al., 2010). Devido à sua elevada concentração e papel central na manutenção do estado redox da célula, a glutatona é um dos antioxidantes celulares mais importantes (MATTILL, 1947).

A GSH é encontrado em altas concentrações na mucosa gástrica atuando diretamente como um potente antioxidante e indiretamente como um substrato para enzimas antioxidantes (CNUBBEN et al., 2001).

Cicatrização da mucosa

A cicatrização de úlceras é um processo complexo, no qual envolve a migração celular, proliferação, replicação das células epiteliais nas margens da úlcera para restabelecer a

arquitetura glandular, angiogênese no tecido de granulação na base da úlcera e deposição da matriz, tudo levando a formação de cicatrizes (TARNAWSKI, 2005; WALLACE, 2005). A restauração da mucosa é alcançada pela rápida migração de células viáveis para o local da lesão e é finalmente concluída pela proliferação celular que repõe o número de células mortas durante a cicatrização de úlceras (TARNAWSKI, 2005). Todos esses eventos são controlados pelas citocinas e fatores de crescimento (EGF, PDGF, KGF, HGF, TGF β , VEGF, angiopoietinas) e fatores de transcrição ativados por lesão tecidual de forma coordenada espacialmente e temporariamente (TARNAWSKI, 2005).

A mucosa da margem da úlcera forma uma característica "zona de cicatrização" (COTRAN; KUMAR; ROBBINS, 1999; TARNAWSKI, 1993; TARNAWSKI, A, 2000). As glândulas gástricas nesta zona tornam-se dilatadas, as células que alinham essas glândulas sofrem desdiferenciação, expressam o receptor do fator de crescimento epidérmico (EGF-R) e proliferam ativamente (TARNAWSKI, 1993; TARNAWSKI, 2000; TARNAWSKI, et al., 1992). O último processo é iniciado dentro de 3 dias após a formação da úlcera e é essencial para a cicatrização, pois fornece células epiteliais cruciais para a re-epitelização da superfície mucosa e a reconstrução das glândulas gástricas (TARNAWSKI, A., 1993; TARNAWSKI, A, 2000; TARNAWSKI, et al., 1990). Essas células migram da margem da úlcera para o tecido de granulação para re-epitelizar a base da úlcera. Além disso, as células epiteliais da base da margem da úlcera formam tubos compostos por linhagem celular associada à úlcera, que invadem o tecido de granulação, migram para a superfície, se ramificam e passam por transformação em glândulas gástricas dentro da cicatriz da úlcera (TARNAWSKI, 2000; TARNAWSKI, et al., 1990; WRIGHT; PIKE; ELIA, 1990). Os principais estímulos para a proliferação celular, divisão, migração e re-epitelização são fatores de crescimento (COTRAN; KUMAR; ROBBINS, 1999; TARNAWSKI, 2000; VANWIJCK, 2001; TAUPIN et al., 1999; WONG; PLAYFORD; WRIGHT, 2000).

Além do conjunto inicial de fatores de crescimento derivados das plaquetas, macrófagos e tecido danificado, a ulceração desencadeia em células que alinham a mucosa da margem da úlcera, genes que codificam os fatores de crescimento (por exemplo, EGF, bFGF, HGF, VEGF e fatores trefoil) e COX-2 de uma maneira espacial e temporal bem sincronizada (TARNAWSKI, A, 2000; WONG; PLAYFORD; WRIGHT, 2000;

TARNAWSKI, A S; JONES, 1998). Esses fatores de crescimento produzidos localmente, ativam a migração e proliferação de células epiteliais através de ações autócrinas e/ou parácrinas (TARNAWSKI, 2005).

Estratégias terapêuticas

Desde a descoberta do ácido clorídrico por William Prout (1823) e da célula parietal como a célula secretora de ácido por Rudolf Heidenhain (1875) e Camillo Golgi (1893), a compreensão dos mecanismos fundamentais envolvidos na secreção ácida provou ser uma área interessante de investigação (AIHARA et al., 2003).

A secreção de ácido gástrico é um processo contínuo e complexo, em que múltiplos fatores centrais e periféricos contribuem para uma meta comum: a secreção de H^+ pelas células parietais (WALLACE; SHARKEY, 2012).

Para evitar danos causados na mucosa induzida pelo ácido, a secreção de ácido gástrico deve ser regulada com precisão através de uma interação altamente coordenada de vias neurais, hormonais e parácrinas (SCHUBERT; PEURA, 2008). Nesse sentido, os principais estimulantes da secreção ácida incluem gastrina, histamina, peptídeo liberador de gastrina (GRP), orexina, grelina e glicocorticoides, enquanto o principal inibidor é a somatostatina, produzidas pelas células delta do pâncreas nas Ilhotas de Langerhans (parácrina) (GOMES SILVA; DE SOUSA, 2011).

A secreção de ácido pela célula parietal envolve o cálcio intracelular, o AMP cíclico ou ambos, seguida de uma cascata que desencadeia a translocação da bomba de prótons, H^+K^+ -ATPase, de tubulovesículas citoplasmáticas para a membrana plasmática apical. Esta bomba é uma proteína integral de membrana que transporta íon hidrogênio do citoplasma para o canalículo da célula parietal em troca de potássio (GOMES SILVA; DE SOUSA, 2011).

Numa tentativa de proteger a mucosa gástrica do ácido gástrico, melhorar a cicatrização da úlcera, e prevenir a sua recorrência, o controle farmacológico da secreção de ácido gástrico tem representado há muito tempo um objetivo desejável (AIHARA et al., 2003). Dentre as opções terapêuticas disponíveis no mercado estão os antiácidos, anticolinérgicos, antagonistas de receptores H_2 , IBP, a erradicação da *H. pylori* e os fármacos citoprotetores (WALLACE; SHARKEY, 2012).

Os antiácidos, tais como bicarbonato de sódio, carbonato de cálcio, hidróxido de alumínio, hidróxido de magnésio, ou a preparação combinada, fornece prontamente um alívio efetivo da dor através de neutralização do ácido intraluminal (FEURLE, 1975). Entretanto, a desvantagem na utilização destes medicamentos é que o tempo efetivo com que os antiácidos duram no estômago humano é muito curto para exercerem um efeito neutralizante a longo prazo. Os antiácidos não são comumente utilizados para o tratamento da úlcera péptica, pois não são capazes de cicatrizar a lesão e apresentam um efeito rebote do ácido (AIHARA et al., 2003).

Para prolongar os efeitos dos antiácidos, fármacos anticolinérgicos, como a propantelina, foram simultaneamente administradas para retardar o esvaziamento destes agentes no duodeno. Os anticolinérgicos também podem inibir a secreção ácida por si só, mas as doses capazes de inibirem a secreção ácida, apresentam como desvantagens os efeitos adversos, tais como boca seca, visão turva, taquicardia e disfunção da vesícula. Em contraste com os anticolinérgicos, os antagonistas seletivos de receptores M_1 , tal como pirenzepina, foram clinicamente utilizados, e seus efeitos colaterais são menos frequentes quando administrados em doses baixas. Tais agentes são úteis tanto para o alívio da dor quanto para a melhora da cicatrização da úlcera, através da inibição da secreção de ácido (AIHARA et al., 2003).

A descoberta de antagonistas dos receptores H_2 em 1972 pelo Sir James Black lhe rendeu um Prêmio Nobel e lançou uma nova luz sobre a secreção ácida e alterou a prática da gastroenterologia para sempre (BLACK et al., 1972). Pela primeira vez, o ácido poderia ser inibido e as úlceras previsivelmente cicatrizadas. A administração contínua da medicação na hora de dormir poderia prevenir a recorrência da úlcera, o primeiro passo no controle dessa condição crônica (SCHUBERT; PEURA, 2008). Com alterações mínimas, Brimblecombe e colaboradores (1975) eventualmente desenvolveram a cimetidina, que foi largamente prescrita no mundo todo para o tratamento da doença péptica relacionada ao ácido, porém, assim como os outros medicamentos causa muitos efeitos colaterais. Por modificação na estrutura química da cimetidina, os potentes antagonistas de receptores H_2 , ranitidina, famotidina, e nizatidina foram desenvolvidos, resultando no tratamento de doenças relacionadas com o ácido, incluindo refluxo gastroesofágico (Fig. 4).

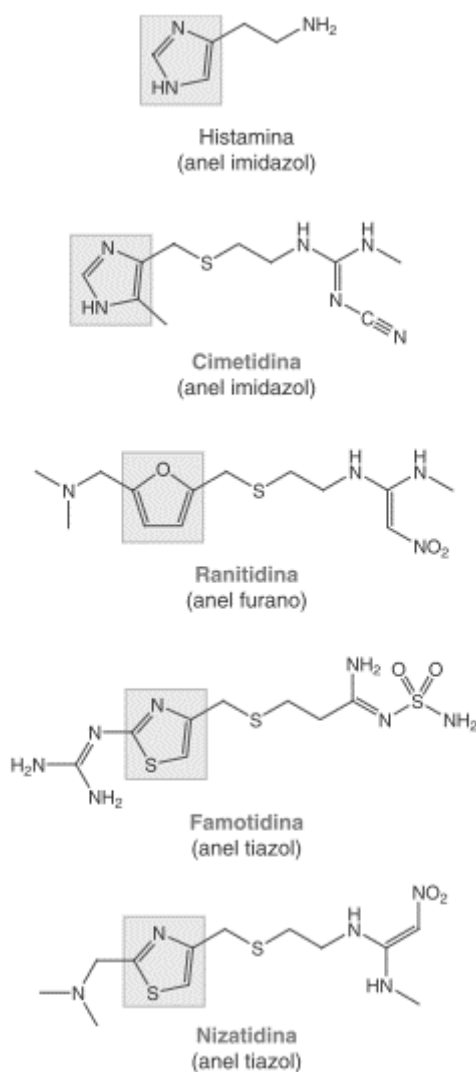


Figura 4: Estrutura química de fármacos com propriedades antagonistas de receptores H₂ (Adaptado de Nagel; Shields, 2009).

A identificação da bomba de prótons, H⁺/K⁺-ATPase, como a via final da secreção ácida forneceu uma oportunidade única de desenvolver uma nova classe de agentes que inibiriam a secreção ácida (AIHARA et al., 2003). Um grupo de pesquisadores da Companhia AB Hassle na Suécia descobriram, por acaso, que derivados do benzimidazol poderiam potencialmente inibir a secreção de ácido gástrico em glândulas gástricas isoladas de coelho ou cobaia pela estimulação da dibutilil-cAMP (FELLENIOUS et al., 1981; 1982). Estas descobertas resultaram na hipótese de que estes compostos poderiam inibir a bomba de prótons na célula parietal. Após triagem de um vasto número de derivados, o primeiro inibidor da bomba de prótons (ex. omeprazol) foi

finalmente descoberto (STRAND; KIM; PEURA, 2017). Subsequentemente, os medicamentos introduzidos incluem lansoprazol, pantoprazol, rabeprazol, tenatoprazol e os compostos estéreo-isoméricos esomeprazol e dexlansoprazol (SACHS; SHIN; HOWDEN, 2006; STRAND; KIM; PEURA, 2017) (Fig. 5).

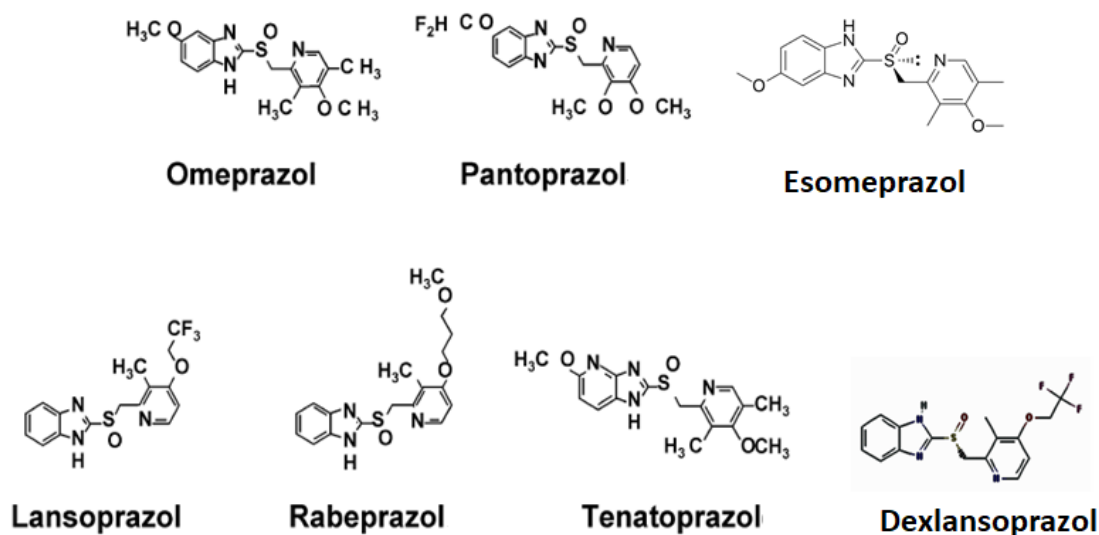


Figura 5. Estrutura química de fármacos inibidores da bomba de prótons (Adaptado de SACHS; SHIN; HOWDEN, 2006).

Os IBPs estiveram entre os medicamentos mais comumente prescritos no mundo desde que o primeiro fármaco ficou disponível na década de 1980 (FORGACS; LOGANAYAGAM, 2008). Entretanto, embora os IBP sejam geralmente considerados seguros, os dados recentes demonstraram vários efeitos adversos associados ao uso prolongado, incluindo fratura óssea (YANG et al., 2006), infecções por *Clostridium difficile* (JANARTHANAN et al., 2012), pneumonia (LAHEIJ et al., 2004), infarto do miocárdio, acidente vascular cerebral (SHERWOOD et al., 2015) e prejudicam a absorção de cálcio, ferro, magnésio e vitamina B12 no intestino (ALBAAYIT et al., 2016). Fox e Muniraj (2016) relataram como efeitos adversos dos IBPs as dores abdominais, diarreia, náuseas, dores de cabeça, erupção cutânea, toxicidade hepática e a osteoporose. Uma meta-análise recente mostrou que o risco de câncer gástrico entre os usuários de IBPs aumentado em 2,4 vezes (CHEUNG et al., 2018).

Vários fatores podem contribuir para a doença ulcerosa péptica, sendo um dos principais a infecção pela bactéria *H. pylori*. Desde que Marshall e Warren (1984) descobriram a bactéria *H. pylori* no estômago, ela tem sido reconhecida por representar um fator de risco não somente para gastrites e úlceras, mas também para recidivas de úlceras já cicatrizadas (MARSHALL; ADAMS, 2008). A erradicação eficaz da *H. pylori* reduz as taxas de recidivas de úlceras (MALFERTHEINER, PETER; CHAN; MCCOLL, 2009). O tratamento mais adequado para a erradicação da bactéria é uma terapia tripla. A terapia tripla geralmente compreende um IBP combinado aos antibacterianos amoxicilina e claritromicina ou metronidazol (MALFERTHEINER et al., 2017).

Outros medicamentos utilizados para a terapia das úlceras pépticas são os fármacos citoprotetores, que recebem este nome por promoverem a defesa da mucosa. Dentre os fármacos citoprotetores podemos citar o misoprostol, o sucralfato e a carbenoxolona.

O misoprostol, um análogo de prostaglandina do tipo E₁, foi usado como agente gastroprotetor durante o uso concomitante de AINEs. O misoprostol é o único análogo de prostaglandina aprovado pelo FDA para a prevenção da doença ulcerosa induzida por AINE (FOX; MUNIRAJ, 2016). Seu efeito gastroprotetor é conseguido pela inibição do ácido gástrico e pepsina e melhora na resistência da mucosa gástrica (DRINI, 2016). Infelizmente, a utilidade clínica do misoprostol é limitada principalmente por seus efeitos adversos gastrointestinais, como cólicas, dor abdominal e diarreia (DRINI, 2016). Também podem ocorrer contrações uterinas, de modo que o fármaco não deve ser utilizado durante a gravidez, pois leva a um abortamento (RANG et al., 2012). Por conta deste último motivo descrito, o fármaco foi retirado do mercado (SHERRIS et al., 2005).

O sucralfato é um complexo de hidróxido de alumínio com sacarose sulfatada que forma uma barreira na mucosa do TGI com excelente propriedade antiulcerogênica (SULOCHANA et al., 2016). Em ambiente ácido (pH<4), o sucralfato sofre extensa ligação cruzada, produzindo um polímero viscoso e pegajoso, que adere as células epiteliais e as úlceras. Além de inibir a hidrólise das proteínas da mucosa pela pepsina, o sucralfato pode ter outros efeitos citoprotetores, incluindo a estimulação da produção local de PGs e fator de crescimento epidérmico. Entretanto, como efeito adverso ele

pode causar constipação, alterar a farmacocinética com modificações na biodisponibilidade (WALLACE; SHARKEY, 2012).

A carbenoxolona é um derivado de ácido glicirrízico com estrutura tipo esteroide, semelhante às substâncias encontradas na raiz da planta de alcaçuz. Esse protótipo tem uma história farmacológica heterogênea. Seu mecanismo exato de ação ainda não foi esclarecido, porém, o fármaco pode alterar a composição e a quantidade de mucina. Infelizmente, a carbenoxolona inibe a 11 β -hidroxiesteróide desidrogenase, que protege o receptor de mineralocorticoide de sua ativação pelo cortisol no néfron distal; por conseguinte, provoca a hipopotassemia e a hipertensão, devido a ativação excessiva do receptor (WALLACE; SHARKEY, 2012; CONNORS, 2012). A carbenoxolona tem sido utilizada como ferramenta farmacológica para tratar úlceras gástricas e outros tipos de inflamação (CONNORS, 2012).

A cicatrização da úlcera gástrica tem sido efetiva com tratamentos convencionais, incluindo antagonistas dos receptores H₂ e os IBP, essenciais para a cicatrização de úlcera e prevenção de complicações. Além disso, a terapia de erradicação da *Helicobacter pylori* é eficaz na redução da recorrência da úlcera e leva a mudanças fisiológicas na mucosa gástrica que afetam o processo de cicatrização da úlcera. No entanto, apesar desses avanços, alguns pacientes sofrem de recorrência ou intratabilidade, apesar da contínua terapia antiulcerogênica (KANGWAN et al., 2014).

Um dos principais problemas relacionados com as úlceras pépticas é que, embora a taxa de cura com os antagonistas de receptores H₂ e os IBPs seja de aproximadamente de 80-100% após 4-8 semanas de terapia, a recorrência da úlcera dentro de 1 ano após a interrupção do tratamento é de 40-80% na maioria dos os estudos (BROOKS, 1985; MILLER; FARAGHER, 1986). Embora a recorrência da úlcera seja um problema multifatorial e, em boa parte, devido à presença da *H. pylori*, sabe-se que uma das razões para a recorrência da úlcera é a má qualidade da cicatrização (SZABO; VINCZE, 2000).

Deste modo, o progresso contínuo na compreensão da secreção de ácido gástrico na saúde e doença é necessário e esse conhecimento será usado para desenvolver novas estratégias mais efetivas para prevenir e gerenciar distúrbios gástricos (GOMES SILVA; DE SOUSA, 2011).

Neste contexto, os medicamentos à base de plantas têm sido uma escolha cada vez mais aceita pela população (TRIBESS et al., 2015). A adoção de plantas para fins terapêuticos também se reflete em números, a Organização Mundial da Saúde (OMS) estima que cerca de 80% da população mundial utiliza plantas para o tratamento de diversas doenças (BUSIA, 2016). Dentre as principais classes de compostos bioativos com capacidade de oferecer gastroproteção estão os alcaloides, saponinas, xantonas, triterpenos, taninos, entre outros (RAMIREZ; ROA, 2003; BAGGIO et al., 2005; MORIKAWA et al., 2006; KLEIN et al., 2010; VASCONCELOS et al., 2010).

***Eugenia punicifolia* (Kunth) DC.**

O gênero *Eugenia* pertence à família Myrtaceae, uma das famílias botânicas de maior expressão nos ecossistemas brasileiros, tanto em número de espécies como em número de indivíduos. No Cerrado, das 6.062 espécies citadas por Mendonça et al. (1998), 214 pertencem à família Myrtaceae, distribuídas em 15 gêneros, o equivalente a 3,5% da flora deste bioma. A espécie *Eugenia punicifolia* (Kunth) DC., popularmente conhecida como “pedra-ume-caá, pedra-ume, murta ou muta”, é um arbusto largamente distribuído na região Amazônica e no bioma Cerrado. Esta espécie é uma das plantas medicinais tradicionais inexploradas do Brasil e foi registrada pelo naturalista francês August de Saint-Hilaire em suas viagens ao território brasileiro de 1816 à 1822 (BRANDÃO et al., 2012). As folhas desta planta são popularmente usadas em decocção ou infusões aquosas como um agente terapêutico natural para tratar a inflamação (MAIA et al., 2001); febre e gripe (SILVA, 1998; CHAVES; BARROS, 2012); diabetes (MAIA et al., 2001; LEITE et al., 2010) e nas infusões alcoólicas para o tratamento de feridas e doenças infecciosas (OLIVEIRA et al., 2005). Estudos fitoquímicos realizados com o gênero *Eugenia* demonstraram a ocorrência de muitas classes de componentes, incluindo os flavonoides, taninos, terpenoides e os óleos essenciais (OLIVEIRA et al., 2006; GALENO et al., 2014). Do ponto de vista farmacológico, estudos utilizados com os extratos brutos deste gênero demonstraram atividade anti-inflamatória, analgésica, antifúngica, antipirética, hipotensora, antidiabética e antioxidante (SLOWING et al., 1994; OLIVEIRA et al., 2006; DONEPUDI et al., 2012). Os estudos farmacológicos com *Eugenia punicifolia* demonstraram que esta espécie possui atividade antioxidante (GONZAGA et al., 2007; GALENO et al., 2014), efeitos benéficos em ratos diabéticos induzidos por estreptozotocina (BRUNETTI et al., 2006), o aumento da

neurotransmissão mediada por receptores nicotínicos de acetilcolina (GRANGEIRO et al., 2006), a liberação de catecolaminas excitatórias que poderiam ser utilizados para tratar pacientes com doença de Alzheimer (PASCUAL et al., 2012) e nosso laboratório caracterizou a ação antinociceptiva e anti-inflamatória do extrato hidroalcoólico (BASTING et al., 2014).

Em continuidade aos estudos farmacológicos do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (HEEP) (Fig. 6), foram realizados ensaios farmacológicos preliminares, frente aos diferentes agentes ulcerogênicos que se assemelham às úlceras gástricas no homem, como as lesões induzidas por etanol, AINEs, aumento da secreção ácida realizados em modelos agudos. Nesses estudos pré-clínicos, o HEEP apresentou um amplo efeito gastroprotetor sobre a mucosa gástrica. Foi possível observar que o HEEP estimula a produção de muco que fortalece ainda mais a barreira mucosa gástrica contra os agentes lesivos (BASTING et al., 2014). Entretanto, estes resultados asseguram somente um efeito citoprotetor do HEEP considerando que o extrato é administrado antes do agente indutor.



Figura 6. *Eugenia punicifolia* (Kunth) DC. Foto tirada por Larissa Lucena Périco no Centro de Divulgação Científica e Cultural da USP São Carlos.

Diante desta imensa potencialidade terapêutica já apontada pela espécie *Eugenia punicifolia* até o presente, almejou-se aprofundar os conhecimentos da ação terapêutica deste extrato em ensaios pré-clínicos *in vivo* visando identificar os principais mecanismos de ação envolvidos na atividade cicatrizante gástrica e duodenal do HEEP em machos e fêmeas e avaliar a possível influência do estrógeno nesta ação.

Os estudos fitoquímicos realizados pelo nosso grupo caracterizaram a presença de flavonoides, taninos, terpenóides, saponinas, como descrito por Oliveira e colaboradores (2006) e também demonstrou a presença de proantocianidinas, galotaninos, quercetina e derivados de miricetina (BASTING et al., 2014). A análise por ESI-MS revelou a presença de ácido gálico, ácido sirínico, ácido elágico, monogaloil-glicose, quercetina, ramnose, miricitrina, mudanoside B, isômero de HHDP glicose, isômero de digaloilglicose, isômero de quercetina galoilhexosídeo e isômero de bis HHDP-glicose (COSTA et al., 2016).

2. OBJETIVOS

2.1 Objetivo Geral

Estudar o extrato hidroalcoólico das folhas de *Eugenia punicifolia* (Kunth) DC. frente às ulcerações pépticas subagudas experimentais, obtendo dados que possam inferir sobre o potencial terapêutico deste extrato.

2.2 Objetivos específicos

- Estudar a participação das atividades antioxidantes e anti-inflamatórias da *Eugenia punicifolia* na cicatrização da mucosa gastroduodenal;
- Avaliar a eficácia e qualidade da cicatrização gastroduodenal promovida pelo tratamento com *Eugenia punicifolia*, através de técnicas bioquímicas e de biologia molecular;
- Analisar se existe diferença entre o efeito do extrato na cicatrização gastroduodenal em ratos Wistar machos e fêmeas e
- Determinar os parâmetros de toxicidade subaguda a fim de obtermos resultados que possam inferir sobre a possível segurança do uso do HEEP.

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CAPÍTULO 2

RESEARCH ARTICLE

Journal of Ethnopharmacology

Can the gastric healing effect of a medicinal plant be the same in males and females? The curative action of *Eugenia punicifolia* (Kunth) DC in NSAID and ethanol experimental models.

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ABSTRACT

Ethnopharmacological relevance: Previous studies have already characterized the anti-inflammatory, antinociceptive and gastroprotective actions of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Kunth) DC., (Myrtaceae), an Amazonian medicinal plant known as “pedra-ume-caá” and popularly used in the treatment of inflammation. Proceeding this study, we evaluated the gastric healing effect of the hydroalcoholic extract from the leaves of *E. punicifolia* (HEEP) in male and female Wistar rats against nonsteroidal anti-inflammatory drugs (NSAIDs) and ethanol.

Materials and methods: The healing effect of HEEP was measured after the subacute application of aggressive factor, such as NSAIDs or ethanol 80% in gastric mucosa of male and female rats. The treatments with HEEP occurred for 2 days (NSAIDs) or 1, 2, 4 and 6 days (ethanol 80%) in male, intact and ovariectomized (OVZ) female rats. Stomachs were analyzed macroscopically for ulcerative lesion (mm²) and healing process were measured by biochemical analysis such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), interferon γ (IFN γ), interleukin 1 β (IL-1 β), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor (TNF- α), reduced glutathione (GSH) and malondialdehyde (MDA) levels as well as the myeloperoxidase (MPO), superoxide dismutase (SOD) and catalase (CAT) activities.

Results: Macroscopic evaluation of gastric mucosa showed that oral treatment with HEEP during 2 days in male rats significantly healed gastric lesions induced by NSAIDs (66%) and also decrease level of pro-inflammatory cytokine IL-5 when compared to the negative control group ($p < 0.05$). However, the healing effect of HEEP against gastric lesion induced by NSAIDs did not happen in female rats ($p > 0.05$). In addition, treatment with HEEP during 4 consecutive days significantly healed the gastric lesions induced by ethanol in male and female rats (63% and 78%, respectively). But OVZ group demanded 6 consecutive days of treatment with HEEP to heal gastric ulcer (67% compared to control group). The HEEP exerts a healing effect against ethanol by significant reduction of neutrophils infiltration into the gastric mucosa by decreasing MPO activity in male (4 days) and OVZ (6 days). Treatment with HEEP also increased a non-enzymatic antioxidant GSH level in intact females when compared to the negative control group ($p < 0.05$).

Conclusion: These findings indicated that hydroalcoholic extract from the leaves of *Eugenia punicifolia* was effective in promoting gastric ulcers healing induced by NSAIDs or ethanol. Based on the healing effects of this extract highlighted in this study, there is a possible female sex hormone interference in the process of gastric healing and demonstrates that studies should be comprehensive and take into account sex differences.

Keywords: *Eugenia punicifolia* (Kunth) DC; Myrtaceae; gastric ulcer healing; rats; sex differences.

Abbreviations: HEEP, hydroalcoholic extract from the leaves of *Eugenia punicifolia*; NSAIDs, nonsteroidal anti-inflammatory drugs; GSH, reduced glutathione; MDA malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; CAT, catalase; GI, gastrointestinal; COX, cyclooxygenase; PGE₂, prostaglandin E₂; TNF- α , tumor necrosis factor alpha; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-5, interleukin 5; OVZ, ovariectomized females; CARB, carbenoxolone; ROS, reactive oxygen species; LPO, lipid peroxidation; PPI, proton pump inhibitor; NET, neutrophil extracellular traps; NADPH, β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate; HTAB, hexadecyltrimethylammonium bromide; DTNB, 5,5'-Dithiobis (2-nitrobenzoic acid); TCA, trichloroacetic acid; NBT, nitrotetrazolium blue chloride; EDTA, ethylenediamine tetraacetic acid; BSA, bovine serum albumin; EGF, epidermal growth factor; IFN- γ , interferon γ ; IL-10, interleukin 10; VEGF, vascular endothelial growth factor; KH₂PO₄, potassium dihydrogen phosphate; KCl, potassium chloride; TBA, 2-thiobarbituric acid; SDS, sodium dodecyl sulfate; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; MAPK, Mitogen-activated protein kinase; CGRP, calcitonin gene related peptide; NO, nitric oxide; PGI₂, prostacyclin; Th2, T helper 2; H₂O₂, hydrogen peroxide; O₂^{•-}, molecular oxygen; OH⁻, hydroxyl.

1. Introduction

The term peptic ulcer refers to acid peptic injury of the digestive tract, resulting in mucosal break reaching the submucosa layer (Lanas and Chan, 2017). Acid peptic disorders are very common in the United States, with 4 million individuals (new cases and recurrences) affected per year (Dell Vale, 2015). Although peptic ulcers affect many people worldwide, this disease occurs more often in men than in women, but these differences are less pronounced after 45 years old, probably because the incidence of ulcers increases in menopausal women (El-Tablawy et al., 2012; Shore et al., 2017). Some studies described the gender-specific protection against gastric ulcer and report that the differences in the incidence of the disease between the sexes are associated with the sex hormones and that the female hormones exert a protective effect against the ulcerations (Smith et al., 2008; Kumral et al., 2014). Therefore, although there are arguments about the beneficial action of estrogen in peptic ulcers, the studies are outdated, scarce and contradictory (Sangma et al., 2014).

Although several antiulcerogenic drugs such as H₂-receptor antagonists (ranitidine), antacids and proton pump inhibitor (PPI) (omeprazole) are readily available for the treatment of ulcers (Albaayit et al., 2016), they generally present adverse effects (abdominal pain, diarrhea, nausea, headache, rash, hepatic toxicity, osteoporosis, and pneumonia), high costs in patients who use the drug continuously and was associated with a 2.4-fold increase in gastric cancer risk in *H. pylori*-infected subjects (Fox and Muniraj, 2016; Cheung et al., 2017). Moreover, this therapeutic approach is insufficient for complete ulcer healing and intimately associated with ulcer recurrence (Kangwan et al., 2014). For this reason, continued research has been stimulated towards new alternative therapies, such as medicinal plants.

Eugenia punicifolia (Kunth) DC., popularly known as “pedra-ume-caá”, “pedra-ume”, “murta” or “muta”, is a shrub largely distributed in the Amazon region and Savanna biome. Different crude extracts of *Eugenia* show several medicinal effects, such as anti-inflammatory (Leite et al., 2010; Basting et al., 2014), antifungal (Costa et al., 2000), neurological (Grangeiro et al., 2006), antimicrobial (Tenfen et al., 2016), antinociceptive and gastroprotective (Basting et al., 2014), among others. The leaves of this medicinal plant are popularly used in decoctions or aqueous infusions as a natural therapeutic agent to treat gastritis, inflammation (Estrella, 1995), fever and flu (Chaves and Barros, 2012; Silva, 1998), diabetes (Maia et al., 2001) and in alcoholic infusions for the treatment of wounds and infectious diseases (Oliveira et al., 2005).

Basting et al. (2014) also reported the gastroprotective effect of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) in male rats. The gastroprotective effects of HEEP by oral route does not exert its role by antisecretory manner but by augmentation of the protective factors such as mucus secretion. Besides gastroprotective effect of this extract against ulcerogenic agents such as NSAIDs or ethanol, it is desirable for any new antiulcer drug that the preventive effect should be accompanied by ulcer healing effects. In fact, the gastroprotective action of a medicinal plant does not ensure its healing effect (Périco et al., 2015). Therefore, due to the therapeutic properties attributed to this medicinal plant by traditional medicine and previous studies that attributed a gastroprotective effect of the extract in male rats (Basting et al., 2014), in this work we propose to study the effect of the HEEP in the

healing of gastric ulcers induced by NSAIDs or ethanol and to analyze the differences in the healing action of the extract in male and female (intact and ovariectomized) rats.

2. Material and Methods

2.1 Chemicals and Reagents

The following drugs were used: absolute ethanol, acetic acid (Labimpex, Brazil), hydrogen peroxide (Dinâmica analitic reagents, Brazil), methanol (Nuclear, Brazil), lansoprazole (Cruz Vermelha Manipulations, Brazil), carbenoxolone, indomethacin, β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), hexadecyltrimethylammonium bromide (HTAB), trichloroacetic acid (TCA), reduced glutathione, RIPA buffer, glutathione reductase from baker's yeast (*S. cerevisiae*), protease inhibitor cocktail, catalase from bovine liver, xanthine oxidase microbial, hypoxanthine, 2-thiobarbituric acid (J.T.Baker, Austria), nitrotetrazolium blue chloride (NBT), o-dianisidine dihydrochloride, Purpald®, potassium hydroxide, potassium periodate, sodium dodecyl sulfate, potassium phosphate monobasic, potassium phosphate dibasic, ethylenediamine tetraacetic acid (EDTA), sodium hydroxide, bovine serum albumin (BSA), potassium chloride (Sigma Chemical Co., St.Louis, USA). To dilute the hydroalcoholic extract from the leaves of *E. punicifolia* (HEEP), a saline solution (NaCl 0.9%) was used as a vehicle and as a negative control.

2.2 Preparation of the extract

The leaves of *Eugenia punicifolia* were collected at the Forest Institute and Experimental Stations - Assis State Forest at the point 22°33' to 22°37' Lat. S-50°21' to 50°24' Long. W, Assis, State of São Paulo, Brazil. Dr. Antônio C. G. Melo identified the species and a voucher (No. 43322) was deposited in the Herbarium D. Bento Pickel, Assis, SP, Brazil. The extract was prepared based on the Brazilian Pharmacopoeia through the dried and crushed leaves (10 g of plant material) and 100 mL of solvent (ethanol: water 70:30 v/v). Dynamic maceration of the solution was performed for 2h at room temperature (25 $\pm$ 2°C). Thereafter, the solution was filtered, and the residue was extracted two more times. For the drying of the solution the rotary evaporator (40°C) was used and the extract yield was 45% (4.49 g) of the original plant material.

2.3 Animals

The experimental procedures conducted *in vivo* used male and female Wistar albino rats (300–350g) obtained from the breeding facility of the University of Campinas (CEMIB - Unicamp/SP), divided into (8-10 individuals/group): negative control (saline solution, NaCl 0.9%, 10 mL/kg), positive control (lansoprazole [30 mg/kg] or carbenoxolone [100 mg/kg]), hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP - 125 mg/kg, the dose was determined by performing a dose-response curve, based on a previous gastric injury induction test (Basting et al., 2014), naive (male and intact females without fasting and treatment) or sham (suffered the stress of fasting and ovariectomy surgery). Male and female rats were kept in separate rooms, fed by Presence[®] diet, with free access to filtrated water under controlled conditions of illumination (12:12 light/dark cycle) and temperature (22 ± 2 °C). Fasting (2h) was used prior all assays since standard drugs were administered orally. The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. To verify the occurrence of sexual maturity, vaginal cytology was performed 60 days after birth. The estrous cycle was verified through a vaginal smear and the material was observed under an optical microscope and the cycle phase was determined by cytology (Long and Evans, 1922; Mandl, 1951; Marcondes et al., 2002). The duration of the estrous cycle was calculated as the number of days between one estrus phase and the next one. Only female rats that showed two consecutive regular estrous cycles of 4 to 5 days were included in the experiment. It was included a group of ovariectomized rats in ethanol-induced gastric ulcer experiment. We performed bilateral ovariectomy in female 10-week-old rats and after 2 weeks we verified the absence of estrous cycle through a vaginal smear. The NSAID-induced gastric ulcer experiment was performed in male and female rats (intact) and ethanol-induced gastric ulcer experiment was performed with male and female (intact and OVZ) rats with 90 days old. All the experimental practices agreed with the protocols, which have already been submitted and approved by the Ethics Committee on the Use of Animals of the Institute of Biosciences of Botucatu (nº. 675 – CEUA).

2.4 Evaluation of healing activity

2.4.1 NSAID induced gastric ulcer

After 2 hours of fasting, rats (males and intact females) from the different experimental groups received indomethacin (15 mg/kg; p.o. - solubilized in sodium carbonate 0.5%, pH 7.4) for three consecutive days. From the fourth day on, the animals received their respective treatments by the oral route: vehicle, lansoprazole (30 mg/kg) and HEEP (125 mg/kg) for 2 days. At the end of the treatments, the animals were euthanized with an overdose of anesthetic and the stomach was removed for examination of the lesion areas and determination of some parameters (Yamagiwa et al., 2001, with modifications). Stomach lesions were analyzed using the AVSoft Bioview Spectra program. For this, the stomachs were opened, washed, pressed into glass plates and scanned. The ulcerative areas obtained were expressed in mm².

2.4.1.1 Multiplex assay for quantification of cytokines

The Multiplex assay was performed on tissue samples from animals submitted to NSAID (indomethacin) gastric ulcer induction. The gastric mucosa fragments were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680g for 15 min at 4°C. It was determined the content of EGF, IFN γ , IL-1 β , IL-6, IL-10, IL-5, TNF α and VEGF by multiplex microspheres immunoassay (Milliplex® MAP Rat Cytokine/Chemokine Magnetic Bead Panel Kit, RECYTMAG-65K, Millipore Corporation, Billerica, MA, USA), according to the manufacturer's instructions. Each set of microspheres, used in the detection of the respective cytokines, was labeled with a specific staining obtained by the combination of two fluorophores. Microspheres are coated with specific monoclonal antibodies that associate with the target cytokines present in the samples. After incubation of the samples with the microspheres, a mixture of biotinylated antibodies which binds to rats cytokines were added. Biotinylated antibodies are then detected by the phycoerythrin-streptavidin conjugate. The assay was analyzed using the Luminex MAGPIX® (Millipore Corporation, Billerica, MA, USA) fluorescence detection system employing two lasers: the first beam excites the microspheres, classifying them by staining; the second beam quantifies the fluorescence emitted by the phycoerythrin molecules. The

concentration of cytokines in the samples is estimated from standard curves constructed with the aid of xPONENT 4.1 software (Millipore Corporation, Billerica, MA, USA).

2.4.1.2 Determination of myeloperoxidase (MPO) activity

Gastric tissue samples from all experiments (NSAIDs and ethanol 80%) were homogenized with an HTAB buffer (hexadecyl trimethyl ammonium bromide) at a 1:20 ratio (w/v). Next, the samples were sonicated for 10s. The aliquots were centrifuged for 10 min at 4°C, 6540g. In a 96-well plate, 50 µL of supernatant from each sample and 150 µL of reaction buffer (o-dianisidine dihydrochloride) were added. The absorbance was determined at 450 nm. The final concentration was multiplied by 400 and the results were expressed as U/g of tissue (Krawisz et al., 1984).

2.4.2 Ethanol-induced gastric ulcer

After 2 hours of fasting, rats (males, intact and OVZ females) from the different experimental groups received ethanol 80% (v/v) at an oral dose of 8 mL/kg for 2 consecutive days. From the third day, the animals received their respective treatments by the oral route: vehicle, carbenoxolone (100 mg/kg) and HEEP (125 mg/kg). After 1, 2, 4 and 6 days of treatment the animals were euthanized with an overdose of anesthetic and the stomach was removed for analysis of the lesion areas and for determination of biochemical parameters (Liu and Cho, 2000, with modifications). Stomach lesions were analyzed using the AVSoft Bioview Spectra program. For this, the stomachs were opened, washed, pressed into glass plates and scanned. The ulcerative areas obtained were expressed in mm².

2.4.2.1 Determination of superoxide dismutase (SOD) activity

The gastric mucosa fragments were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680 g for 15 min at 4 °C. The supernatant was diluted in phosphate buffer (0.1 M; pH 7.4) in the ratio of 1:20 (w/v). In 100 µL of the homogenate were added 150 µL of cocktail containing: hypoxanthine, xanthine oxidase and nitrobluetetrazolium (NBT) in a ratio 1:1:1. The absorbance was read every minute for 10 min, 37 °C, at 560 nm. The integral was multiplied by 800 and results were expressed as U/g of tissue (Winterbourn et al., 1975).

2.4.2.2 Determination of catalase (CAT) activity

The determination of catalase activity was performed according to the method described by Aebi (1984). The gastric mucosa fragments were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680 *g* for 15 min at 4 °C. The supernatant was diluted in sample buffer [KH₂PO₄ (potassium dihydrogen phosphate), 25 mM, pH7.5; EDTA 1 mM; BSA 1%] in a ratio of 1:20 (w/v). The plate contained 2-wells with catalase, used as a positive control. A 20 µL of the homogenate was placed in a 96-well plate with 100 µL of assay buffer (KH₂PO₄, 250 mM, pH 7.0); 30 µL of methanol and 20 µL of hydrogen peroxide (35.3 mM). The plate was incubated for 20 min at room temperature. After this period, 30 µL of potassium hydroxide (10 mM) and 45 µL of Purpald® was added and the plate was incubated for more 10 min at room temperature. After that, 15 µL of potassium periodate (65.2 mM) was added and the plate was incubated for more 5 min at room temperature. At the end, the absorbance determination was done in a spectrophotometer at 540 nm. The concentration was multiplied by 4000 and results were expressed as U/g of tissue.

2.4.2.3 Determination of reduced glutathione (GSH) levels

The strips of the gastric mucosa were homogenized with a solution of 5% trichloroacetic acid (TCA) in the ratio of 1:20 (w/v). After homogenization, the samples were centrifuged for 10 min at 4 °C, 1520 *g*. In a 96-well plate, 20 µL of the sample supernatant, 5 µL of PBS, 20 µL of 5,5'-Dithiobis (2-nitrobenzoic acid) and 140 µL of β-Nicotinamide adenine dinucleotide 2'- phosphate reduced tetrasodium salt hydrate (NADPH) were added. In a spectrophotometer the plate was incubated at 30 °C for 5 min. After incubation 15 µL of glutathione reductase was added and the absorbance was recorded at 412 nm. The concentration was multiplied by 200 and the results were expressed as nmol/g of tissue (Anderson, 1985).

2.4.2.4 Quantification of malondialdehyde (MDA)

Gastric tissue fragments (80% ethanol) were homogenized with 1.15% potassium chloride (KCl) (1:5 – w/v). The samples were centrifuged for 10 min at 4 °C, 7690 *g*. In the test tubes were added 1500 µL of acetic acid (20%) pH 3.5, 400 µL of distilled

water, 1500 μ L of 2-thiobarbituric acid (TBA) (0.8%) diluted in acetic acid (20%), 400 μ L of supernatant and 200 μ L of sodium dodecyl sulfate (SDS) (8.1%). The test tubes of standard curve contained 100 μ L of an MDA solution of known concentration, 1500 μ L of acetic acid (20%) pH 3.5, 700 μ L of distilled water, 200 μ L of sodium dodecyl sulfate (SDS) (8.1%) and 1500 μ L of 2-thiobarbituric acid (TBA) (0.8%) diluted in acetic acid (20%). The test tubes were incubated for 1h at 95 °C water bath and then were centrifuged for 10 min at 1520 *g*. In a 96-well plate, 200 μ L aliquot of each sample was added. The absorbance was read at 532 nm and the results were multiplied by 50 and were expressed as the nmol of MDA/g of tissue (Ohkawa et al., 1979)

2.5 Statistical analysis

The results were expressed as the means $\pm$ standard error of the mean (S.E.M) of the parameters obtained. The values obtained were subjected to Student's *t*-test (comparisons between two groups) or one-way ANOVA followed by Dunnett's test (compare three or more groups). Data were analyzed using the software GraphPad Prism 6.0. The minimal significance level considered was $p < 0.05$.

3. Results and discussion

Previous studies have already demonstrated antinociceptive, anti-inflammatory and gastroprotective effect of HEEP in rodents. The antinociceptive and anti-inflammatory effects are related to the inhibition of the glutamatergic system, the syntheses of nitric oxide and the inhibition of the phosphorylation of p38 α MAPK (Basting et al., 2014). The effective anti-inflammatory effect of HEEP was followed by the protective effect against exogenous aggressive factors such as NSAIDs or absolute ethanol. But besides gastroprotective action of HEEP, we have previously observed that the gastroprotective effect of a medicinal plant not ensure its healing effect as is desirable in anti-ulcer therapy (Périco et al., 2015).

NSAIDs are widely used nowadays to treat inflammation, fevers and pain in clinical medicine and can be divided into non-selective cyclooxygenases inhibitors (COX-1 and COX-2) and COX-2 selective inhibitors (Marjoribanks et al., 2015). Epidemiologic studies indicate that patients medicated with non-selective NSAIDs have a higher risk of ulcers in the GI tract (Luigi et al., 2016). Non-selective NSAID induction of peptic

ulcers is a multifaceted process that includes the activation of phospholipases A, downregulation of COX-1, upregulation of COX-2 and reactive oxygen species (ROS) and the production of leukotriene B₄, 5-lipoxygenase, PGE₂, TNF- α , IL-1 β and IL-6 (Cheng et al., 2017). Moreover, ROS may also cause oxidative damage to biological macromolecules and react with proteins, lipids and mitochondrial DNA, leading to cells death by affecting various apoptotic pathways in the GI tract (Musumba et al., 2009). Furthermore, NSAIDs also downregulates angiogenesis, inhibits mucosal cellular restitution and promotes extracellular matrix degradation (Tarnawski and Ahluwalia, 2012).

Therefore, in continuity with previous studies with this medicinal plant, we evaluate the healing effect of HEEP using the model of gastric ulcer induced by NSAIDs. This drug cause a gastric mucosal damage due to its ability to decrease the production of prostaglandins (PG) through the inhibition of the enzyme cyclooxygenase (COX) (Musumba et al., 2009).

Figure 1 shows the healing effect of HEEP administered during 2 days after injury induced by indomethacin (15 mg/kg for three consecutive days) in the male and intact female animals, with a normal and preserved estrogen cycle.

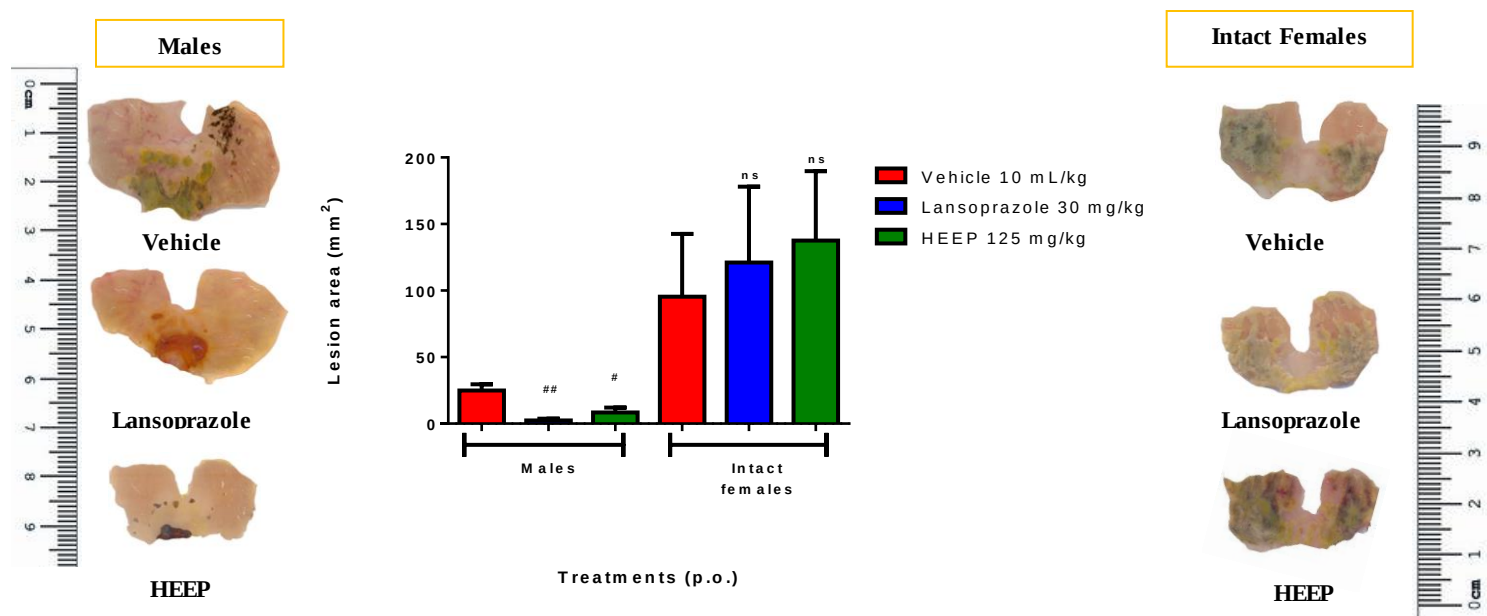


Figure 1: Effect of HEEP administered during two days after injury induced by NSAIDs (indomethacin) in male and female rats (n=5-7). The bars represent the means $\pm$ S.E.M. and statistical significance was determined by ANOVA followed by Dunnett's test; # $p < 0.05$ and ## $p < 0.01$ when compared to the vehicle group of males. ns = not significant.

Treatment with HEEP in male rats, inhibits significantly the gastric lesions by 66% when compared to the vehicle (Figure 1). But, in spite of the expressive curative result of the treatment in male rats, this same extract was not able to heal gastric mucosa against NSAIDs in intact females. In figure 1, we can see that female rats showed no significant difference in the treatment with HEEP as well as lansoprazole ($p > 0.05$).

Our results do not corroborate with the findings of Aguwa (1984) and Borekci et al. (2008) who studied the effects of estrogen and progesterone (alone or in combination), on experimental models of indomethacin-induced gastric ulcer and described the decrease in the lesion area by hormonal effect. In these both studies, male or female rats were used, however, the authors did not report whether there was control over the estrous cycle. In our results, we verify clearly that there is a large variation on the lesion area in the intact female rats and we suppose that it was due to the fact that, although they are cycling, there is no synchronization of the phases of this cycle. The same results occurred with Shore and collaborators (2017) that used male and female rats to evaluate sex difference and the effects of estrogen administration in rat gastric mucosal defense. We supposed that this fact may have generated great interference with our results and based on these we decided to include a new group in the next experiment, ovariectomized rats (OVZ).

In human, estrogen induces vasodilation in a major artery in females but not in males, concluding that there may be gender differences in the effects of estrogen therapy on endothelial functions and nitric oxide (NO) production/release (Kawano et al., 1997). Estrogen has been shown to increase levels of calcitonin gene related peptide (CGRP) in sensory nerve-endings leading to increased blood flow through endothelial production of NO, COX-1 and prostacyclin (PGI₂) which in turn inhibits neutrophil activation reducing inflammatory response (Ham and Kaunitz, 2007; Shore et al., 2017). On the other hands, Sangma et al. (2014) evaluated the effect of ovarian sex hormones on NSAIDs-induced gastric lesions in female rats and concluded that estrogen or progesterone neither worsen nor protect against NSAIDs lesion.

The mechanisms of indomethacin induced gastric mucosal injury include: 1) inhibition of COX-1 and COX-2 enzymes causing a reduction in PGE₂ and PGI₂ generation and a shift of arachidonic acid metabolism to the alternative 5-lipoxygenase pathway resulting in increase in leukotrienes production and 2) increased gastric motility,

resulting in compression of blood vessels thus limiting blood flow thorough microcirculation (Tarnawski et al., 2012). All these factors together probable contributed to remove the protective effect of estrogen as described by Shore and collaborators (2017) and explain our results in intact females submitted to administration of NSAIDs for 3 consecutive days.

Cheng et al., (2017) reported that non-selective NSAID-induced ulcer increase the levels of pro-inflammatory mediators, neutrophil adherence, among other parameters. Based on the healing of the gastric lesion induced by NSAID in male rats we decided to measure some biochemical parameters such as cytokines (pro and anti-inflammatory), myeloperoxidase (neutrophil infiltration marker) and growth factors presented in table 1.

The concentrations of EGF, VEGF, MPO, IL-10, TNF- α , IL-6, IL-1 β and IFN- γ (Table 1) in the gastric mucosa of the male rats treated with HEEP and subjected to NSAID-induced ulcer were not statistically significant when compared to the control group (vehicle) indicating, therefore, that the degree of inflammation promoted by indomethacin was not high enough to significantly alter these cytokines. The same results occur with the group naïve.

Another cytokine that was evaluated in the model of NSAID-induced ulcer was IL-5. Interleukin-5 is a Th2 (T helper) cell-derived hematopoietic cytokine that plays key roles in the proliferation, maturation, migration and survival of eosinophils as well as some modulatory role in the development and function of basophils and mast cells (Molfino et al., 2012). IL-5 and inflammatory immune responses characterizing numerous diseases, such as eosinophilic gastrointestinal diseases (Molfino et al., 2012). The data obtained in table 1 showed that there is reduction of IL-5 cytokine in the gastric mucosa of male rats treated with HEEP and subjected to indomethacin-induced gastric ulcer ($p < 0.05$; Table 1).

Table 1: Effect of HEEP administered during two days after injury induced by NSAIDs (indomethacin) in male rats. Evaluation of biochemical parameters, cytokines and growth factors.

Treatment	EGF (pg/mL)	VEGF (pg/mL)	TNF- α (pg/mL)	IL-1 β (pg/mL)	IL-6 (pg/mL)	IFN- γ (pg/mL)	IL-5 (pg/mL)	IL-10 (pg/mL)	MPO (U/g of tissue)
Vehicle	0.21 $\pm$ 0.05	0.07 $\pm$ 0.01	8.49 $\pm$ 2.32	0.12 $\pm$ 0.01	0.03 $\pm$ 0.00	9.31 $\pm$ 1.21	0.26 $\pm$ 0.03	3.86 $\pm$ 0.35	6419 $\pm$ 708.9
Lansoprazole	0.29 $\pm$ 0.04 ^{ns}	0.08 $\pm$ 0.01 ^{ns}	9.84 $\pm$ 2.23 ^{ns}	0.15 $\pm$ 0.02 ^{ns}	0.04 $\pm$ 0.00 ^{ns}	7.97 $\pm$ 1.47 ^{ns}	0.17 $\pm$ 0.02 *	3.76 $\pm$ 0.46 ^{ns}	5893 $\pm$ 464.4 ^{ns}
HEEP	0.25 $\pm$ 0.04 ^{ns}	0.06 $\pm$ 0.00 ^{ns}	10.40 $\pm$ 1.60 ^{ns}	0.13 $\pm$ 0.01 ^{ns}	0.04 $\pm$ 0.00 ^{ns}	9.8 $\pm$ 1.47 ^{ns}	0.18 $\pm$ 0.01 *	4.41 $\pm$ 0.45 ^{ns}	5242 $\pm$ 309.9 ^{ns}
Naive	0.31 $\pm$ 0.05 ^{ns}	0.11 $\pm$ 0.04 ^{ns}	13.44 $\pm$ 0.89 ^{ns}	0.18 $\pm$ 0.01 ^{ns}	0.05 $\pm$ 0.00 ^{ns}	8.45 $\pm$ 1.1 ^{ns}	0.16 $\pm$ 0.00 *	3.81 $\pm$ 0.38 ^{ns}	4818 $\pm$ 370.1 ^{ns}

The results were expressed as means $\pm$ S.E.M (n = 5-7) and statistical significance was determined by ANOVA followed by Dunnett's test; * $p < 0.05$ when compared to the vehicle control group or by the Kruskal–Wallis followed by Dunn's (VEGF). ns = not significant.

Besides HEEP is effective in the healing of the gastric mucosa of male rats in the model of NSAID-induced gastric ulcer, we decided to continue the healing studies of this extract against the more damage exogenous aggressive factor, ethanol 80%. We evaluated the curative effect of HEEP on gastric ulcers induced by ethanol in rats (male, intact females and OVZ females) during 1, 2, 4, and 6 consecutive days after injury.

Globally, harmful use of ethanol causes approximately 3.3 million deaths every year (or 5.9% of all deaths), and 5.1% of the global burden of disease is attributable to ethanol consumption (WHO, 2014). Among the systems of the body that are affected by prolonged ethanol exposure, the gastrointestinal tract deserves special attention because provokes gastric lesions (Chai, 2011; dos Reis Lívero et al., 2016).

The ethanol-induced gastric mucosal injury model is considered one of the widely used experimental models of ulcer disease (Shawon & Gautam, 2012). Ethanol easily penetrates the gastric mucosa and the ulceration is due to a decrease in gastric mucus, PG levels, glutathione, mucosal blood flow, and bicarbonate secretion as well as an increase in lipid peroxidation, oxidative stress, leukotriene production, and generation of free radicals leading to cell and membrane damage (Szabo et al., 1985; Bhattacharyya et al., 2014).

In virtue of the lesions induced by ethanol 80% (8 mL/kg for two consecutive days) on the gastric mucosa of the rats, we observed that treatment with carbenoxolone (CARB) or HEEP were not able to heal the gastric lesions within 1 (Figure 2a) or 2 days (Figure 2b).

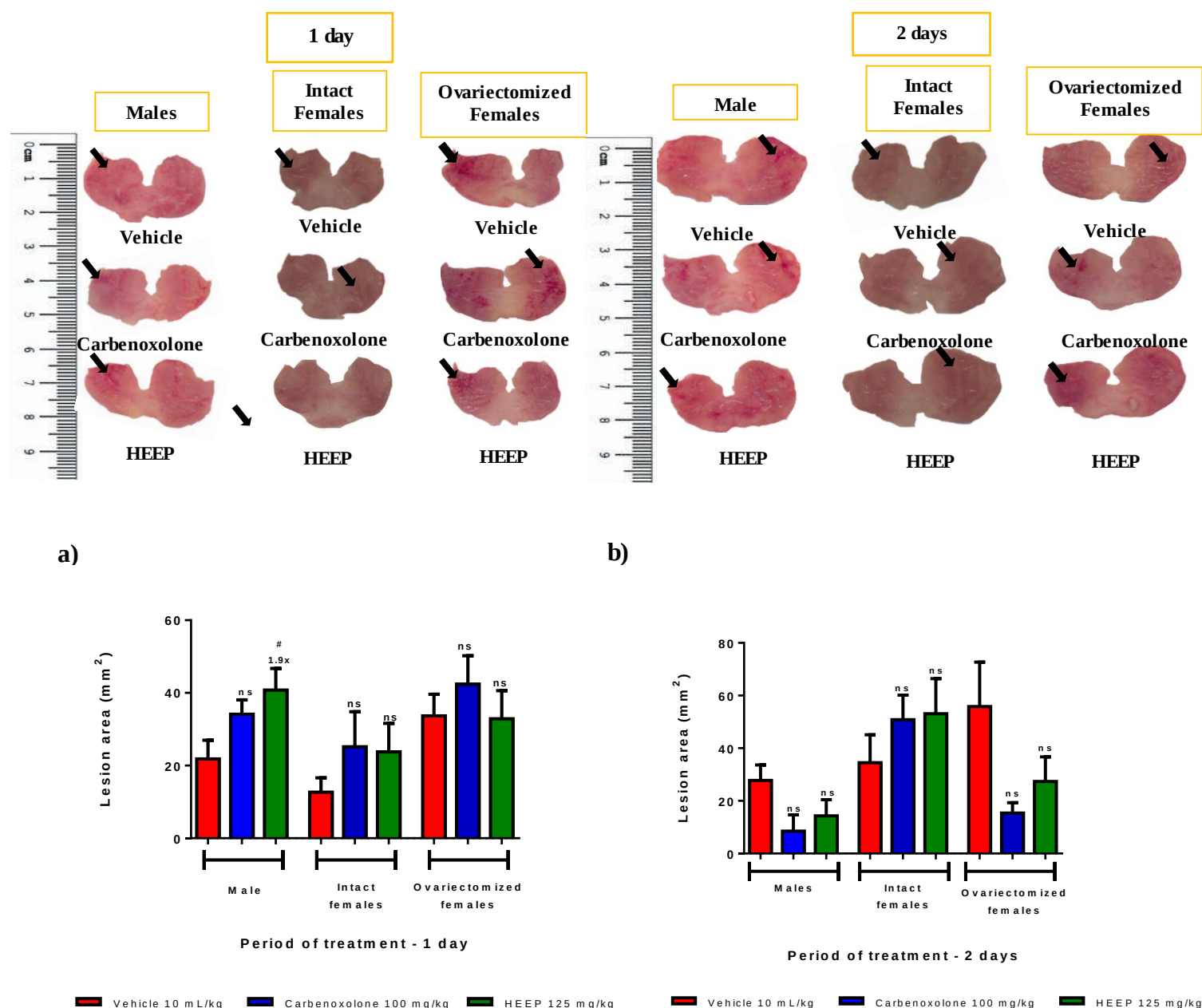


Figure 2: Effect of HEHP administered through one (panel a) or two days (panel b) after damage induced by ethanol 80% in rats (n=8-10). The bars represent the means $\pm$ S.E.M. of lesion area and statistical significance was determined by ANOVA followed by Dunnett's test; # $p < 0.05$ when compared to the vehicle group of males. ns = not significant.

However, the result presented in Fig. 3a shows that the treatments with CARB or HEHP during four consecutive days could heal the gastric ulcers by reducing the lesion area in male (82.52% and 63.23%, respectively) and intact female rats (69.47% and 78.10%, respectively) when compared with the vehicle (control group). From six days of treatment with CARB and HEHP, we can see that there is no difference in any of the treatments in male and intact females, since there aren't lesions in any of the groups that were exposed to ethanol (Fig. 3b).

Despite the healing effect of HEEP on gastric lesions induced by ethanol in males and intact females, the same result was not observed in the OVZ rats (Fig. 3a). It is possible to observe in OVZ rats (fig. 3b) that the healing effect of treatment with CARB or HEEP occurred only after six days of treatment. Both treatments could reduce significantly the lesion area at 65.24% (CARB) and 67.35% (HEEP) when compared to the control group treated with vehicle. So, it is clear that the profile of OVZ female rats differed in healing evolution when compared to male and intact female rats. We can observe that there is an interference of the female sex hormones in the healing of the ethanol-induced lesion since the intact (cycling) rats had healing of the lesion before the OVZ rats.

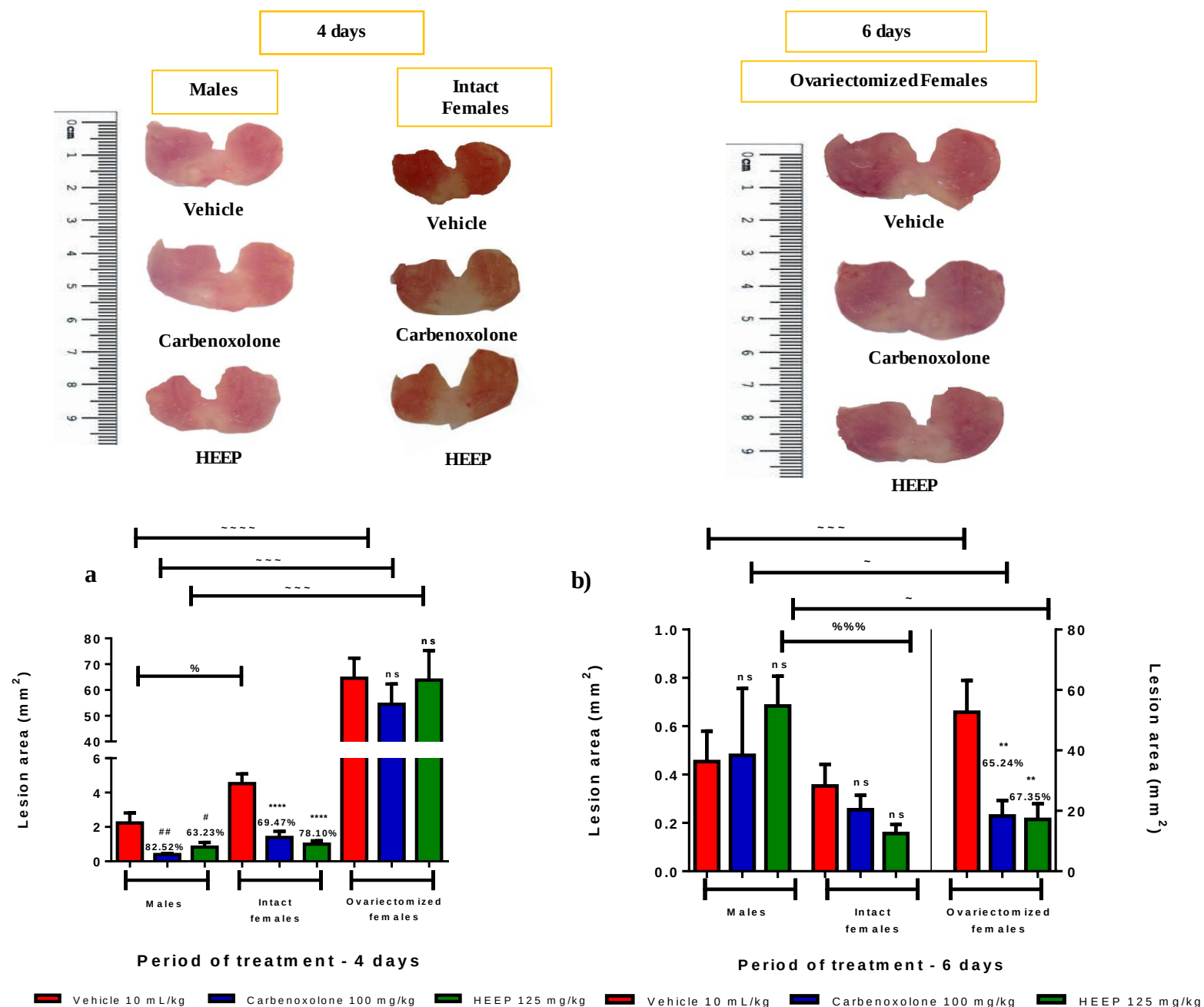


Figure 3: Effect of HEPP administered through four (panel a) or six days (panel b) after damage induced by ethanol 80% in rats (n=8-10). The bars represent the means $\pm$ S.E.M. of lesion area and statistical significance was determined by ANOVA followed by Dunnett's test; # $p < 0.05$ and ## $p < 0.01$ when compared to the vehicle group of males; **** $p < 0.0001$ when compared to the vehicle group of the intact females; ++ $p < 0.01$ when compared to the vehicle group of the ovariectomized females or by the "t" test; % $p < 0.05$ and %%% $p < 0.001$ when compared to vehicle or HEPP group of intact females and males and ~ $p < 0.05$; ~~~ $p < 0.001$ and ~~~~ $p < 0.0001$ when compared to vehicle, carbenoxolone and HEPP groups of ovariectomized females and males. ns = not significant.

Based on the fact that the ethanol induces oxidative stress and increase the infiltration of neutrophils and lipid peroxidation, we decided to evaluate biochemical parameters such as SOD, CAT, GSH, MDA and MPO from gastric tissue of rats after ethanol-induced gastric ulcer (Figures 4, 5, 6, 7 and 8).

SOD is an antioxidant enzyme that catalyzes the conversion of the free radical superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and to molecular oxygen (O_2) (Bhattacharyya et al., 2014). The activity of the SOD enzyme decreases in response to the decomposition of the superoxide anion produced and may lead to deleterious effects while increased activity reduces tissue damage (Bhattacharyya et al., 2014). This response illustrates the detrimental effects of reactive oxygen species (ROS) on tissue damage and the importance of antioxidant activity in promoting health (Bhattacharyya et al., 2014). SOD activity was not involved in the gastric ulcer healing of the rats because treatment with HEEP or CARB in male and female (intact or OVZ) rats after ethanol 80% induced gastric lesion were not able to increase SOD activity (Fig. 4a, b and c) when compared to control group (vehicle).

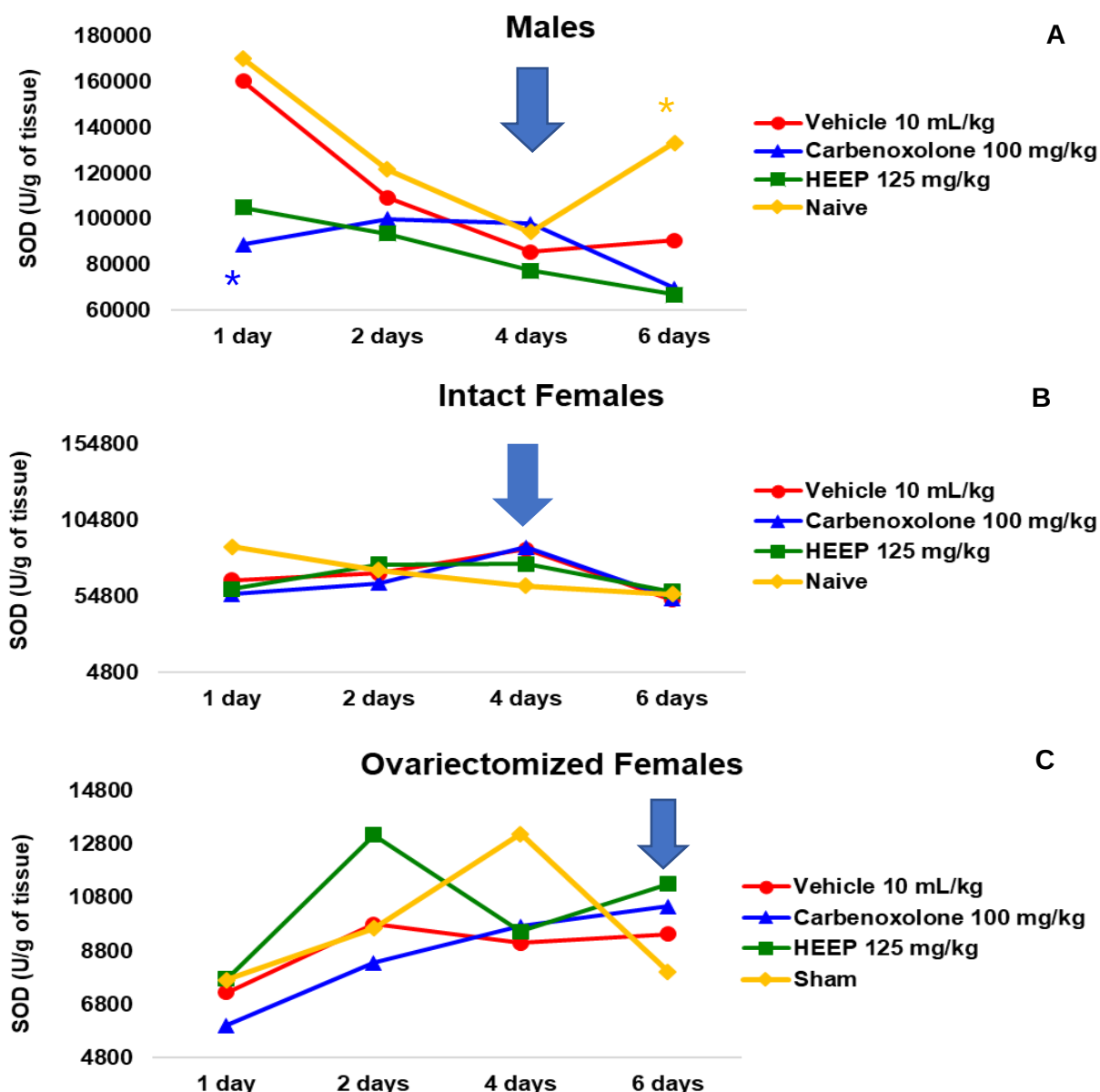


Figure 4: Superoxide dismutase (SOD) activity in the gastric tissues of male (A), intact female (B) and ovariectomized female (C) rats treated with HEEP during 1, 2, 4 or 6 days after injury induced by ethanol 80%. The asterisks denote the significance level when compared with the vehicle control group (ANOVA followed by Dunnett's test) and full arrow indicates when gastric ulcers have healed in HEEP and carbenoxolone groups.

CAT is an antioxidant enzyme that exerts a remarkable protective role in the gastric epithelium against mucosal injury induced by ethanol. It has been demonstrated that ethanol induces oxidative stress and leads to a decrease in CAT activity and glutathione levels (Das and Vasudevan, 2007). In addition, the increase in the enzymatic antioxidant activity of CAT rapidly converts the peroxy radicals to biologically safe substances, such as water (Bhattacharyya et al., 2014). CAT is involved in the healing of OVZ rats, since their activity was increased after 4 days of treatment with HEEP (Fig. 5c, $p < 0.05$). Probably this enzyme is acting in the removal of ROS present in the tissue after

injury and considering that healing only occurs after 6 days of treatment. In the intact female rats, their activity was reduced after six days of treatment of the animals with the extract (figure 5b), data that corroborate that CAT returned to basal levels, because when compared to the naive animals, there was no significant difference between them. This antioxidant enzyme is not involved in the healing effect of HEEP on gastric lesion of male rats (Fig. 5a).

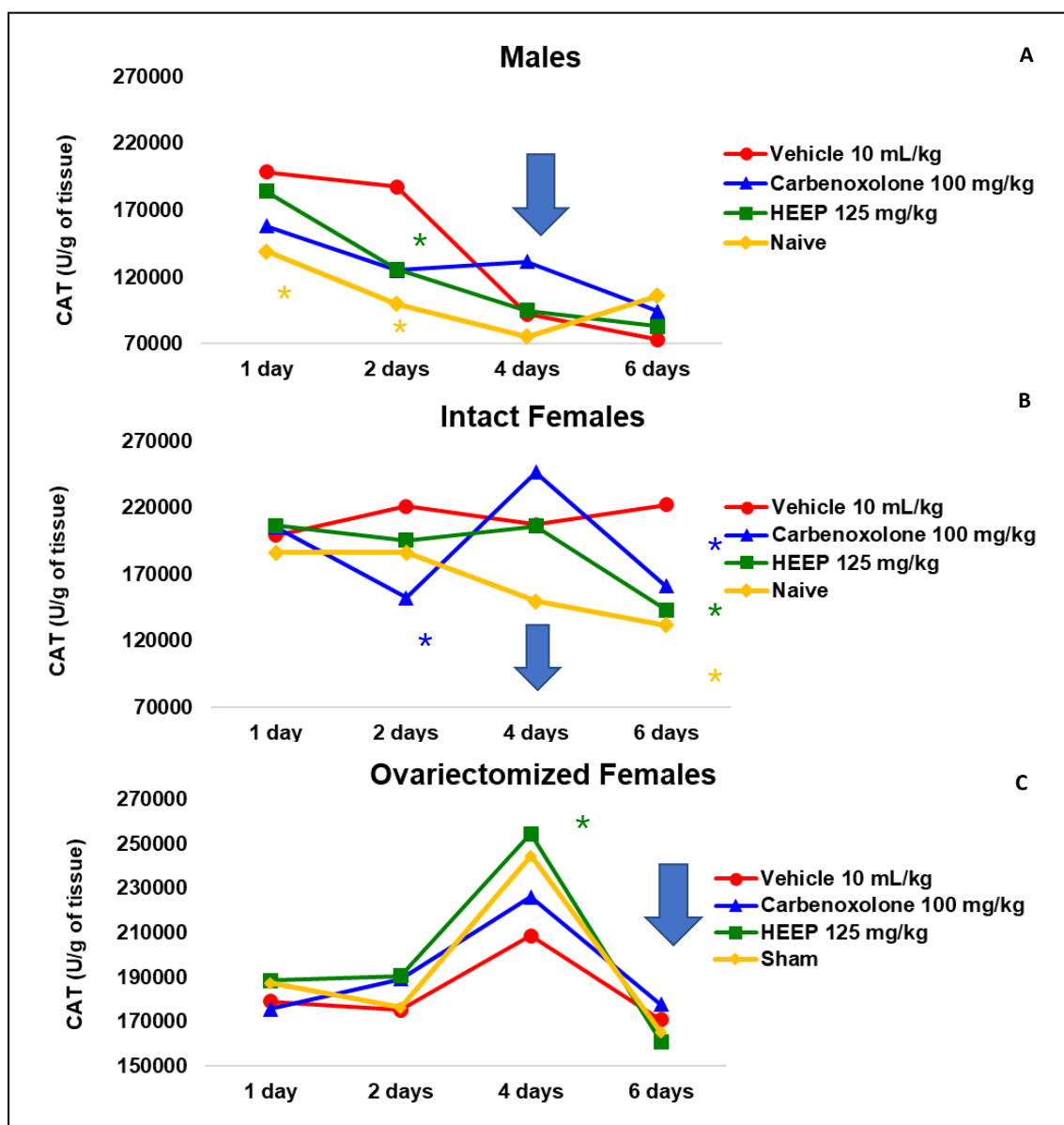


Figure 5: Catalase (CAT) activity in the gastric tissues of male (A), intact female (B) and OVZ female (C) rats treated with HEEP during 1, 2, 4 or 6 days after injury induced by ethanol 80%. The asterisks denote the significance level when compared with the vehicle control group (ANOVA followed by Dunnett's test) and full arrow indicates when gastric ulcers have healed in HEEP and carbenoxolone groups.

The main physiological function of GSH is to eliminate free radicals in the body. It is an important antioxidant because it can protect molecules containing the thiol group in its structure, such as proteins and enzymes (Bhattacharyya et al., 2014). Elevated levels of GSH are essential to maintain the integrity of the gastric mucosa and depletion can induce mucosal ulceration (Bhattacharyya et al., 2014; Hoppenkamps et al., 1984). GSH is not involved in the healing of gastric lesion of HEEP on male rats (Fig 6a), instead GSH is involved in the healing of intact and also OVZ female (Fig 6b and 6C). First there is a decrease in GSH levels previously the healing effect of HEEP in both female groups.

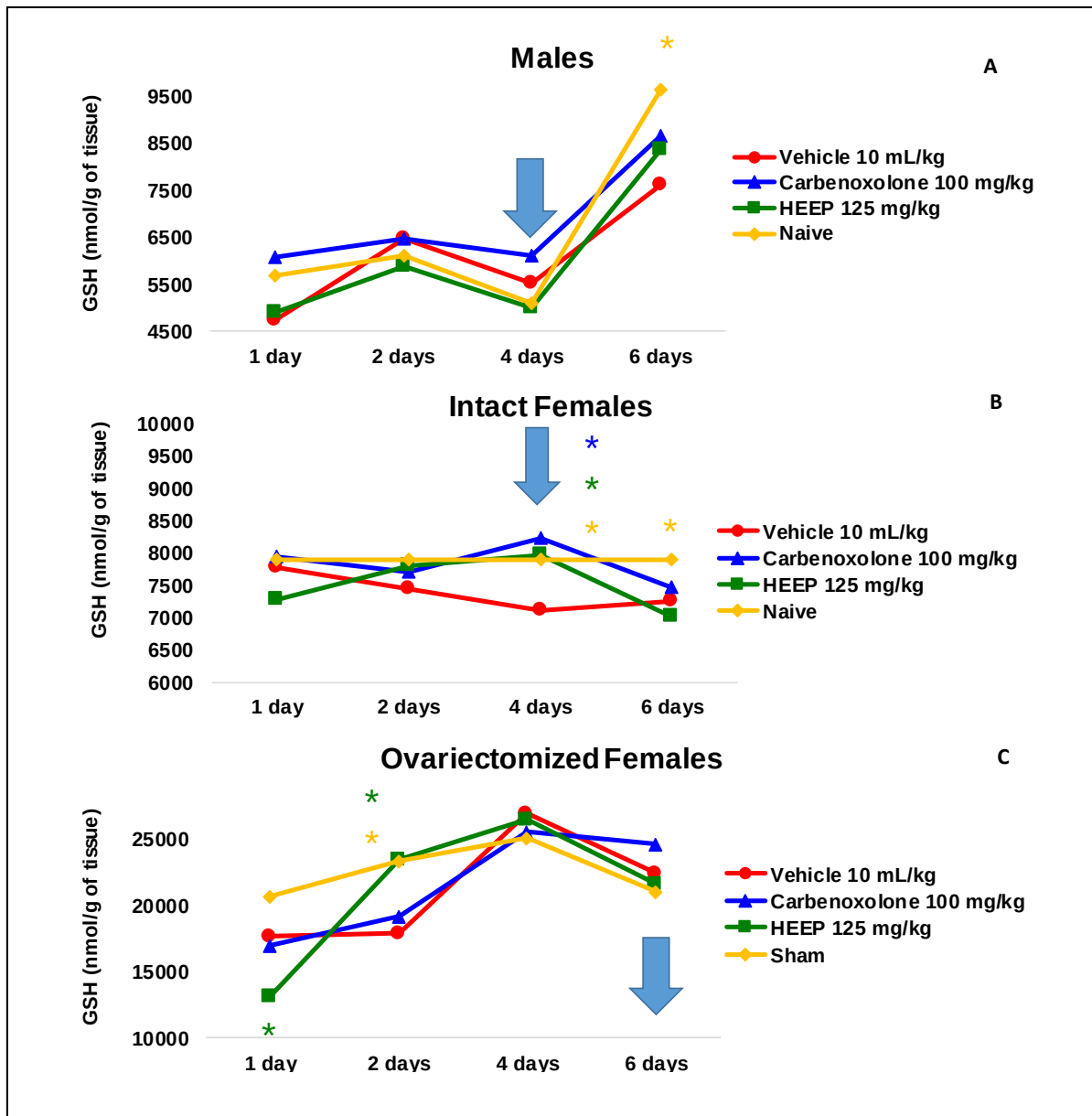


Figure 6: Reduced glutathione (GSH) level in the gastric tissues of male (A), intact female (B) and OVZ female (C) rats treated with HEEP during 1, 2, 4 or 6 days after injury induced by ethanol 80%. The asterisks denote the significance level when compared with the vehicle control group (ANOVA followed by Dunnett's test) and full arrow indicates when gastric ulcers have healed in HEEP and carbenoxolone groups

Ethanol also induces lesion in the vascular endothelium of the mucosa, causing changes in the microcirculation and generating ischemia, which results in the exacerbated production of free radicals (Pan et al., 2008). Ethanol causes a constriction in the submucosa venules with subsequent paralysis in the blood flow in the mucosal microcirculation, as well as dilation of the artery and extravasation of plasma from the vascular bed, resulting in the formation of erosions in the gastric mucosa (Santos and Rao, 2001; Oates and Hakkinen, 1988).

In addition, as previously reported, in human, estrogen was seen to induce vasodilation in a major artery in females but not in males and this estrogen effect was related with a nitric oxide (NO) production/release (Kawano et al., 1997). Recent studies indicate that estrogen can preserve endothelial function by inhibiting smooth muscle proliferation, improving muscle reactivity and its antioxidant actions (Chakrabarti et al., 2014). The results of oxidant pathway analysis demonstrated a discrepancy between the groups of male and intact female rats with the OVZ female rats group. Since the absence of estrogen predisposes to oxidative stress, our results suggest that ethanol could be exacerbated this pro-oxidant response promoted by the absence of the hormone, which may have contributed to increased lesions in the OVZ group when compared to the intact female group (Fig. 2).

Since accumulation of ROS increases lipid peroxidation (LPO), its content in the gastric mucosa can be used as a biomarker for development of ROS-mediated gastric mucosal lesions (Tandon et al., 2004). MDA is the end product of LPO which leads to further cell damage (Ayala et al., 2014). The low levels of MDA shows a reduction of LPO and ROS, which are results from oxidative gastric damage (Tandon et al., 2004; Ayala et al., 2014).

Lipid peroxidation is not involved in the healing process exerted by HEEP in intact and OVZ female rats, considering that this parameter remained unchanged in all treatment periods (Fig. 7b and c). Unlike females, the reduction of lipid peroxidation is involved in the healing process of gastric lesions in male rats (Fig. 7a, $p < 0.01$) after six days of treatment confirming the improvement of the gastric tissue after the healing induced by the extract.

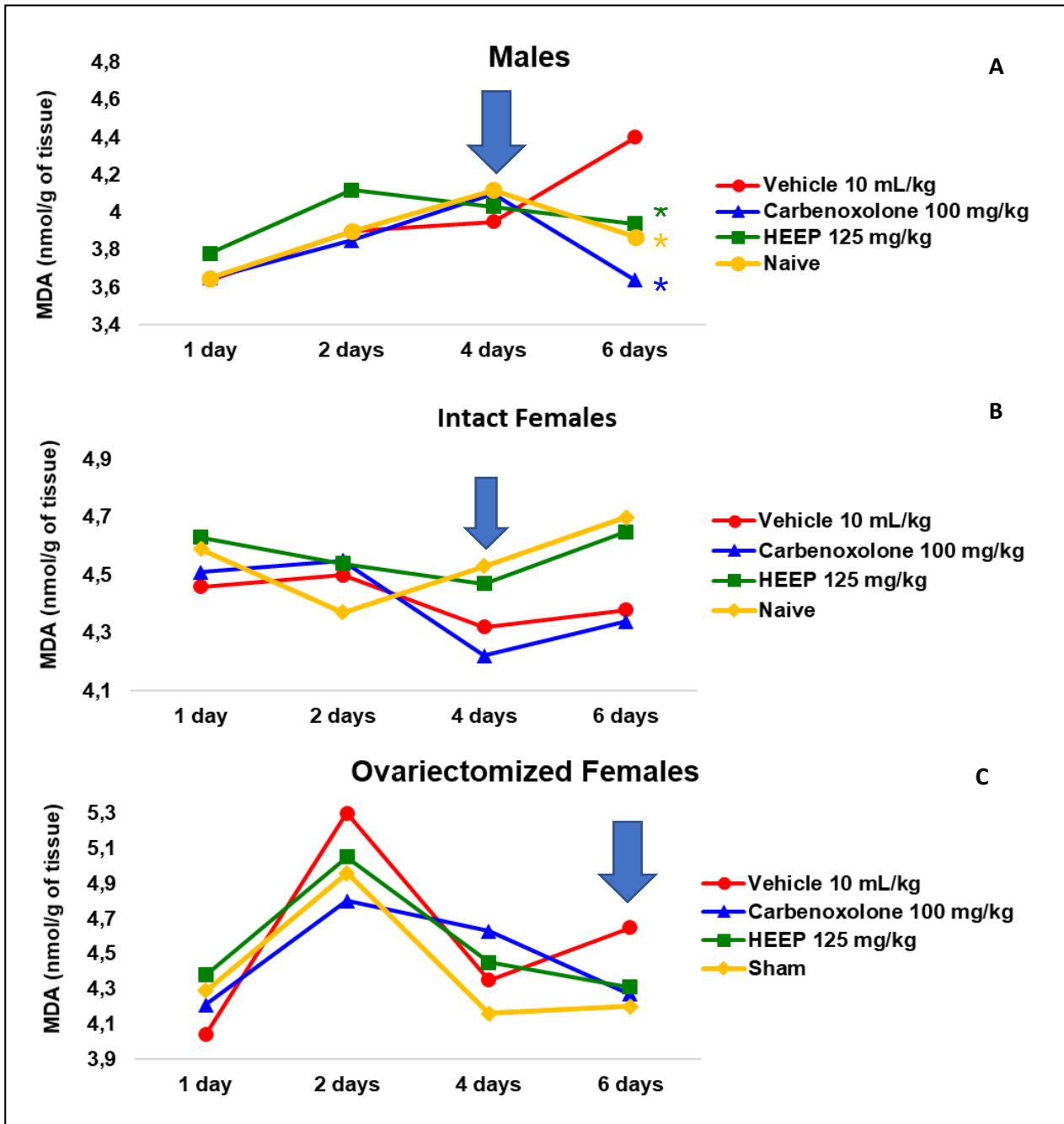


Figure 7: Malondialdehyde (MDA) level in the gastric tissues of male (A), intact female (B) and OVZ female (C) rats treated with HEEP during 1, 2, 4 or 6 days after injury induced by ethanol 80%. The asterisks denote the significance level when compared with the vehicle control group (ANOVA followed by Dunnett's test) and full arrow indicates when gastric ulcers have healed in HEEP and carbenoxolone groups.

Ethanol harshly damages the stomach glandular epithelium leading to augmented neutrophils access into the disrupted stomach mucosa (Kwiecien et al., 2014). The presence of oxygen free radicals resulting from the penetration of neutrophils in the wounded gastric wall can damage stomach gastric mucosa in rats (Parra-Cid et al., 2011). Neutrophils are the main sources of inflammatory mediators and can release potent ROS, which are cytotoxic and can promote tissue injury (Parra-Cid et al., 2011;

Kwiecien et al., 2014). The MPO activity is an index of neutrophil-dependent inflammatory response and neutrophil infiltration in various gastric injuries (Nishida et al., 1997). The interaction of MPO with radicals such as O_2^- , H_2O_2 , and hydroxyl (OH^-) released as a result neutrophil activation produces hypochlorous acid and N-chloramine that lead to tissue damage (Davies, 2011). Our results indicate that HEEP could significantly reduce the area of gastric injury and this healing effect was accompanied the reduction of MPO activity in the gastric tissue of male and OVZ female rats (Fig. 8a and c, $p < 0.01$). MPO is not involved in the healing of the mucosa of intact female rats (Fig. 8b).

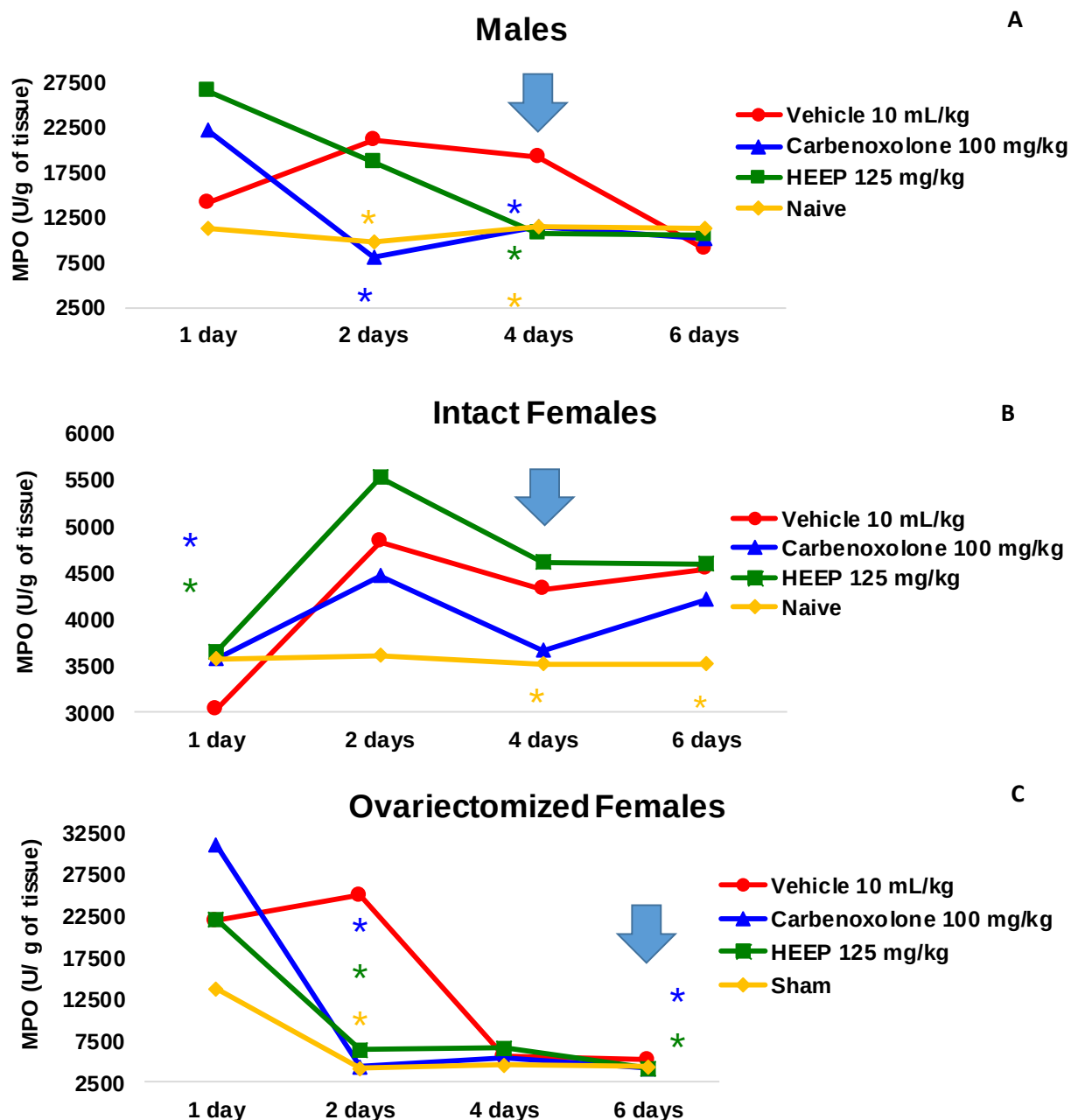


Figure 8: Myeloperoxidase (MPO) activity in the gastric tissues of male (A), intact female (B) and OVZ female (C) rats treated with HEEP during 1, 2, 4 or 6 days after injury induced by ethanol 80%. The asterisks denote the significance level when compared with the vehicle control group (ANOVA followed by Dunnett's test) and full arrow indicates when gastric ulcers have healed in HEEP and carbenoxolone groups.

Therefore, the results presented show that treatment with HEEP in male and female (intact and OVZ) rats after gastric damage induced by ethanol promoted reduction of gastric lesion area after 4 (male and intact female - cycling) or 6 days (OVZ female) of consecutive treatment with significant reduction of the inflammatory process

represented by the decrease of MPO activity, performance of the antioxidant systems (CAT and GSH) and decrease of LPO.

The estrogen participates in healing process of ethanol-induced lesion, since intact rats (cycling) showed a faster recuperation of the lesion area in relation to OVZ female rats. Our results corroborate with the findings of Hajrezaie et al. (2015), which uses females in the model of induction of gastric ulcers by ethanol and shows the reduction of the lesion area. However, in our studies, we included a control group of intact female rats (we used only cycling rats) and OVZ rats and used subacute ethanol induction treatments. The same does not occur with the study presented by Hajrezaie and collaborators (2015) that besides using intact female rats, they perform the gastric induction and the treatment of the injuries acutely.

The ethanol lesion predominantly affects the glandular portion of the stomach (Sidahmed et al., 2013) and can also be attributed to rupture of endogenous mucus and gastric acid secretion, as well as damage to the gastric mucosa with hemorrhagic lesions (Rajasekaran et al., 2012). The mucus bicarbonate barrier is the first line of defense of the gastric mucosa (Laine et al., 2008), serving as barrier against autodigestion caused by acid and pepsin (Allen and Flemström, 2005). Therefore, any quantitative or qualitative disturbance of this secretion can be deleterious. Carbenoxolone, a drug used as a positive control in this model, is a pharmacological tool with secreting properties of mucus (Henman, 1970), which certainly contributed to the reduction of lesion area in all experimental groups (male rats, intact females and OVZ). The results of Basting et al. (2014) clearly demonstrate that the gastroprotective activity of HEEP is dependent on the production of adhered mucus (in an acute model), so it should not be ruled out that, like carbenoxolone, HEEP healing activity on ethanol-induced ulcer model, may be related to the production of gastric mucus.

4. Conclusion

The present study demonstrates the ulcer-healing action of the hydroalcoholic extract from the leaves of *Eugenia punicifolia*. The HEEP could heal the gastric lesions, protecting the mucosa of highly damaging agent such as NSAIDs and ethanol 80% and this effect is modulated by female sex hormones, since the intact rats showed healing before the OVZ rats. The curative effect of the extract is also mediated by a reduction of

IL-5, neutrophils infiltration, a decrease of lipid peroxidation, increase in catalase activity and reduced glutathione levels in gastric mucosa.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the conception and design of the study, performed most of the experiments, coordinated the study, participation in writing the manuscript and approved the final version of the article to be published.

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CAPÍTULO 3

Research Article

International Journal of Molecular Sciences

Antioxidant and anti-inflammatory potentials of *Eugenia punicifolia* in healing gastric and duodenal injury induced by ischemia-reperfusion in male and female rats

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Abstract: *Eugenia punicifolia* (Kunth) DC. (Myrtaceae), popularly known as “murta”, is a shrub largely distributed in the Amazon region and Cerrado biome. The leaves of this medicinal plant are popularly used as a natural therapeutic agent to treat inflammation, wounds and infections. This study evaluated the healing effect of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) in rodent models. The HEEP was evaluated against the gastric or duodenal ulcers induced by ischemia and reperfusion (IR). We assessed the levels of reduced glutathione (GSH) and malondialdehyde (MDA), as well as superoxide dismutase (SOD), myeloperoxidase (MPO) and catalase (CAT) activities. The oral administration of HEEP (125 mg/kg) for 6 consecutive days significantly decreased the severity of gastric [male (73.86%); intact female (52.83%); ovariectomized female (43.13%)] and duodenal [male (57.03%); intact female (56.04%); ovariectomized female (54.83%)] damages induced by IR when compared to the control group (vehicle). The HEEP exerts its curative effects by increasing the CAT and SOD activities in the gastric mucosa and decreasing the MPO activity and GSH levels in the duodenal mucosa. Treatment with HEEP administered during six consecutive days in male and female (intact and ovariectomized) rats after gastric/duodenal injury induced by IR, could heal the mucosa acting as antioxidants and anti-inflammatory.

Keywords: *Eugenia punicifolia*; Myrtaceae; ischemia-reperfusion; healing effect.

1. Introduction:

Ischemia-reperfusion (IR) injury often occurs in many clinical and surgical procedures, especially those involving the gastrointestinal tract [1]. Ischemic injury occurs when the blood supply to an area of tissue is interrupted. Both experimental studies using several animal models as well as clinical studies involving patients undergoing surgery show that damage to the mucosa occurs during reperfusion [2]. Reperfusion of ischemic tissues is often associated with microvascular dysfunction [3].

Ischemia causes formation of xanthine oxidase which breaks down with the excess of hypoxanthine produced during reperfusion, resulting in the formation of toxic reactive oxygen species (ROS) [4]. This increase in ROS levels causes a breakage in the cell membrane integrity, thereby resulting in ulcer formation [5]. Antioxidants control mechanisms and their activity depend on the type, the site and process of generation of ROS, as well as if it prevents or delay oxidative damage [6].

Antioxidant agents have demonstrated to protect the gastrointestinal mucosa against IR-induced damages in many reports [7-9]. The mechanisms involved in the protection include the scavenge of free radicals, the reduction of lipid peroxidation, and the maintenance of glutathione levels [9], suggesting that rich natural sources of antioxidants may be useful in mitigating IR injury [1].

Recently, studies showed that phenolics compounds from plants have proved to be more potent antioxidants than Vitamins C, E and even carotenoids [11-12]. Extracts from plants are composed by a great diversity of compounds including flavonoids, tannins, etc. that have antioxidant properties [1], so it is reasonable to suggest that an extract could have biological activity due to the combined action of all compounds [12].

Our hypothesis is that the extract of *Eugenia punicifolia* with its high content of flavonoids, tannins, terpenoids, saponins, as described by Oliveira et al. (2006) and proanthocyanidins, galotannins, quercetin, gallic acid, ellagic acid and myricetin derivatives [14-15], should offer healing actions in clinical situations involving IR injury.

There is no information on the role of *Eugenia punicifolia* during healing of IR injury. Therefore, the aim of our study was to investigate the curative role of hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP) in gastric and intestinal ischemia-reperfusion injury in male and female Wistar rats.

2. Results:

HEEP (125 mg/kg) given orally once daily for six days in male rats in the IR model decreased the gastric lesion area ($p < 0.01$) (Figure 1) when compared to vehicle group. Animals treated with lansoprazole (30 mg/kg) were also able to significantly heal the gastric mucosa ($p < 0.05$).

Gastric healing was also evaluated in the IR induction model in intact and ovariectomized female rats treated for 6 consecutive days with HEEP and lansoprazole. Treatment with HEEP showed a reduction in the lesion area in the intact (52.83%) and ovariectomized (43.13%) female rats when compared to the control group (vehicle), as well as lansoprazole (Figure 1). When we compare the group of intact females with ovariectomized females, there is no significant difference in relation to the lesion area for the groups treated with HEEP or lansoprazole (Figure 1).

In Figure 1, we can observe that males when compared to intact females subjected to gastric ulcers induced by IR, showed a reduction of the lesion area in the treatment with HEEP ($p < 0.01$). There is no significant difference between male and ovariectomized females treated with HEEP in relation to lesion area. Among the three groups evaluated, the males showed better gastric healing in the IR injury when treated with HEEP.

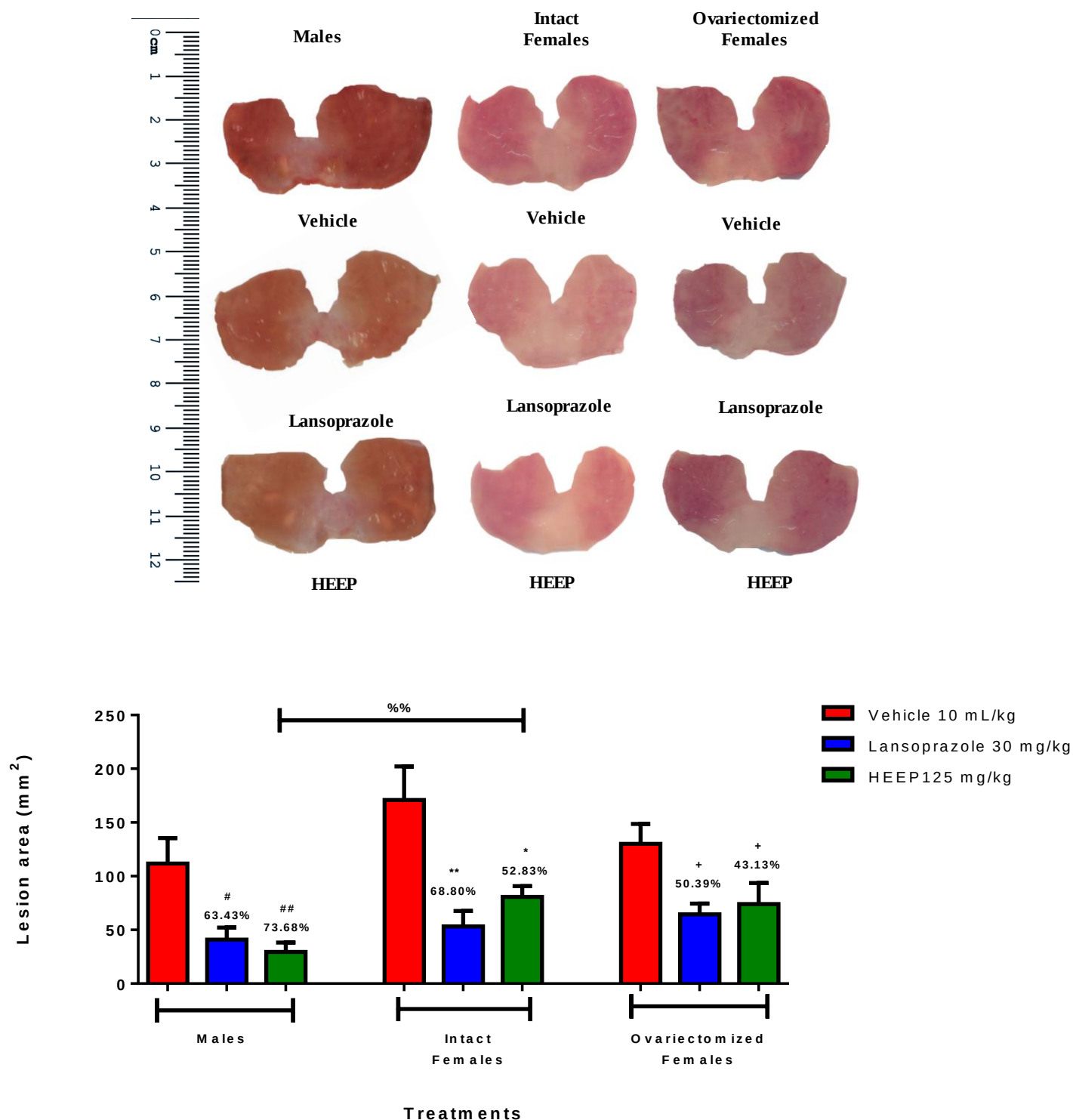


Figure 1: Effect of the oral administration of the HEEP and lansoprazole on the healing of ulcers produced by ischemia-reperfusion in the stomach of Wistar rats. The results were expressed as mean $\pm$ S.E.M (n = 7-8) and statistical significance was determined by ANOVA followed by Dunnett's test; # $p < 0.05$ and $p < 0.01$ when compared to the vehicle group of males; * $p < 0.05$ and ** $p < 0.01$ when compared to the vehicle group of intact females (cycling); + $p < 0.05$ when compared to the vehicle group of ovariectomized females or Student's t-test; %% $p < 0.01$ when compared HEEP group of males and intact females. The percentage indicates healing in relation to the vehicle group. ns = not significant.

In addition to the lesion area, we also evaluated the biochemical parameters that determine the neutrophils infiltration (activity of MPO), the lipid peroxidation (MDA level) and the antioxidant enzymes (GSH, SOD and CAT).

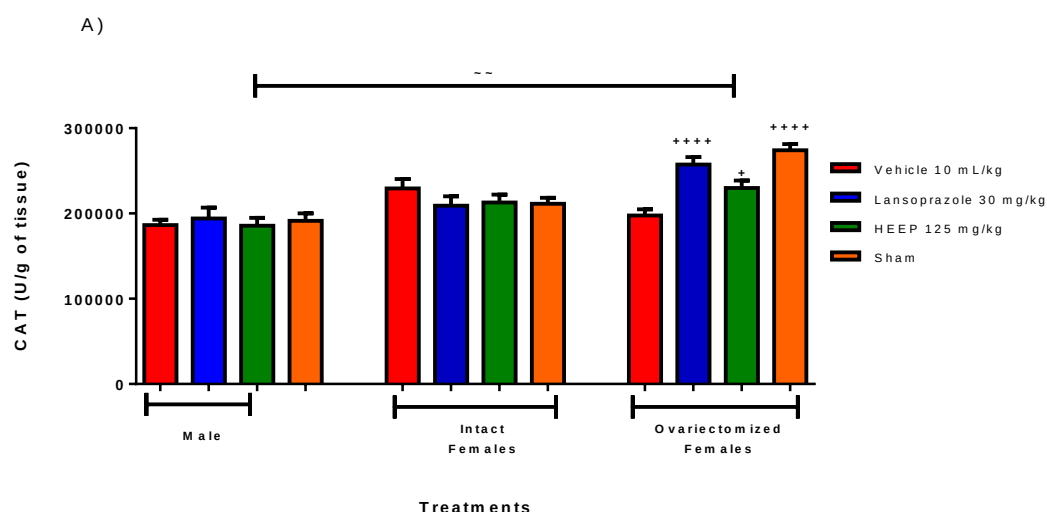
Treatment during six days with HEEP or lansoprazole did not change any evaluated biochemical parameters in males. The same results occurred with the group of sham animals when compared to vehicle (Figure 2).

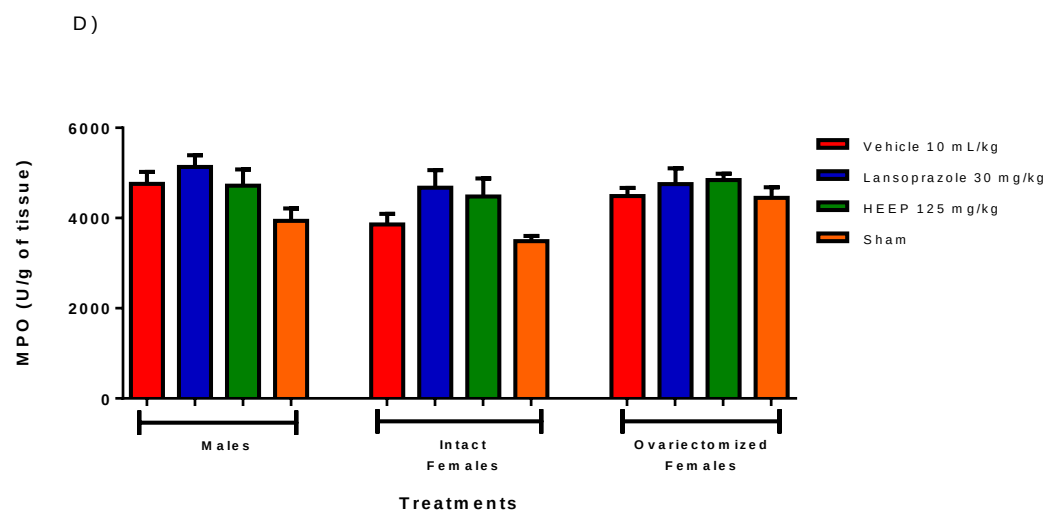
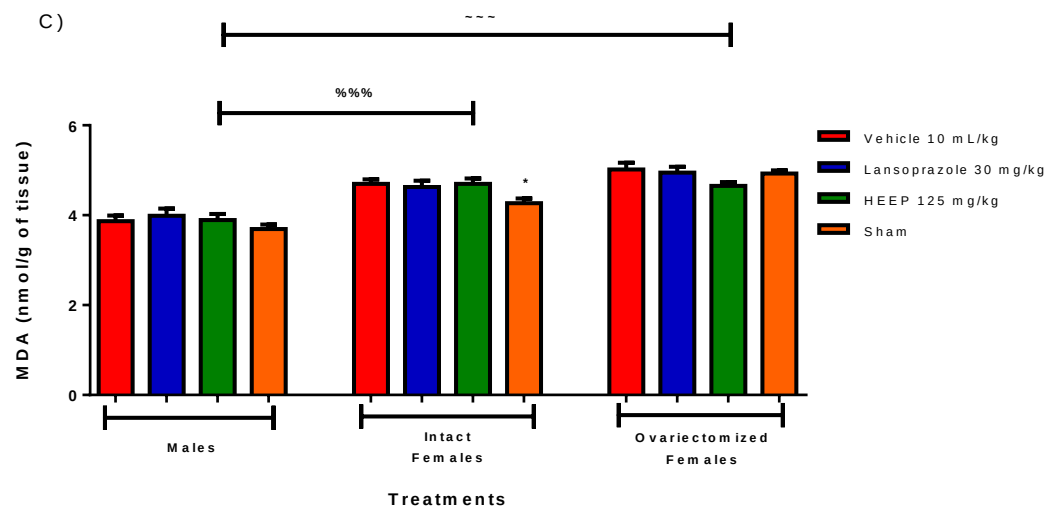
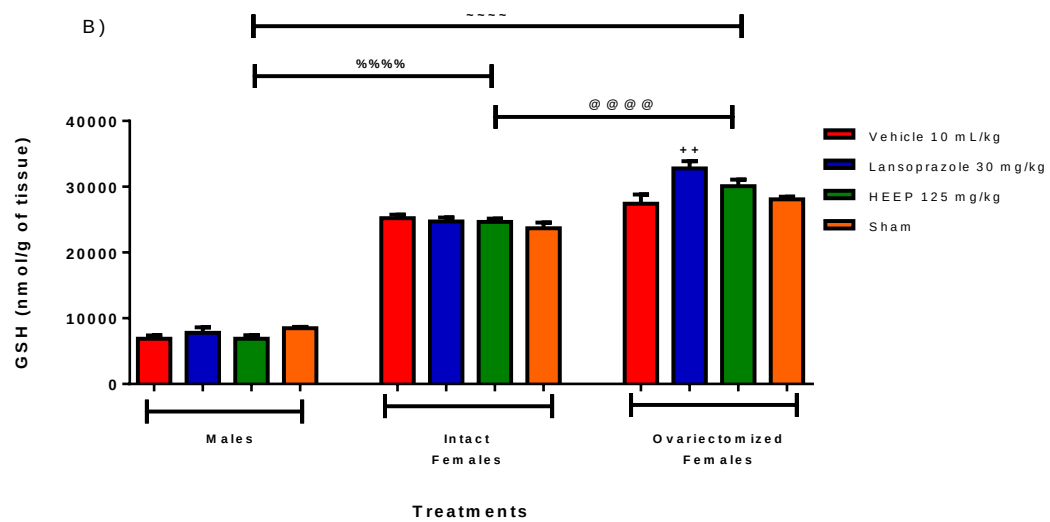
Similar to the group of male rats, the intact female group have no change in the biochemical parameters in the group of animals that received HEEP or lansoprazole during six consecutive days. As expected, intact female rats treated with vehicle had an increase in MDA levels and decrease in SOD activity when compared to the sham group (Figure 2 C and E).

Treatment during six days with HEEP was able to increase CAT and SOD activities ($p < 0.05$) in the ovariectomized female rats when compared to the control (vehicle) group. The lansoprazole group also increased GSH levels ($p < 0.01$) and CAT ($p < 0.0001$) and SOD ($p < 0.01$) activities when compared to the vehicle group of ovariectomized female rats. As expected, group treated with vehicle had a significant reduction in CAT activity (Figure 2 A, B and E) when compared to sham animals. In figure 2A, we can observe another difference between the group of male and ovariectomized females treated with HEEP in which CAT activity is increased in the latter group ($p < 0.01$).

When we evaluated the GSH levels, we can note a significant increase in the group of females (intact and ovariectomized) that received HEEP in relation to the group of males ($p < 0.0001$; figure 2B). After six days of treatment with HEEP, we also can observe that MDA levels in males is decreased in comparison with both group of females (intact and ovariectomized) ($p < 0.001$; figure 2C).

There is an increase in SOD activity between the groups of ovariectomized and intact females ($p < 0.001$) that was treated with HEEP during six days (figure 2E).





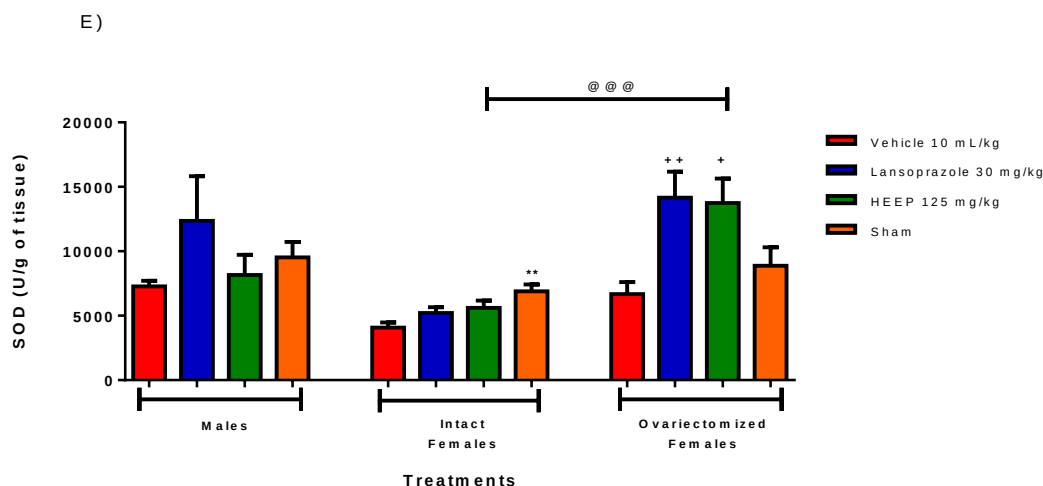


Figure 2. Effects of the HEEP on the gastric IR lesions. A) Catalase (CAT) activity; B) Reduced glutathione (GSH) levels; C) Malondialdehyde (MDA) levels; D) Myeloperoxidase (MPO) activity and E) Superoxide dismutase (SOD) activity in the gastric tissues of male, intact female and ovariectomized female rats. The results were expressed as mean $\pm$ S.E.M (n = 7-8) and statistical significance was determined by ANOVA followed by Dunnett's test; * $p < 0.05$ and ** $p < 0.01$ when compared to the vehicle group of intact females (cycling); + $p < 0.05$; ++ $p < 0.01$ and +++ $p < 0.0001$ when compared to the vehicle group of ovariectomized females or Student's t-test; %%% $p < 0.001$ and %%% $p < 0.0001$ when compared HEEP group from males to intact females; ~ $p < 0.01$, ~ ~ $p < 0.001$ and ~ ~ ~ $p < 0.0001$ when compared HEEP group from males to ovariectomized females; @@@ $p < 0.001$ and @@@@ $p < 0.0001$ when compared HEEP group from intact females to ovariectomized females.

Male rats treated with HEEP administered for six consecutive days healed the duodenal mucosa submitted to IR, when compared to vehicle group. As expected, there was an increase in the duodenal lesion area of vehicle treated animals. Oral treatments with lansoprazole and HEEP significantly reduced the extent of ulcerative lesions by 62.29% and 57.03%, respectively, in relation to the vehicle treated group (Figure 3).

Duodenal healing in the IR induction model was evaluated in intact (cycling) and ovariectomized female rats treated for six consecutive days with HEEP and lansoprazole. HEEP showed a reduction in the lesion area in the intact (56.04%) and ovariectomized (54.83%) female rats when compared to the control group (vehicle). Lansoprazole was also able to heal gastric mucosa in intact ($p < 0.05$) and ovariectomized rats ($p < 0.0001$). There was a reduction in the lesion area of vehicle and lansoprazole groups of intact ones when compared to ovariectomized rats (Figure 3).

In figure 3, we can observe an increase in lesion area of all treatments of intact females when compared to males submitted to IR induced duodenal ulcers. There was a decrease in the lesion area of the lansoprazole group of ovariectomized females when compared to males.

Such as gastric lesion, among the three groups evaluated, the males group showed better duodenal healing in the IR injury when treated with HEEP.

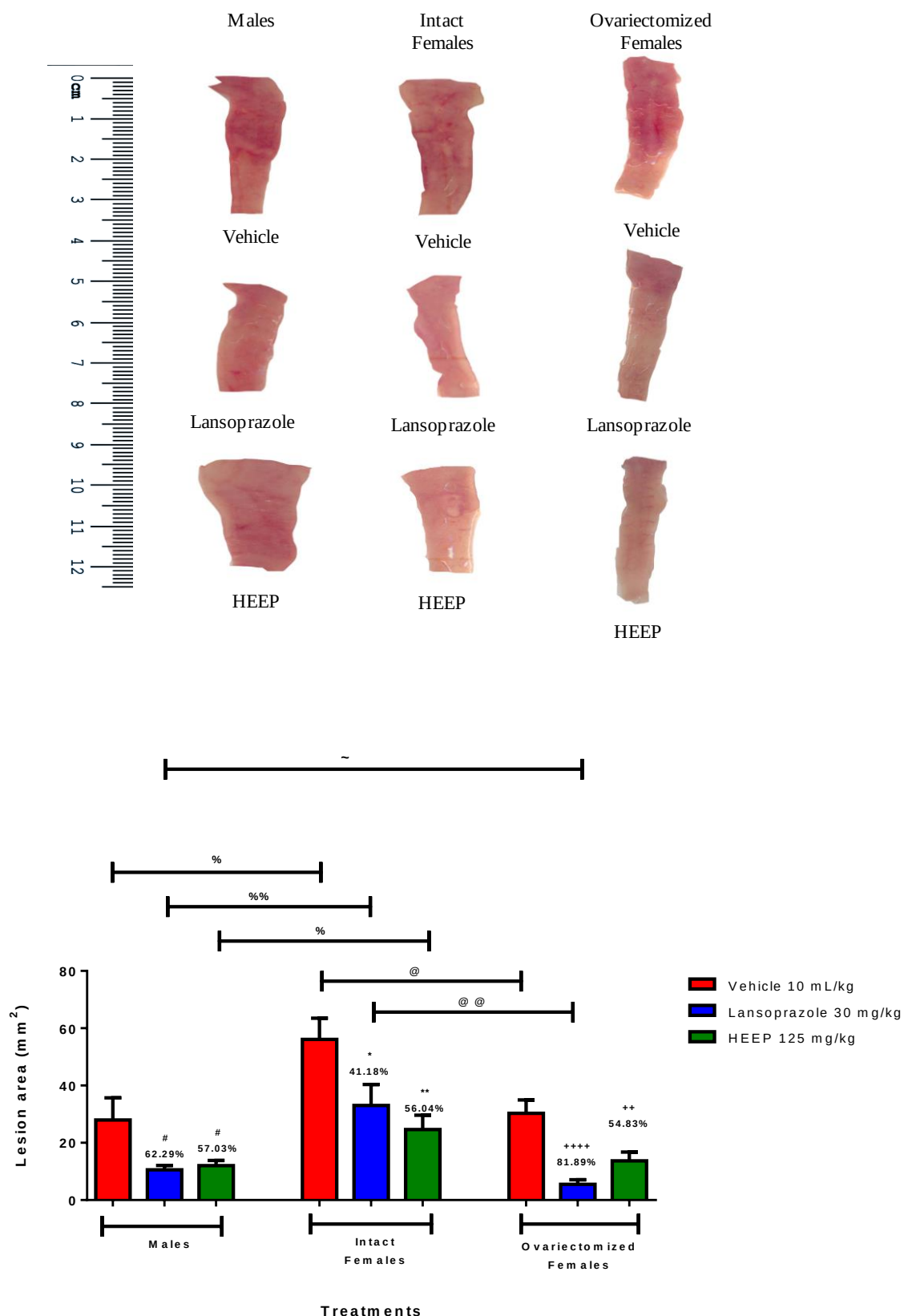


Figure 3: Effect of the oral administration of the HEEP and lansoprazole on the healing of ulcers produced by ischemia-reperfusion in the duodenum of Wistar rats. The results were expressed as mean $\pm$ S.E.M ($n = 7-8$) and statistical significance was determined by ANOVA followed by Dunnett's test; # $p < 0.05$ when compared to the vehicle group of males; * $p < 0.05$ and ** $p < 0.01$ when compared to the vehicle group of intact females (cycling); ++ $p < 0.01$ and ++++ $p < 0.0001$ when compared to vehicle group of ovariectomized females or Student's t-test; % $p < 0.05$ and %% $p < 0.01$ when compared vehicle, HEEP and lansoprazole groups of males and intact females; ~ $p < 0.05$ when compared

lansoprazole group of males and ovariectomized females; @ $p < 0.05$ and @@ $p < 0.01$ when compared vehicle and lansoprazole groups of intact females and ovariectomized females. The percentage indicates healing in relation to the vehicle group. ns = not significant.

Figure 4 shows the results obtained with biochemical parameters in the duodenal ulcer model induced by IR in rats. The biochemical parameters analyzed were the levels of GSH and MDA, the activity of MPO, SOD and CAT in the duodenum mucosal tissue.

Treatment for six days in males with HEEP and lansoprazole was not able to change the biochemical parameters when compared to the control group (vehicle). In male rats, vehicle group decreased MDA levels when compared to sham (Figure 4 C).

The treatment with HEEP and lansoprazole in intact female rats for six days reduced the MPO ($p < 0.01$) activity when compared to the control (vehicle) group. Animals that received vehicle treatment had an increase in MPO, MDA, CAT and SOD activities when compared to sham animals (Figure 4 A, C, D and E).

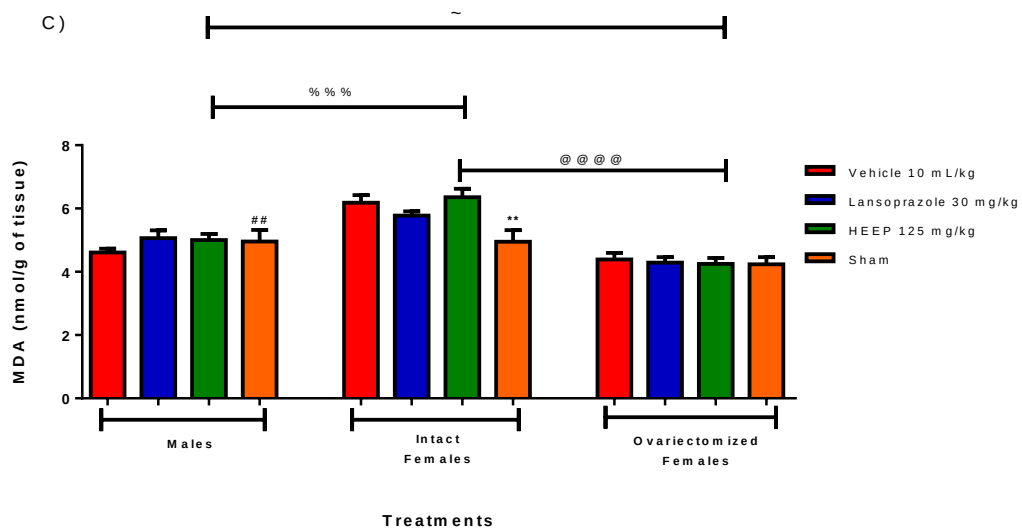
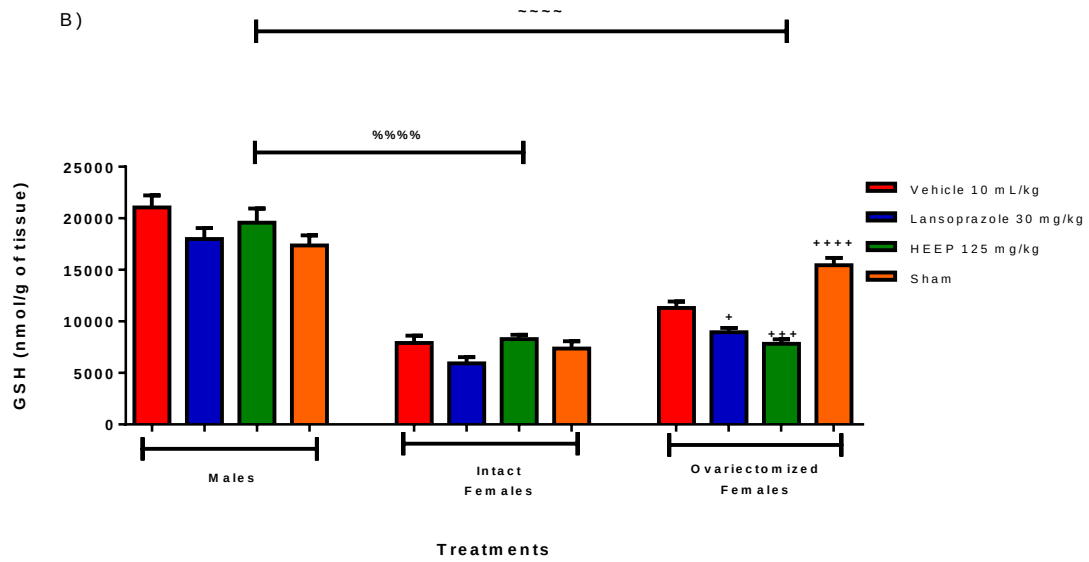
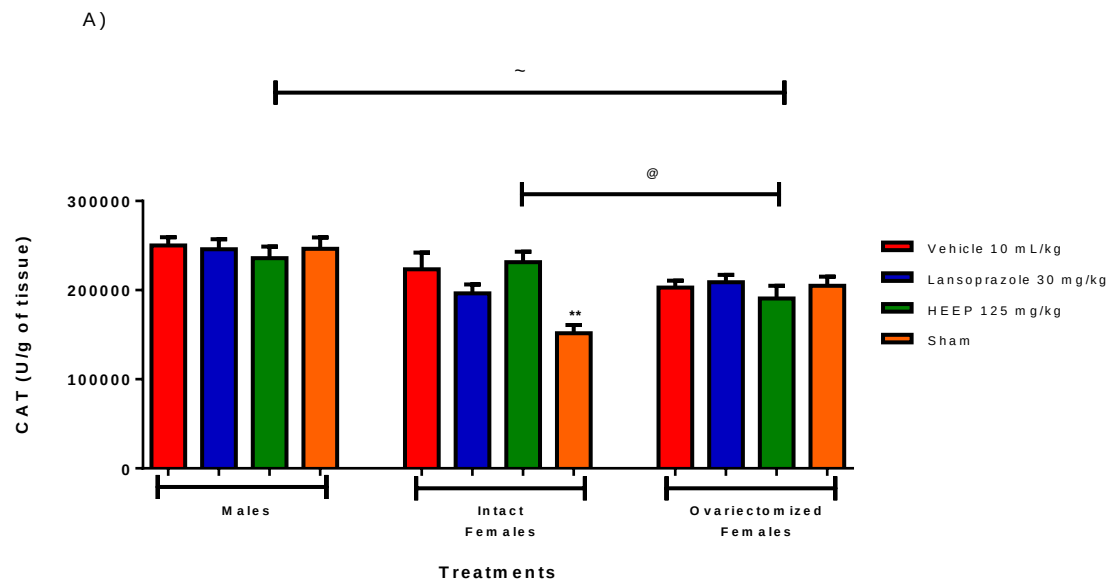
Differently, ovariectomized female rats showed a significant reduction in GSH levels in the groups treated with HEEP and lansoprazole for six consecutive days when compared to the control group (vehicle). Animals that were treated with vehicle had a reduction in GSH levels and increase in MPO activity when compared to the sham group (Figure 4 B and D).

In figure 4A, we can see a difference between the group of animals treated with HEEP between male and ovariectomized females when CAT activity is increased in males ($p < 0.05$). The same results occurred with intact females when compared to ovariectomized females ($p < 0.05$) when CAT activity is increased in intact female group. Therefore, among the three groups treated with HEEP, ovariectomized females presented lower CAT activity.

When we evaluated the GSH levels, we can observe a decrease in the group of females (intact and ovariectomized) in relation to the group of males that received HEEP ($p < 0.0001$; figure 4B). Same results were observed in relation to MPO activity. There is an increase in MPO activity between the groups of males in relation to the female rats (intact and ovariectomized) that were treated with HEEP for six days (figure 4D).

After six days of treatment with HEEP, we can observe that MDA levels in males is decreased in comparison with intact females ($p < 0.001$; figure 4C). The same result was observed in the group of intact in comparison with ovariectomized females ($p < 0.0001$). MDA levels is high in intact female that was treated with HEEP in relation to male or ovariectomized female ($p < 0.05$; figure 4C).

Our results highlight the SOD activity is increased in ovariectomized females ($p < 0.0001$) in relation to males and intact females that received HEEP for six days (figure 4E).



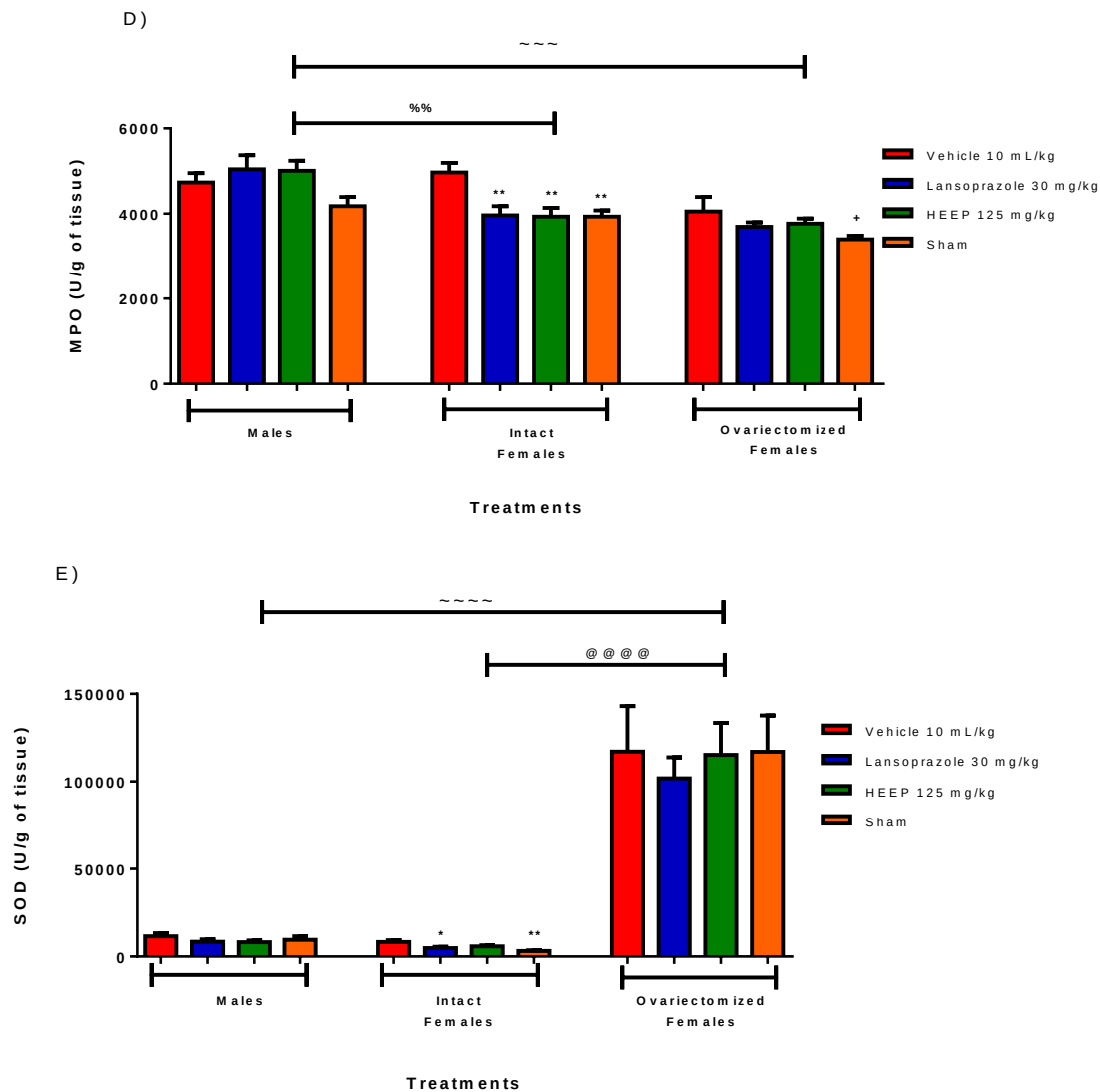


Figure 4. Effects of the HEEP on the IR lesions. A) Catalase (CAT) activity; B) Reduced glutathione (GSH) levels; C) Malondialdehyde (MDA) levels; D) Myeloperoxidase (MPO) activity and E) Superoxide dismutase (SOD) activity in the duodenal tissues of male, intact female and ovariectomized female rats. The results were expressed as mean $\pm$ S.E.M (n = 7-8) and statistical significance was determined by ANOVA followed by Dunnett's test; ## $p < 0.01$ when compared to the vehicle group of males; * $p < 0.05$ and ** $p < 0.01$ when compared to the vehicle group of intact females (cycling); + $p < 0.05$; +++ $p < 0.001$ and ++++ $p < 0.0001$ when compared to the vehicle group of ovariectomized females or Student's t-test; %% $p < 0.01$, %%% $p < 0.001$ and %%%% $p < 0.0001$ when compared HEEP groups of males and intact females; ~ $p < 0.05$, ~~~ $p < 0.001$ and ~~~~ $p < 0.0001$ when compared HEEP groups of males and ovariectomized females; @ $p < 0.05$ and @@@@ $p < 0.0001$ when compared HEEP groups of intact females and ovariectomized females.

3. Discussion:

IR-induced gastric injury is a common clinical problem that can lead to acute lesions in the gastric mucosa [16]. Many clinical conditions, including vascular rupture or surgery, gastrointestinal ischemia, hemorrhagic shock and stress may contribute to gastric IR injury [17]. However, there are few satisfactory methods for the clinical treatment of injury [18]. The gastric IR is caused by obstruction of the celiac artery, mainly by the arched medial ligament [21,22]. IR is a well-recognized pathological condition characterized by an initial deprivation of blood supply to an area or organ, followed by

vascular restoration and tissue reoxygenation [21]. The pathophysiology of IR is associated with inflammatory responses, characterized by the release of inflammatory cytokines, neutrophil infiltration and generation of free oxygen radicals, which cause tissue damage [22]. Ischemia weakens the mucosal barrier, increases the diffusion of acid and predisposes the mucosa to damage [17,18]. After reperfusion, ROS are produced, which can lead to tissue lipoperoxidation, induce cell death and mucosal injury.

Antioxidants have been documented to override ROS deleterious effects by direct scavenging, reducing the number of oxidants in and around the cells, preventing ROS from reaching their biological targets, limiting the propagation of oxidants such as the ones occurring during lipid peroxidation hence inhibiting oxidative stress [25]. Basting *et al.* described the phytochemical composition of HEEP and revealed the presence of some compounds that can act as antioxidants [14].

For the first time in the literature, our results show that the HEEP was able to heal the gastric mucosa of male and female (intact and ovariectomized) rats with six days of treatment, after the IR process in the celiac artery (fig. 1). In this work, lesion areas and biochemical parameters (GSH, CAT, SOD, MDA and MPO) were evaluated in animals submitted to different treatments and exposed to gastric IR (Figure 1 and 2).

Although the area of gastric lesion was reduced by treatment with HEEP, in male and intact females (figure 2), the antioxidant and anti-inflammatory biochemical parameters analyzed did not show relationship with gastric IR-induced healing. But in ovariectomized female rats, HEEP acts by removing ROS from tissue after injury (figure 2) when we observed that there is a significant increase in CAT and SOD activities (antioxidant enzymes) after treatment with HEEP (figure 2A and E).

SOD is an antioxidant enzyme that can be found in the cytosol, mitochondria and extracellularly [26]. It catalyzes the conversion of excess superoxide anions and converts them to H_2O_2 [27]. CAT then scavenges H_2O_2 and converts it to water [6]. Decreased SOD/CAT activity may lead to deleterious effects [28], while increased activity reduces tissue damage [29]. This response illustrates the detrimental effects of ROS on tissue damage and the importance of antioxidant activity in promoting health [30].

SOD and CAT have an important role in the redox balance. In normal cell metabolism, the ROS are generated at a low velocity and these oxidative radicals are eliminated by antioxidant defense system [26]. When gastric mucosa lesion occurs, there is a reduction in SOD and CAT activity, which leads to an increase in ROS formation [25].

In Figures 1 and 3, we can observe that intact females showed a greater lesion area when compared with males subjected to ulceration induced by IR process (gastric and duodenal). The comparison between the lesion area of ovariectomized females and male was not altered significantly. Some studies have been developed about the sex differences in gastric and duodenal mucosa defense and there is an unexplained difference between the sexes regarding the incidence of peptic ulcer disease. Some studies have tried to show that these sex differences could be due to the protective effect of female sex hormone.

Our results have shown the opposite that has been speculated. Bülbül *et al.* (2008) used females in their study of gastric IR, which showed a reduction in injury in female rats, but this study not present control of the estrous cycle of the rats which certainly affect the final results. Du *et al.* (2010) shown that the severity of gastric ulcers induced by the IR process is attenuated by the effect of estradiol. However, these authors used male rats treated with estradiol as animal model and our study used both male and female (intact and ovariectomized) rats. In contrast, Drago *et al.*, 1999 demonstrated that estradiol aggravated ulcer healing in female rats [33].

Recently Shore *et al.*, (2017) found a sex difference in gastric corpus mucosa blood flow of male rats. Gastric mucosal blood flow is higher in male than in female rats and is reduced in male rats by estrogen administration [34]. Probable this low blood flow in female rats can contributed to the aggravation of process of gastric and duodenal IR when compared to the male rats. Additionally, Paré and Redei (1993) have described that females are more vulnerable to stress and that the female sex hormone influences susceptibility to ulcers. Further studies are necessary to evaluate possible mechanisms related to the role of sex hormones in ulcerogenic diseases.

In addition to IR induced gastric lesions, we also evaluated the intestinal lesion in this study. IR-induced intestinal damage is a potentially fatal condition that can be induced by organ transplantation, shock, thrombotic or embolic disease in the mesenteric artery. Duodenal IR occurs very frequently in elderly [36]. Despite recent improvements in diagnosis and health care, the mortality rate of patients with intestinal IR remains high [29-31].

Complete ischemia by temporary vascular obstruction (with clamps) or permanent vascular occlusion (by ligature) of the superior mesenteric artery in rodent models is currently the most commonly used method to induce intestinal ischemia [40]. After passing through an IR process in the superior mesenteric artery (duodenal IR), treatment with HEEP reduced the hyperemic lesions formed (Figure 3) in both male and female (intact and ovariectomized) rats.

IR can cause severe intestinal damage leading to subsequent destruction of organs such as the liver, lungs and kidneys. In the process of IR occurs the intestinal barrier dysfunction and bacterial translocation, resulting in the release of inflammatory cytokines, overproduction of ROS and even apoptosis [41].

From the results presented on HEEP healing in the IR-induced duodenal ulcer model, the next step was to evaluate the participation of the antioxidant and anti-inflammatory system in this process (Figure 4).

In the duodenal IR model, the biochemical parameters analyzed (CAT, GSH, MDA MPO and SOD) corroborate with the results obtained in the gastric IR model for male rats (figure 4), since these parameters are not directly related with healing of the lesion. In intact female rats, HEEP acts by decreasing MPO activity after injury (figure 4D), an enzyme that indicates inflammatory process.

MPO is an inflammatory enzyme found in neutrophils that acts on the reaction between H_2O_2 and chloride ion (Cl^-). The products of this enzyme modify biomolecules during an exacerbated inflammatory process, indicating that the action of MPO may be

beneficial or harmful. Some phytochemicals inhibit MPO activity [42]. Considering the phytochemical composition of HEEP, where we have gallic acid, ellagic acid, myricetin derivatives, among others constituents, some studies have reported that gallic acid and its derivatives has proven anti-inflammatory action in ulcerative colitis by significantly reducing the MPO activity and the expression of inflammatory mediators such as COX-2 and inducible nitric oxide synthase (iNOS) [43] and myricetin significantly alleviated I/R-induced rat intestinal injury by reducing the generation of pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6 and by reducing MPO activity [44]. Our results corroborate with the findings of these authors, since we have a reduction in MPO activity.

The treatment with HEEP in ovariectomized female rats acts reducing GSH levels in the duodenal tissue (figure 4B). The decrease observed in GSH levels may be the result of an unbalanced turnover involving increased consumption of GSH in detoxifying peroxides, a reduction in gamma-glutamyl-cysteinyl synthetase activity, and a lack of availability of cysteine, glycine, and methionine, the limiting amino acids for GSH synthesis [45]. The main physiological function of GSH is to eliminate free radicals of the body. It is an important antioxidant because it can protect molecules containing the thiol group in its structure, such as proteins and enzymes [46]. Elevated GSH levels are essential to maintain intestinal mucosal integrity and depletion can induce mucosal ulceration [47].

There are a great number of evidence for the protective effects of estrogen against IR injury in the heart (increase Ca²⁺-independent NOS activity and cGMP content) and brain (decreases the extent of cell death), our results additionally evidence this effect of estrogen in the intestinal IR [48].

Figure 3 indicates that intact female rats showed an increase in lesion area after treatment with HEEP when compared to males subjected to duodenal IR ulceration procedure. The lesion area of males and ovariectomized females, which were treated with HEEP for six days, was not altered (figure 3). We can infer the participation of female sex hormones in the healing of the duodenal lesion. These results corroborate with the findings of Liu and colleagues (2015), in which the severity of IR-induced duodenal ulcers in female rats was shown to be attenuated after treatment with the test substance. We did control the estrus cycle of the female rats, the area of injury of the intact female rats was greater than that of the male rats. The same control did not occur in the studies presented by Liu and collaborators. The results of our study indicate that further studies are needed to delineate and clarify the mechanisms by which female sex hormones are effective in the treatment of gastrointestinal lesions.

Therefore, the results showed that treatment with HEEP for six consecutive days in male and female (intact and ovariectomized) rats after duodenal IR induced injury promoted healing effect with a significant reduction of the anti-inflammatory process represented by the decrease in MPO activity. The reduced GSH levels are also related to the healing of HEEP. Other possible mechanisms by which HEEP would be acting on duodenal healing would be the modulation of pro and anti-inflammatory cytokines and apoptosis. Female sex hormones play a role in the healing of the duodenal IR-induced lesion since intact (cycling) female rats obtained a larger lesion area when compared to males.

Current research has been focused on the development of new therapies that aim at the period of reperfusion, inhibiting the oxidant lesion and neutrophil infiltration [32]. Our results corroborate with this research when indicate a probable effect of HEEP in the reperfusion period, acting on the metabolites of the ROS.

Our results showed for the first time that treatment with HEEP for six consecutive days can heal the gastric and duodenal mucosa after injury induced by IR and that there are an antioxidant and anti-inflammatory involvement in this action. The female sex hormones are involved in this healing effect, but further studies are necessary to clarify these mechanisms.

4. Materials and Methods:

Chemicals and Reagents

The following drugs were used: hydrogen peroxide (Dinâmica analitic reagents, Brazil), methanol (Nuclear, Brazil), lansoprazole (Cruz Vermelha Manipulations, Brazil), β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), hexadecyltrimethylammonium bromide (HTAB), trichloroacetic acid (TCA), reduced glutathione, RIPA buffer, glutathione reductase from baker's yeast (*S. cerevisiae*), protease inhibitor cocktail, catalase from bovine live, Xanthine Oxidase microbial, Hypoxanthine, 2-thiobarbituric acid (J.T.Baker, Austria), Nitrotetrazolium Blue chloride (NBT), o-dianisidine dyhydrochloride, Purpald®, Potassium hydroxide, Potassium periodate, sodium dodecyl sulfate, Potassium phosphate monobasic, Potassium phosphate dibasic, Ethylenediaminetetraacetic acid (EDTA), Sodium hydroxide, Bovine serum albumin (BSA), potassium chloride (Sigma Chemical Co., St.Louis, USA). To dilute the hydroalcoholic extract from the leaves of *E. punicifolia* (HEEP), a saline solution was used as a vehicle and as a negative control.

Plant material

In December 2009, Dr. Catarina dos Santos collected the leaves of *Eugenia punicifolia* at the Forest Institute and Experimental Stations - Assis State Forest at the point 22°33 'to 22°37' Lat. S-50°21 'to 50°24' Long. W, Assis, State of São Paulo, Brazil. Dr. Antônio C. G. Melo identified the species and a voucher (No. 43322) was deposited in the Herbarium D. Bento Pickel, Assis, SP, Brazil.

Preparation of plant extract

The extract was prepared from the dried and crushed leaves (10 g of plant material) and 100 mL of solvent (ethanol: water 70:30 v/v). Dynamic maceration of the solution was performed for 2h at room temperature (25 $\pm$ 2°C). Thereafter, the solution was filtered, and the residue was extracted two more times. For the drying of the solution the rotary evaporator (40°C) was used and the extract yield was 45% (4.49g) of the original plant material.

Animals

The experimental procedures conducted *in vivo* used male and female Wistar albino rats (300–350g) obtained from the breeding facility of the State University of Campinas (CEMIB - Unicamp/SP), divided into (8-10 individuals/group): negative control (saline solution - 0.9% NaCl – 10 mL/kg, positive control (lansoprazole - 30 mg/kg), hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP - 125 mg/kg, the dose was determined by performing a dose-response curve, based on a previous gastric injury induction test [14] or sham (animals that did not undergo injury, but who went through the same stress of fasting and surgery). Male and female rats were kept in separate rooms, fed by Presence[®] diet, with free access to filtrated water under controlled conditions of illumination (12:12 light/dark cycle) and temperature (22 ± 2 °C). The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. The estrous cycle was verified through a vaginal smear. The material was observed under an optical microscope and the estrous cycle phase was determined by cytology [46-48]. The vaginal cytology of the female rats was checked daily starting on postnatal day (PND) 60. The duration of the estrous cycle was calculated as the number of days between one estrus phase and the next one. Only female rats that showed two consecutive regular strains cycles of 4 to 5 days were included in the experiment. It was included a group of ovariectomized rats. All the experimental practices agreed with the protocols, approved by the Ethics Committee on the Use of Animals of the Institute of Biosciences of Botucatu (nº. 675 – CEUA).

Gastric Ischemia and reperfusion (I/R) lesions – healing model.

Male and female Wistar rats (n=8-10) were divided into different groups after 16h of fasting. Then, the animals were anesthetized with a solution of ketamine (80mg/kg) and xylazine (8 mg/kg) injected intraperitoneally. An incision of approximately 3 cm was made on the left side of the abdomen and the celiac trunk was isolated with a clamp, which remained on the animal for 1 hours. After the period of ischemia, the clamp was removed to allow reperfusion, the animals were sutured and returned to their respective environments. Elapsed 24 hours of injury induction, treatments with vehicle (saline - 0.9% NaCl, 10 mL/kg), HEEP (125 mg/kg) or lansoprazole (30 mg/kg) started. After six days of daily treatments, the animals were euthanized with an overdose of anesthetic and the stomach removed for examination of the lesion areas (AvSoft Bioview software) and determination of biochemical parameters [52], with modifications.

Intestinal Ischemia and reperfusion (I/R) lesions – healing model.

Male and female Wistar rats (n=8-10) were divided into different groups after 16h of fasting. Then, the animals were anesthetized with a solution of ketamine (80mg/kg) and xylazine (8 mg/kg) injected intraperitoneally. An incision of approximately 3 cm was made on the right side of the abdomen and the superior mesenteric artery was isolated with a clamp, which remained on the animal for 1 hours. After the period of ischemia, the clamp was removed to allow reperfusion, the animals were sutured and returned to their respective environments. Elapsed 24 hours of injury induction, treatments with vehicle, HEEP or lansoprazole started. After six days of daily treatments, the animals were euthanized with an overdose of anesthetic and the duodenum removed for

examination of the lesion areas (AvSoft Bioview software) and determination of biochemical parameters [52], with modifications.

Determination of myeloperoxidase (MPO) activity

Tissue samples were homogenized with an HTAB buffer (hexadecyl trimethyl ammonium bromide) at a ratio of 1:20 (w/v). Next, the samples were sonicated for 10s. The aliquots were centrifuged for 10 min at 4 °C, 6540 g. In a 96-well plate, 50 µL of supernatant from each sample and 150 µL of reaction buffer (o-dianisidine dihydrochloride) were added. The absorbance was determined at 450 nm. The final concentration was multiplied by 400 and the results were expressed as U/g of tissue [53].

Determination of superoxide dismutase (SOD) activity

The gastrointestinal fragments were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680 g for 15 min at 4 °C. The centrifuged samples were diluted in phosphate buffer (0.1 M; pH 7.4) in a 1:20 ratio. In 100 µL of the homogenate were added 150 µL of cocktail containing: hypoxanthine, xanthine oxidase and nitrobluetetrazolium (NBT) in a ratio 1:1:1. The absorbance was read every minute for 10 min, 37°C, at 560 nm. The integral was multiplied by 800 and results were expressed as U/g of tissue [54].

Determination of catalase (CAT) activity

The gastrointestinal fragments were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680 g for 15 min at 4 °C. The centrifuged samples were diluted in sample buffer (KH₂PO₄, 25 mM, pH7.5; EDTA 1 mM; BSA 1%) in a 1:20 ratio. The plate contained 2wells with catalase, used as a positive control. A 20 µL of the homogenate was placed in a 96-well plate with 100 µL of assay buffer (KH₂PO₄, 250 mM, pH 7.0); 30 µL of methanol and 20 µL of hydrogen peroxide (35.3 mM). The plate was incubated for 20 min at room temperature. After this period, 30 µL of potassium hydroxide (10 mM) and 45 µL of Purpald® was added and the plate was incubated for more 10 min at room temperature. After that, 15 µL of potassium periodate (65.2 mM) was added and the plate was incubated for more 5 min at room temperature. At the end, the absorbance determination was done in a spectrophotometer at 540 nm. The concentration was multiplied by 4000 and results were expressed as U/g of tissue [55].

Determination of reduced glutathione (GSH) levels

The strips of the mucosa were homogenized with a solution of 5% trichloroacetic acid (TCA) in a 1:20 ratio (w/v). After homogenization, the samples were centrifuged for 10 min at 4 °C, 1520g. In a 96-well plate, 20 µL of the sample supernatant, 5 µL of PBS, 20 µL of 5,5'-Dithiobis (2-nitrobenzoic acid) and 140 µL of β-Nicotinamide adenine dinucleotide 2'- phosphate reduced tetrasodium salt hydrate (NADPH) were added. In a spectrophotometer the plate was incubated at 30 °C for 5 min. After incubation 15 µL of glutathione reductase was added and the absorbance was recorded at 412 nm. The

concentration was multiplied by 400 and the results were expressed as nmol/g of tissue [56].

Quantification of malondialdehyde (MDA)

Gastrointestinal fragments were homogenized with 1.15% potassium chloride (KCl) (1:5 – w/v). The samples were centrifuged for 10 min at 4 °C, 7690 g. In the test tubes were added 1500 µL of acetic acid (20%) pH 3.5, 400 µL of distilled water, 1500 µL of 2-thiobarbituric acid (TBA) (0.8%) diluted in acetic acid (20%), 400 µL of sample and 200 µL of sodium dodecyl sulfate (SDS) (8.1%). The test tubes of standard curve contained 100 µL of an MDA solution of known concentration, 1500 µL of acetic acid (20%) pH 3.5, 700 µL of distilled water, 200 µL of sodium dodecyl sulfate (SDS) (8.1%) and 1500 µL of 2-thiobarbituric acid (TBA) (0.8%) diluted in acetic acid (20%). The test tubes were incubated for 1h at 95 °C water bath and then were centrifuged for 10 min at 1520 g. In a 96-well plate, 200 µL aliquot of each sample was added. The absorbance was read at 532 nm and the results were multiplied by 50 and were expressed as nmol of MDA/g of tissue [57].

Statistical analysis

The results were expressed as the means $\pm$ standard error of the mean (S.E.M) of the parameters obtained. The values obtained were subjected to Student's t-test (comparisons between two groups) or analysis of variance (ANOVA) followed by Dunnett's test (compare three or more groups). The minimal significance level considered was $p < 0.05$.

5. Conclusions:

In conclusion, the results of our study showed that the hydroalcoholic extract from the leaves of *Eugenia punicifolia* administered during six consecutive days in male and female rats (intact and ovariectomized) after gastric or duodenal injury induced by IR, can heal the mucosa, acting via antioxidant and anti-inflammatory, with a significant increase in CAT and SOD activities and a reduce in MPO activity and GSH levels. Other possible mechanisms of HEEP healing properties should be investigated, like pro- and anti-inflammatory cytokines. Further studies are needed to delineate and clarify the mechanisms by which female sex hormones are effective in the treatment of gastrointestinal lesions

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Conflicts of Interest: The authors declare no conflict of interest

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CAPÍTULO 4

Effects of *Eugenia punicifolia* in gastric ulcer healing and differences between sexes

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Abstract

AIM

To evaluate the healing effect of hydroalcoholic extract obtained from the leaves of *Eugenia punicifolia* (HEEP) and analyze the sex differences in this action.

METHODS

The gastric ulcers were induced by acetic acid model in male and female rats (intact and ovariectomized). Lesion area, biochemical, zymographic, molecular and in vitro parameters were assessed. Moreover, the subacute toxicity (*in vivo*) and cytotoxicity (*in vitro*) effect of HEEP were also evaluated.

RESULTS

HEEP (125 mg/kg) demonstrated a great healing capacity by reducing the ulcerative lesion area in male (52.44%), intact (85.22%) and ovariectomized (65.47%) female rats when compared to the control group (vehicle). HEEP accelerates the healing of acetic acid induced gastric lesions and this effect is modulated by female sex hormones. The curative role of HEEP is due to an increase in PGE₂, inhibition of MMP-9 and maintenance of MMP-2 in gastric mucosa. Furthermore, HEEP healing properties were also confirmed by enhancement of proliferation and coverage of scratched wounds in fibroblast monolayer (*in vitro*). No sign of subacute toxicity and cytotoxicity was observed in this study.

CONCLUSION

The data indicate healing activity. This effect involves participation of prostaglandin, remodelling of extracellular matrix, proliferation and migration of cells in gastric mucosa.

Key-Words: *Eugenia punicifolia*; gastric ulcer healing; acetic acid; sex difference; Myrtaceae; toxicity.

Core tip:

Gastric ulcer is a chronic disease that affects millions of people around the world with high rates of relapse and is related to an imbalance between the protective and aggressive agents into the gastric mucosa. Their conventional treatment is related to several side effects and a poor healing of the gastric mucosa. Therefore, alternative therapies are needed. *Eugenia punicifolia* is a specie used to treat inflammation and wounds. Thus, the present study was designed to evaluate the gastric ulcer healing effect in rat model and analyze the sex difference, contributing to the safe use and collaborating with the scientific knowledge of natural products.

INTRODUCTION

Eugenia punicifolia (Kunth) DC. (Myrtaceae), popularly known as “pedra-ume-caá”, “pedra-ume”, “murta” or “muta” is a shrub largely distributed in the Savanna biome and Amazon region. The leaves of this medicinal plant are popularly used as a natural therapeutic agent to treat inflammation [1], wounds, infections [2], diabetes [3], fever and flu [4,5].

The gastric ulcer is a chronic disease that affects millions of people around the world with high rates of relapse and related to an imbalance between the protective and aggressive agents into the gastric mucosa [6, 7]. Alcohol consumption, prolonged treatment with non-steroidal anti-inflammatory drugs (NSAIDs), stress and *Helicobacter pylori* infection favor this imbalance and are pivotal etiological factors to this disease [8]. The anti-secretory drugs, including antagonists of type 2 histamine receptor (ARH2, for example ranitidine) and proton pump inhibitors (PPIs, for example lansoprazole), as well as antibiotics for the eradication of *H. pylori*, are the current treatment of gastric ulcers. Although effective, prolonged use of anti-secretory drugs, especially ranitidine and PPIs, is related to several side effects [9] and a poor healing gastric process [10].

Tarnawski *et al* [11] proposed the concept of quality of ulcer healing (QOUH) in 1991 for the first time, which considers the fact that tissue regeneration is often incomplete within ulcer scars. QOUH suggests that the evaluation of gastric ulcer healing should focus on whether the structure and function of mucosal and submucosal tissue have been completely regenerated in addition to endoscopic examination and evaluation of ulcer size. It has been shown that ulcer recurrence is closely related to QOUH.

Ulcers frequently recur following antacid drug treatment [12, 13]. Therefore, alternative therapies for gastric ulcer are desirable [14]. Herbal combination preparations are popular within traditional herbal medicine. The rationale behind such combinations is frequently questioned and it remains challenging

to assess the individual contribution of each of the combination partners to the overall activity of the preparation. This holds especially true when the preparation is used in the treatment of a chronic multifactorial disease, such as gastric ulcers [15].

The gastroprotective activity of hydroalcoholic extract of leaves of *Eugenia punicifolia* (Kunth) DC. (HEEP) [16] against ethanol-induced/NSAIDs ulcer in rodents was already been described. However, the gastroprotective activities of a extract do not ensure their gastric healing effects in installed gastric ulcers [17]. Therefore, the present research aims to evaluate the effect of HEEP in the healing of gastric ulcers induced by acetic-acid and analyze the differences in the curative action of this extract between males and female rats.

MATERIALS AND METHODS

Chemicals and Reagents

The following drugs were used: acetic acid, methanol (Dinâmica Analitic Reagents, Brazil), lansoprazole (Cruz Vermelha Manipulations, Brazil), RIPA buffer, protease inhibitor cocktail, ethylenediaminetetraacetic acid (EDTA), N, N, N', N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), sodium chloride, acrylamide 40%, Triton X-100, Tris-HCl, calcium chloride, zinc chloride, coomassie brilliant blue, tris base, glycine, sodium dodecyl sulfate, glycerol, Tween 20, β -mercaptoethanol, bromophenol blue, bis-acrylamide 30 %, Ponceau (Sigma Chemical Co., St.Louis, USA) gelatin (Merck, KGaA, Darmstadt, Germany). To dilute the hydroalcoholic extract from the leaves of *E. punicifolia* (HEEP), a saline solution was used as a vehicle and as a negative control.

Plant material

In December 2009, Dr. Catarina dos Santos collected the leaves of *Eugenia punicifolia* at the Forest Institute and Experimental Stations - Assis State Forest at the point 22°33 'to 22°37' Lat. S-50°21 'to 50°24' Long. W, Assis, State of São

Paulo, Brazil. Dr. Antônio C. G. Melo identified the species and a voucher (No. 43322) was deposited in the Herbarium D. Bento Pickel, Assis, SP, Brazil.

Preparation of plant extract

The extract was prepared through the dried and crushed leaves (10 g of plant material) and 100 mL of solvent (ethanol: water 70:30 v/v). Dynamic maceration of the solution was performed for 2h at room temperature ($25 \pm 2^{\circ}\text{C}$). Thereafter, the solution was filtered, and the residue was extracted two more times. For the drying of the solution the rotary evaporator (40°C) was used and the extract yield was 45% (4.49 g) of the original plant material.

Animals

The experimental procedures conducted *in vivo* used male and female Wistar albino rats (300–350g) obtained from the breeding facility of the State University of Campinas (CEMIB - Unicamp/SP), divided into (7-10 individuals/group): negative control (saline solution - 0.9% NaCl - 10 mL/kg, positive control (lansoprazole - LZ - 30 mg/kg), hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP - 125 mg/kg, the dose was determined by performing a dose-response curve, based on a previous gastric injury induction test [16]. Male and female rats were kept in separate rooms, fed by Presence® diet, with free access to filtrated water under controlled conditions of illumination (12:12 light/dark cycle) and temperature ($22 \pm 2^{\circ}\text{C}$). The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. The estrous cycle was verified through a vaginal smear. the vaginal cytology of the female rats was checked daily starting on postnatal day (PND) 60. The material was observed under an optical microscope and the estrous cycle phase was determined by cytology [16-18]. The duration of the estrous cycle was calculated as the number of days between one estrus phase and the next one. Only female rats that showed two consecutive regular strains cycles of 4 to 5 days were included in the experiment. It was included a group of ovariectomized (OVZ) rats. All the experimental practices agreed with the protocols approved by the Ethics

Committee on the Use of Animals of the Institute of Biosciences of Botucatu (nº. 675 – CEUA).

Healing effect of HEEP on acetic acid-induced gastric lesions

Three groups (n=8-10) of male and female (intact and OVZ) Wistar rats were fasted for 16h before this experiment. Under anesthesia, a laparotomy was done in all animals through a midline epigastric incision. A plastic 4.2 mm internal diameter tube was firmly applied to the serosal surface of the stomach wall, and 70 µL of an 80% solution of acetic acid was applied for 20s on the serosal surface of the stomach and then completely removed. The stomach was bathed with saline to avoid adherence to the external surface of the ulcerated region and then the abdomen was closed, and all the animals were fed normally. HEEP (125 mg/kg), LZ (30 mg/kg) or saline (10 mL/kg) were administered for the determination of the healing effects by the subacute treatment for 14 days. All treatments were done orally once a day beginning one day after surgery. One day after the last drug administration, the rats were euthanized through decapitation and the stomachs were removed to evaluate the area of gastric lesions, expressed as mm², using the AvSoft Bioview software [5, 6].

Cell viability assay (MTT)

L929 cells (1x10⁵) were cultured in 96-well plates (in triplicates) in the presence of a vehicle (culture medium with 0.1% of DMSO), 10% DMSO (control) or HEEP (0.3 - 30 µg/mL) at 37 °C for 24h. MTT (0.5 mg/mL) was added to cells, the cultures were incubated for 3 h at 37 °C, and the absorbance was analyzed at 570 nm after solubilization of reduced formazan crystals with pure DMSO [23]. The percentage of cell viability was calculated as the ratio (Abs. sample - Abs. blank/ Abs. vehicle x 100).

Cell proliferation activity

The proliferation ability of fibroblasts exposed to HEEP was assessed using the scratch wound assay as previously described by Balekar *et al.* (2012)^[24] with few

modifications, which measures the expansion of a cell population on surfaces. Monolayers of L929 cells were allowed to form in a 24-well plate containing an enriched medium of Dulbecco's Modified Eagle Medium (DMEM)/supplemented with 10% fetal bovine serum (FBS). When nearly confluent, the plate was taken out and artificial wounds were created in the monolayers by making a linear scratch in the center of each well using the tip of a sterile 200 μ L plastic pipette tip. Any cellular debris created from the scratch was removed by gently washing the wells with phosphate buffered saline (PBS). Once ready, the scratched wounds were divided into groups. The cells were treated with a lower (3 μ g/mL), an intermediate (10 μ g/mL) or a high (30 μ g/mL) concentration of HEEP, DMEM/supplemented with 5% FBS was used as negative control. The plates were then incubated at 37 °C in a humidified incubator with 5% CO₂ atmosphere. The plates were evaluated after 24 and 48h of incubation to assess the closure of the scratched wounds. Micrographs were used to record the wound closure activity, which was captured under an inverted microscope (Olympus, CK40) with magnification of 100x. All the experiments were performed in triplicate.

Determination of prostaglandin E₂ (PGE₂) levels

Serum from animals that underwent gastric lesion induction by acetic acid and treated for 14 consecutive days with vehicle, lansoprazole and HEEP were used for the determination of PGE₂ levels. The PGE₂ level was quantified with an immune-enzymatic dosage kit from R&D Systems (USA).

Extraction of total protein and zymography

Tissue with gastric ulcer from each experimental group described previously was used to extract total protein. The extraction was carried out by crushing with the Polytron type homogenizer in a ripa buffer and protease inhibitor cocktail in a 1:5 ratio. The homogenate was centrifuged at 18620 g for 45 min at 4 °C. The supernatant was collected and then centrifuged again at 13680 g for 15

min at 4 °C, after this process, protein content was quantified by the method of biuret [7,8].

The complete protocol for gelatin zymography was described by Justulin-Jr *et al.* [27]. Samples of extracted proteins (35 µg) of the gastric ulcer from different experimental groups treated for 14 days were subjected to electrophoresis under non-reducing conditions on 8% polyacrylamide gel copolymerized with 0.1% purified gelatin. After electrophoresis, the gels were subjected to two washes of 15 min in a solution of 2.5% Triton X-100 to remove SDS, and two washes of 5 min in 50mM pH 8.0 Tris-HCl buffer. Subsequently, gels were incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM of CaCl₂ and 1 µM de ZnCl₂ for 20 h at 37 °C. Finally, the gels were stained with Coomassie Brilliant BlueR-250. The relative molecular weight of the bands was determined according to the molecular weight standard (Precision Plus Protein™, BIO-RAD) used in electrophoresis. The bands obtained through zymography were scanned and analyzed by densitometry. The bands representative of the gelatinase activity of MMP-2 and -9 were analyzed by obtaining the integrated optical density (IOD) of the bands using ImageJ software. Values were plotted in a histogram showing the ratio of the IOD of the treated groups to the control group IOD.

Western blot

The protein extracts obtained as described above for zymography were used in this test.

After extraction, samples were treated with Laemmili buffer (PB buffer 0.5 M, pH 6.8; glycerol, sodium dodecylsulfate (SDS) 10%, bromophenol 0.1%, β-mercaptoethanol) in a 1:1 proportion. Equal amounts of protein from samples (70 µg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, proteins were electrophoretically transferred onto a nitrocellulose membrane or PVDF membrane (EGF), blocked with 5% non-fat milk diluted in PBS, and incubated

with the following primary antibodies: anti-COX-1 (1:10.000, ab133319); anti-COX-2 (1:1000, ab52237); anti-VEGF (1:1.000, ab46154); anti-EGF (1:800, ab77851), anti-Bcl-2 (1:800, ab59348), anti-Caspase-3 (1:10.000, ab32499), anti-Caspase-8 (1:2.000, ab25901) and anti-Caspase-9 (1:5.000, ab32539) Abcam®, Cambridge, USA). The membranes were washed with PBS and incubated with a specific secondary antibody for 2h (HRP, 1:10.000, ab 97051, Abcam®, Cambridge, USA). After washing, the reactions were detected using an ECL kit (Amersham, USA) and the signals were captured using a GBOX (Syngene™). The integrated optical densities (IODs) of the targeted protein bands were measured using Image J (<http://rsb.info.nih.gov/ij/>). The expression levels were normalized to that of actin (1:10000; ab179467, Abcam®, Cambridge, USA).

Toxicological evaluation

Some toxicological parameters were also obtained from three groups of animals subjected to the healing gastric ulcer model and treated orally during 14 consecutive days once a day with: HEEP (125 mg/kg), LZ (30mg/kg) and vehicle (10mL/kg). Body weight was recorded daily throughout the experimental period, and the macroscopic analyses and weight of vital organs (liver, kidneys, heart, spleen and lungs) were compared between either the HEEP or lansoprazole and the vehicle-treated groups to evaluate subacute toxicity. On the day after the last drug administration, the rats from each treatment group were euthanized through decapitation, and their blood was collected. The blood samples were then centrifuged (3000 g for 15 min), and the serum obtained was frozen at -80 °C until biochemical analysis. Serum biochemical parameters, including glucose, urea, creatinine, γ -glutamyl transpeptidase (γ -GT), aspartate aminotransferase (AST) and alanine amino transferase (ALT), were measured using an automated biochemical analyzer.

Statistical analysis

The results were expressed as the means $\pm$ standard error of the mean (S.E.M) of the parameters obtained. The values obtained were subjected to Student's *t*-test (comparisons between two groups), one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's or two-way ANOVA followed by Bonferroni's test (compare three or more groups). The minimal significance level considered was $p < 0.05$.

RESULTS

Healing effect of HEEP on acetic acid-induced gastric lesions

In male rats, the treatment with HEEP during 14 consecutive days significantly decreased the gastric lesion area when compared with control group treated with vehicle ($P < 0.01$). The same occurred with the group treated with the LZ ($P < 0.05$), we can see a decrease in the lesion area when compared to vehicle (Figure 1).

The gastric healing process was also evaluated in intact (cycling) and OVZ female rats. HEEP presented a significant reduction in lesion area in cycling rats ($P < 0.0001$) and OVZ rats ($P < 0.001$). The same decrease occurred with the group LZ with significance levels of $P < 0.0001$ and $P < 0.01$, respectively (Figure 21).

In Figure 1, we can observe that intact females when compared to males, submitted to acetic acid-induced gastric ulcer model, showed a greater reduction of gastric lesion area both HEEP and LZ treatment ($P < 0.01$). Comparison of the same parameters with OVZ females and males did not present a significant difference ($P > 0.05$). When we observe the intact and OVZ females, we can see that the intact females have a greater decrease in the lesion area between the groups of HEEP and LZ ($P < 0.01$ and $P < 0.001$), when compared to the OVZ females.

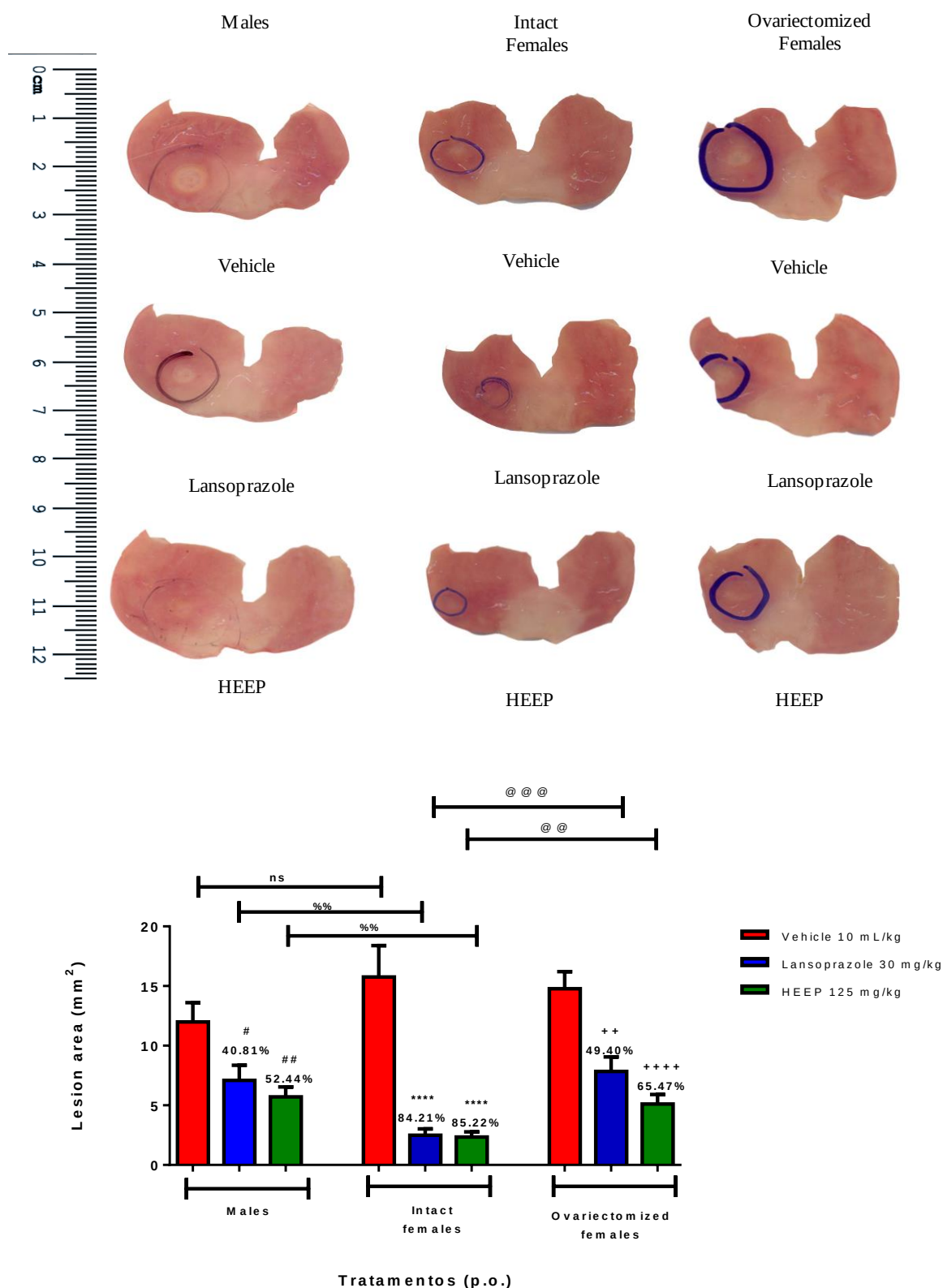


Figure 1 Evaluation of HEPP and LZ against gastric ulcer model induced by acetic acid - treatment for 14 consecutive days. Results were expressed as mean $\pm$ S.E.M (n=8-10) and statistical significance was determined by one-way ANOVA followed by Dunnett's test; # $P < 0.05$ and ## $P < 0.01$ when compared to the vehicle group of males; **** $P < 0.0001$ when compared to the vehicle group of intact females (cycling); ++ $P < 0.01$ and ++++ $P < 0.0001$ when compared to the vehicle group of OVZ or Student's t-test; %% $P < 0.01$ when compared HEPP and LZ groups of intact females and males; @@ $P < 0.01$ and @@@ $P < 0.001$ when compared HEPP and LZ groups of intact females and OVZ females. The percentage indicates

the healing against the vehicle group. ns = not significant.

Cell viability assay (MTT)

Based on the results obtained from the MTT assay, no cytotoxicity signal has been found after 24h of incubation of L929 cells with HEEP (0.3–30 $\mu\text{g/mL}$) when compared to control group (basal) (Figure 2).

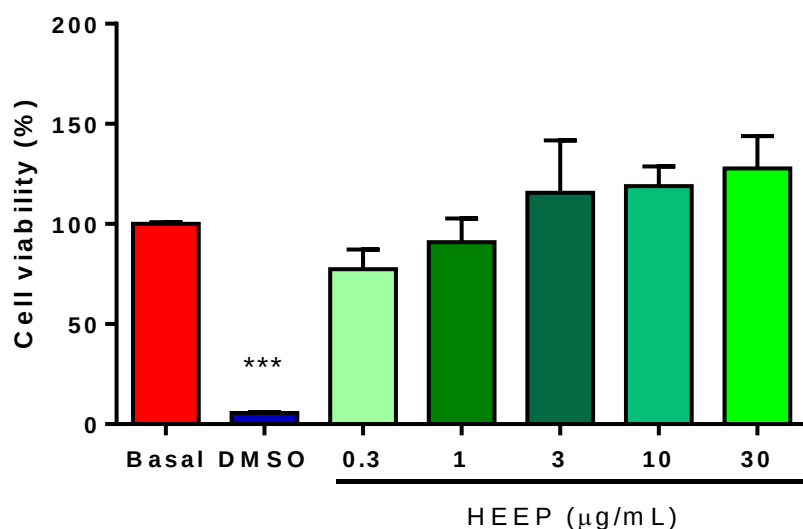


Figure 2 Effects of HEEP on the cell viability of L929 fibroblasts as measured by MTT assay. L929 cells were treated with different concentrations of HEEP. After 24h incubation MTT assay was performed. The results represent mean $\pm$ S.E.M in triplicated experiments. Statistical significance was determined by one-way ANOVA followed by Dunnett's test; *** $P < 0.001$ compared to basal group.

Cell proliferation activity

In figure 3A, we can see that HEEP was able to cover scratched wound areas made on the L929 cell monolayer. The enhancement of proliferation and coverage of scratched wounds by HEEP at 3 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ was found to be 92.14%, 74.31% and 75.25%, respectively, compared with the negative control (vehicle) (Figure 3B).

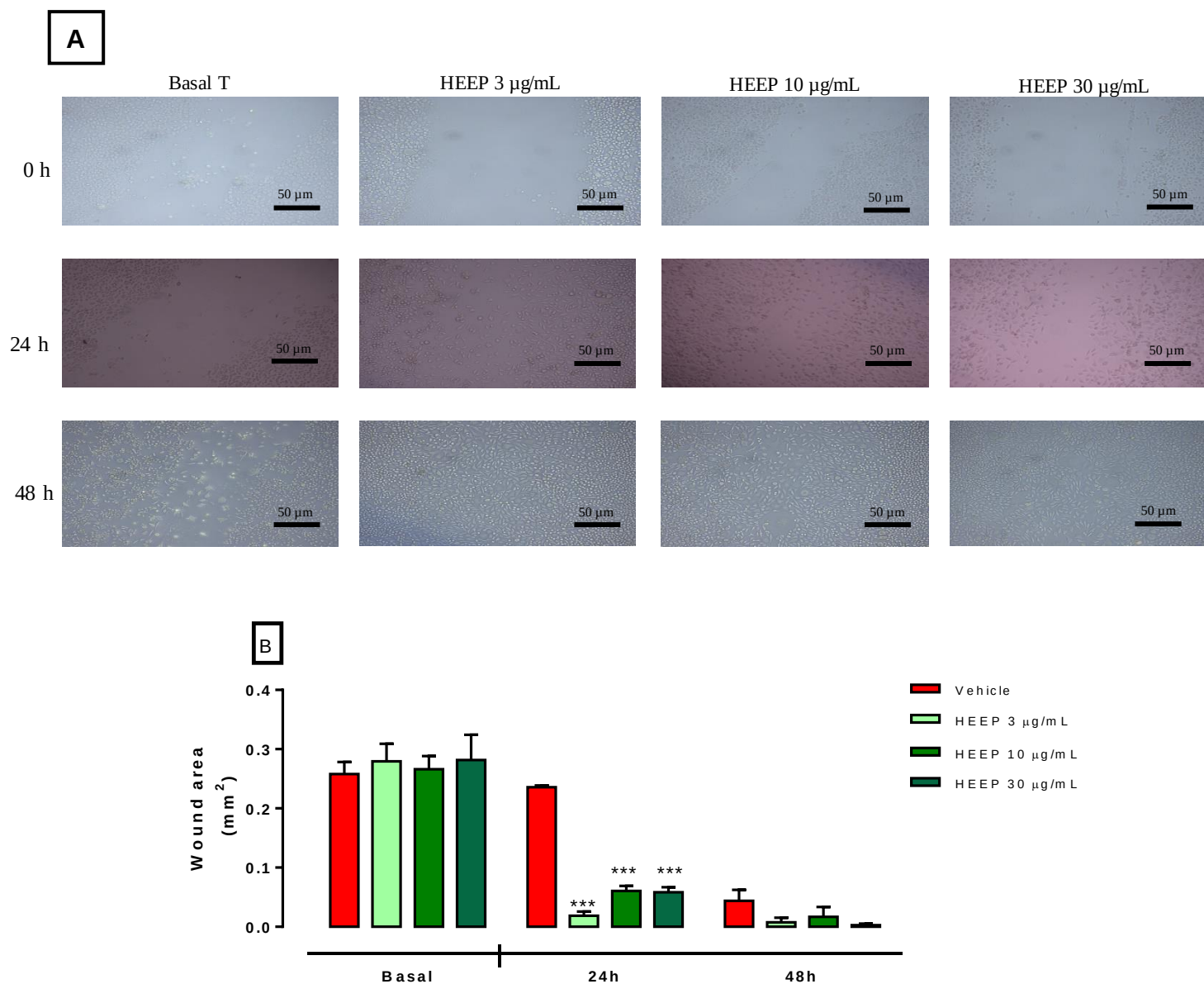


Figure 3 Effects of HEEP on cell migration in the *in vitro* scratch assay. A fibroblast L929 layer subjected to scratch and treated with HEEP 3 µg/mL, HEEP 10 µg/mL, HEEP 30 µg/mL and with vehicle. In Panel A: Images captured at 100x magnification at hour 0, 24 and 48 after incubation. In Panel B: Measurement the rate of migration by quantifying the total distance that the cells moved from the edge of the scratch toward the center. The results represent mean $\pm$ S.E.M in triplicated experiments. Statistical comparison was performed using analysis of variance (two-way ANOVA) followed by Bonferroni's test; *** $P < 0.001$ compared to the matched-time vehicle group.

Determination of prostaglandin E₂ (PGE₂) levels

Treatment of males for 14 days showed elevation of serum PGE₂ levels in the group treated with HEEP ($P < 0.05$), LZ ($P < 0.001$) when compared to the control group (vehicle) (Figure 4). The same result did not occur with the group of intact and OVZ female rats.

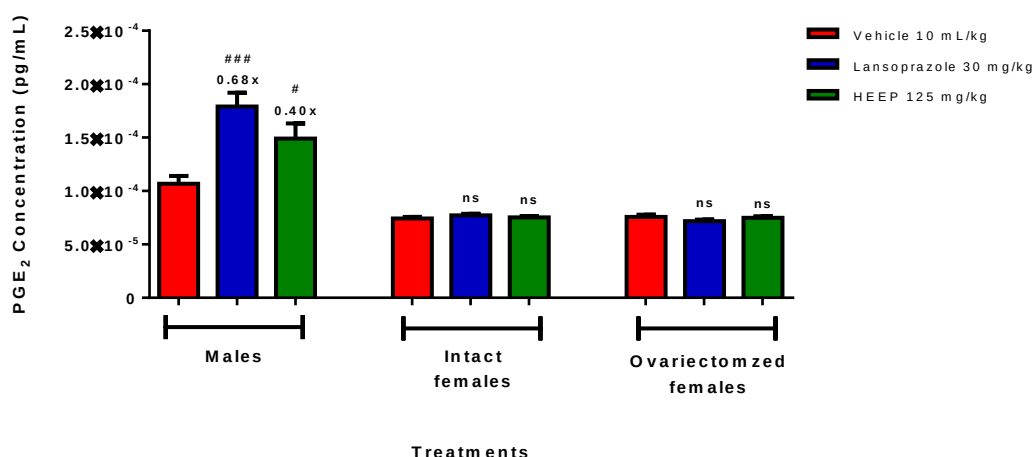


Figure 4 Quantification of PGE₂ levels in animals submitted to the model of gastric ulcer induced by acetic acid with treatment for 14 days. The results are expressed as mean $\pm$ S.E.M ($n = 6-8$) and statistical significance are determined by one-way ANOVA followed by Dunnett's test; # $P < 0.05$ and ### $P < 0.001$ when compared to the vehicle group of males. ns = not significant.

Zymography

To determine in better detail the healing process of HEEP, we also analyzed the stomach by zymography, presenting the activities of MMP-2 and MMP-9 on the gastric mucosa after treatment with vehicle, LZ and HEEP. Our results show that MMP-9 activity is absent in all groups, the bands was not detected. In contrast, MMP-2 activity was present in all treated groups. The treatment of male (fig. 5), intact female (fig. 6) and OVZ female (fig. 7) rats exhibited pro, intermediate and active MMP-2 activity. There is no difference between the activity of MMP-2 in the animals treated with HEEP and LZ in relation to the vehicle ($P > 0.05$).

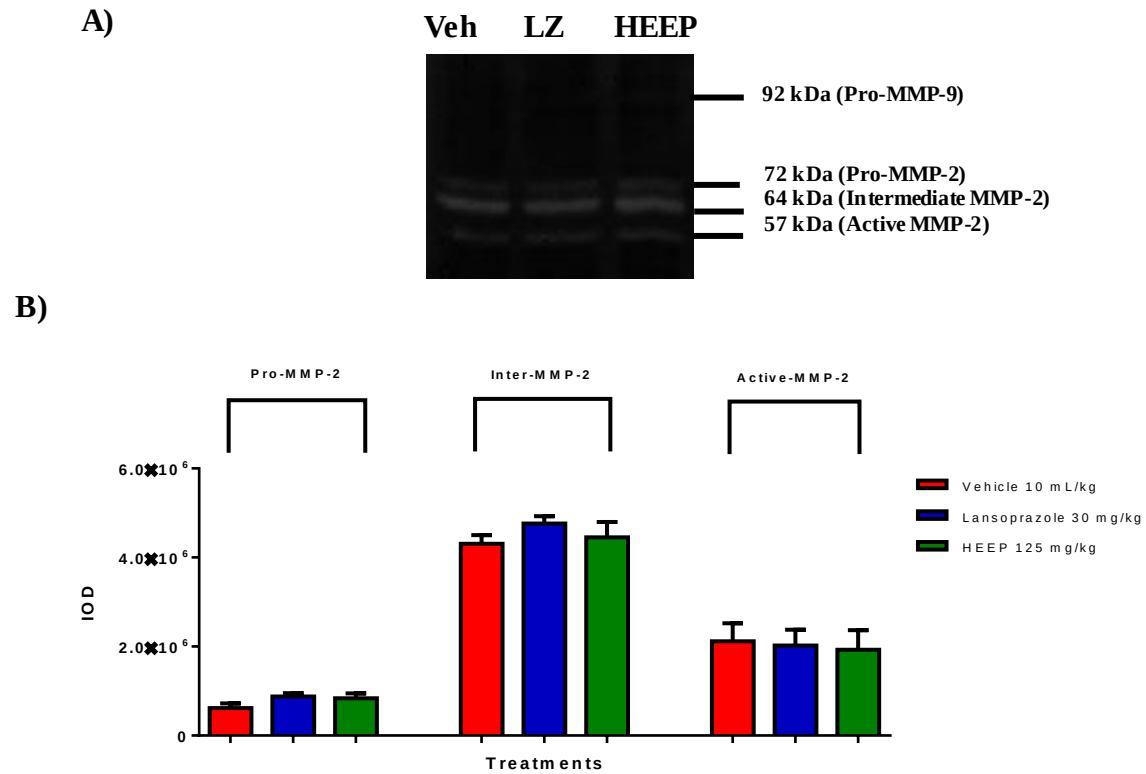
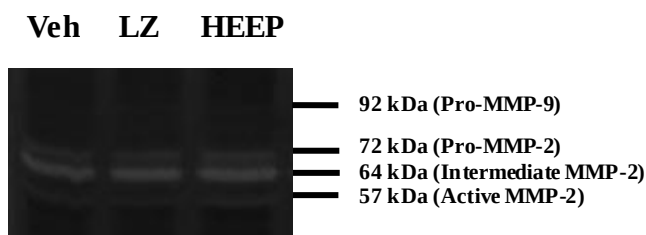


Figure 5 Representative zymography results of the gastric ulcers from the vehicle, LZ or HEEP in male rats. Panel A: Zymography gel showing the typical clear bands of MMP-2: proenzyme (72 kDa), intermediate (64 kDa) and active enzyme (57 kDa). In the gel are represented the treatment of male for 14 days: vehicle (Veh), lansoprazole (LZ) and *Eugenia punicifolia* (HEEP). Panel B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 days of treatment. The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test. Values show the ratio of the IOD from the treatment groups to the control group (vehicle) IOD value.

A)



B)

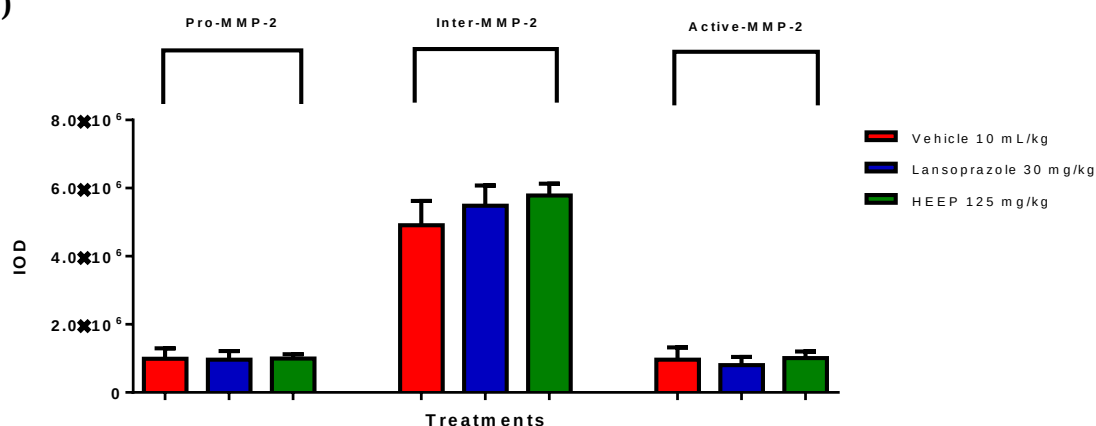


Figure 6 Representative zymography results of the gastric ulcers from the vehicle, LZ or HEEP in intact female rats. Panel A: Zymography gel showing the typical clear bands of MMP-2: proenzyme (72 kDa), intermediate (64 kDa) and active enzyme (57 kDa). In the gel are represented the treatment of intact female for 14 days: vehicle (Veh), lansoprazole (LZ) and *Eugenia punicifolia* (HEEP). Panel B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 days of treatment. The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test. Values show the ratio of the IOD from the treatment groups to the control group (vehicle) IOD value.

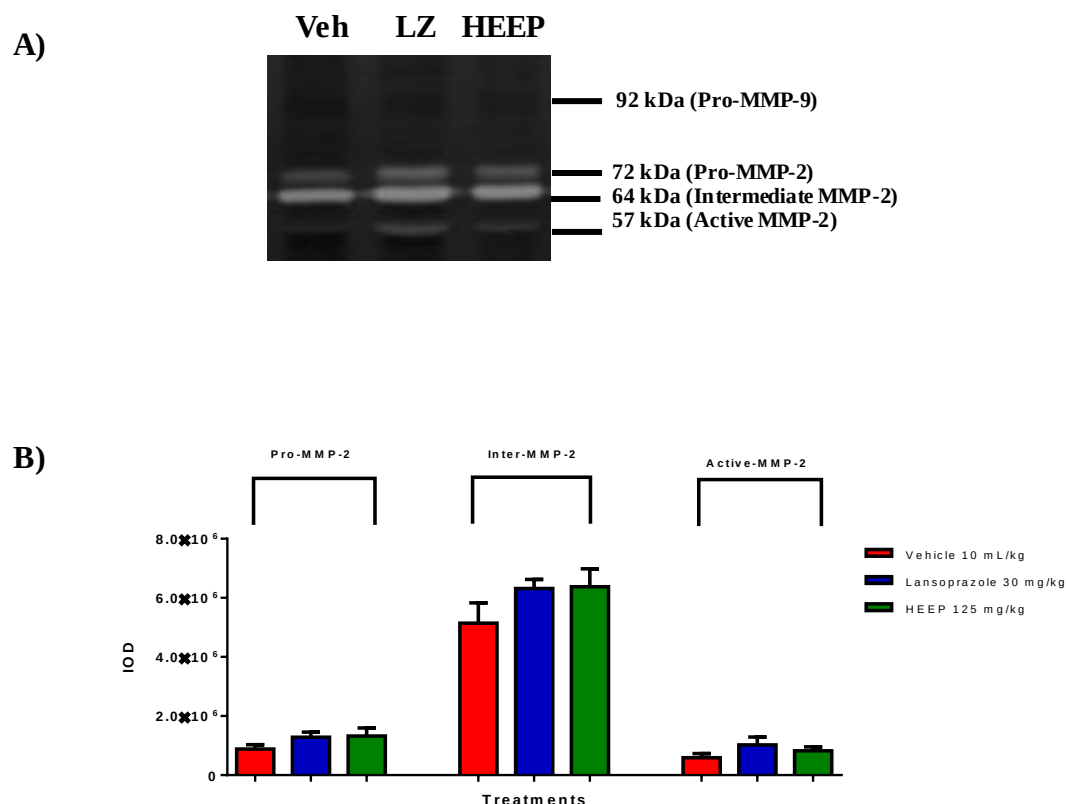


Figure 7 Representative zymography results of the gastric ulcers from the vehicle, LZ or HEEP in OVZ female rats. Panel A: Zymography gel showing the typical clear bands of MMP-2: proenzyme (72 kDa), intermediate (64 kDa) and active enzyme (57 kDa). In the gel are represented the treatment of OVZ female rats for 14 days: vehicle (Veh), lansoprazole (LZ) and *Eugenia punicifolia* (HEEP). Panel B: Gelatinolytic activity of MMP-2 in the gastric mucosa after 14 days of treatment. The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test. Values show the ratio of the IOD from the treatment groups to the control group (vehicle) IOD value.

Western blot

Analysis of western blotting (COX-1) revealed that oral treatment with HEEP and LZ after gastric exposure to absolute acetic acid remains unchanged in all group of animals when compared to vehicle (Figure 8).

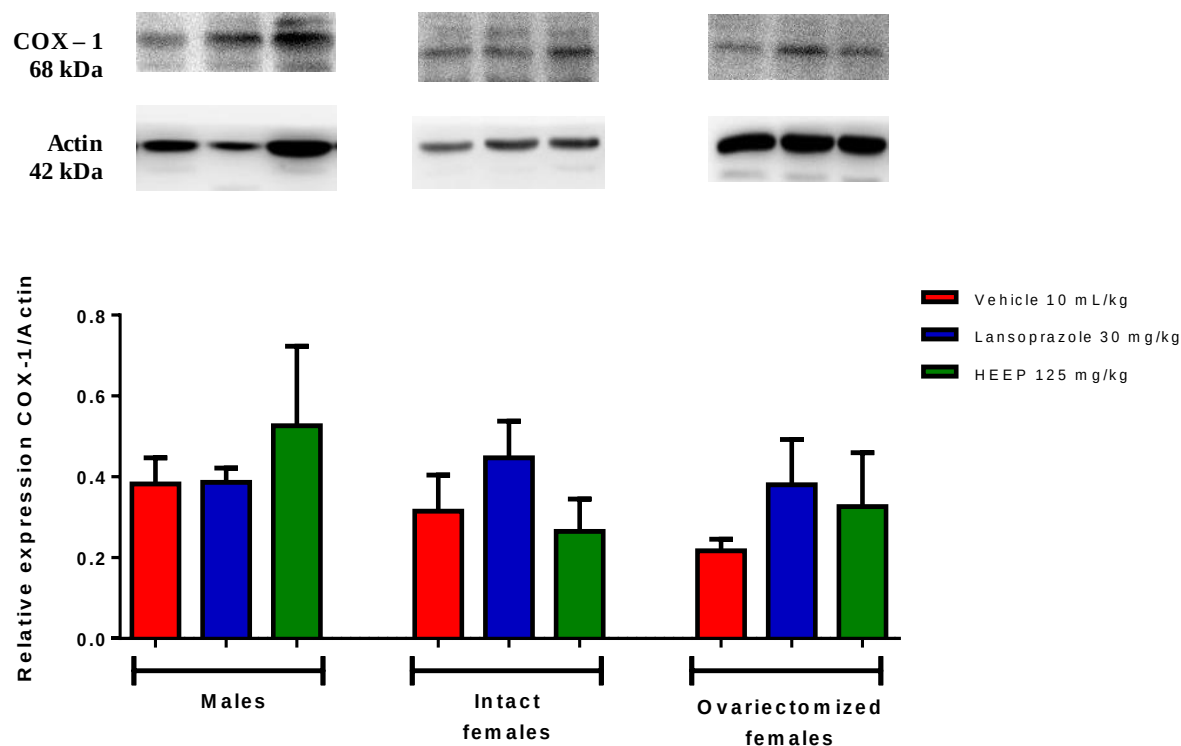


Figure 8 Effects of oral administration during 14 days of HEEP and LZ on COX-1 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test, no statistical differences are found.

On 14th day, COX-2 also did not have its expression altered in males, intact and OVZ females when compared to their respective control group (vehicle) (Figure 9).

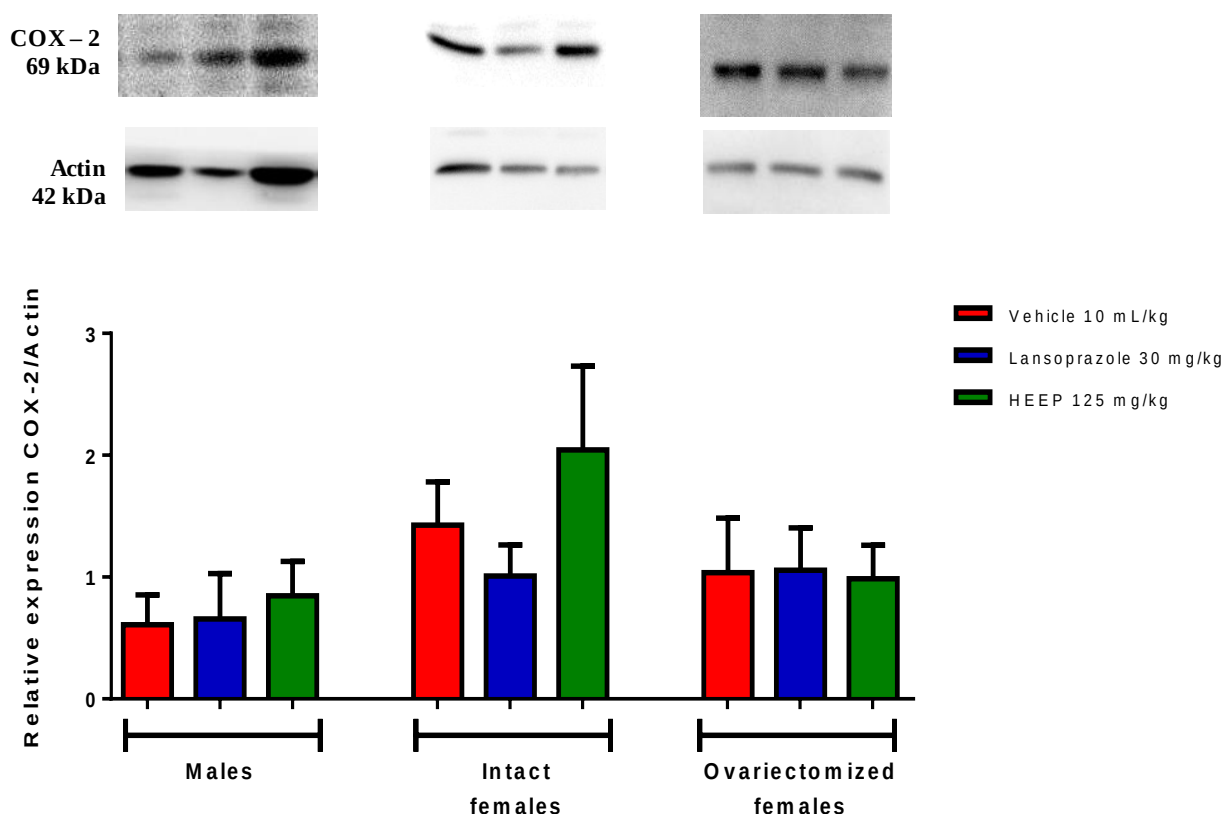


Figure 9 Effects of oral administration during 14 days of HEEP and LZ on COX-2 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test, no statistical differences are found.

However, a rise of EGF expression in LZ ($p < 0.01$) groups of males and also OVZ female rats were found when compared to vehicle (Figure 10). We can observe that there is a difference between LZ group of intact females and males and between intact and OVZ females. Males and OVZ females have an increased expression of EGF when compared to intact females. HEEP did not altered this expression in none of the groups.

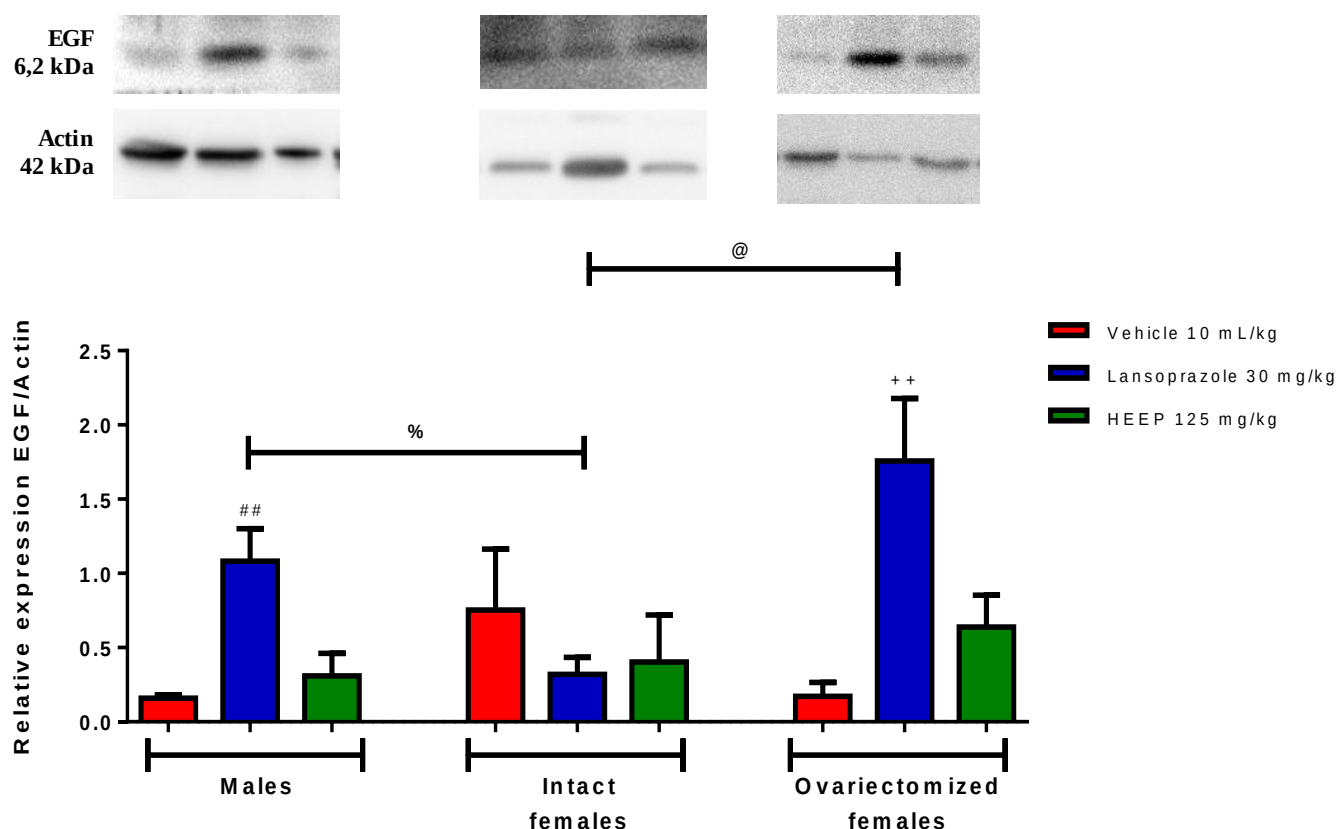


Figure 10 Effects of oral administration during 14 days of HEEP and LZ on EGF expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test; ** $P < 0.01$ when compared to control group (vehicle) or Student's t-test; % $P < 0.05$ when compared LZ group of males and intact females or @ $P < 0.05$ when compared LZ group of intact and OVZ females.

Western blot analysis reveals that VEGF protein was characterized, although low signal of VEGF band was observed in all groups on day 14 (Figure 11). There is no change in the protein expression of the animals treated with HEEP and LZ when compared to vehicle ($P > 0.05$), but there is an increase in the expression of VEGF of intact females treated with HEEP and LZ when compared to OVZ females ($P < 0.05$). Considering that intact females treated with HEEP have a greater healing (85.22%, fig. 1), the VEGF data are corroborating with this cure process, since they present higher VEGF expression than OVZ females.

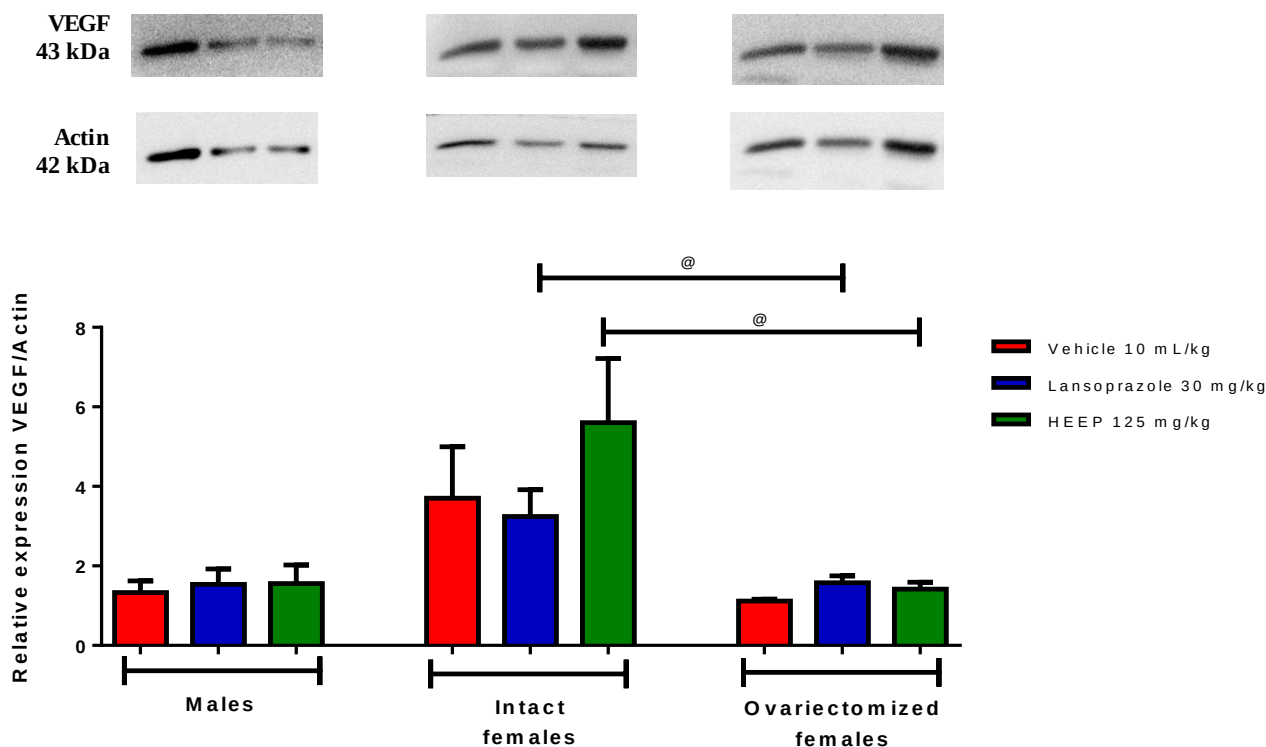


Figure 11 Effects of oral administration during 14 days of HEEP and LZ on VEGF expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test or Student's t-test; @ $P < 0.05$ when compared LZ or HEEP groups of intact and OVZ females.

In addition to growth factors, we evaluated apoptotic and anti-apoptotic factors. As apoptotic factors we investigated caspase-8 (fig.12), 9 (fig.13) and 3 (fig.14) and as anti-apoptotic factor Bcl-2 (fig. 15). Both treatments with HEEP and LZ were not able to alter the protein expression of these factors when compared to the vehicle group ($P > 0.05$). When we observe the expression of caspase-8 (fig. 12), we can see that there is a significant reduction between HEEP and LZ groups of intact females when compared to males and between vehicle, HEEP and LZ groups of intact and OVZ females. In Bcl-2 expression (fig.15), we can note that the HEEP group of intact females have a higher expression when compared to OVZ females.

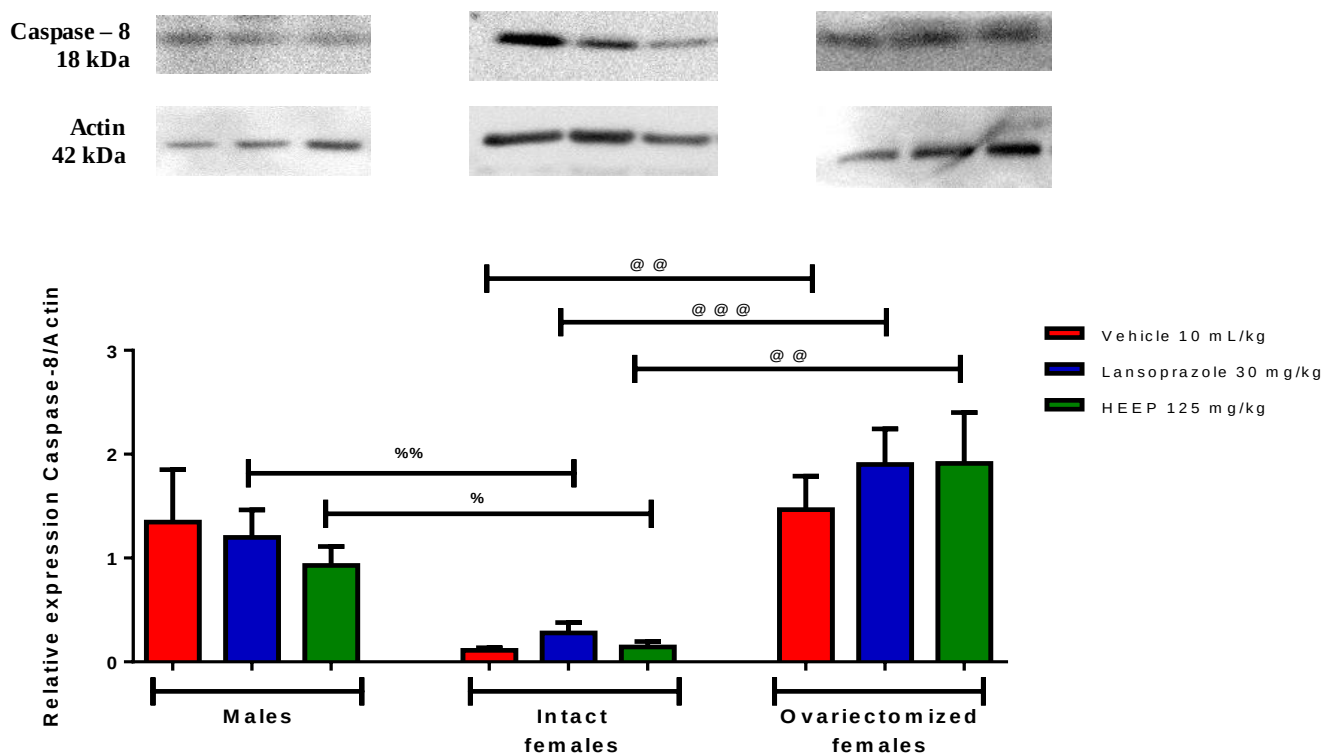


Figure 12 Effects of oral administration during 14 days of HEEP and LZ on Caspase - 8 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test or Student's t-test; % $P < 0.05$ and %% $P < 0.01$ when compared LZ or HEEP groups of intact females and males or @ $P < 0.01$ and @@@ $P < 0.001$ when compared vehicle, LZ and HEEP groups of intact and OVZ females.

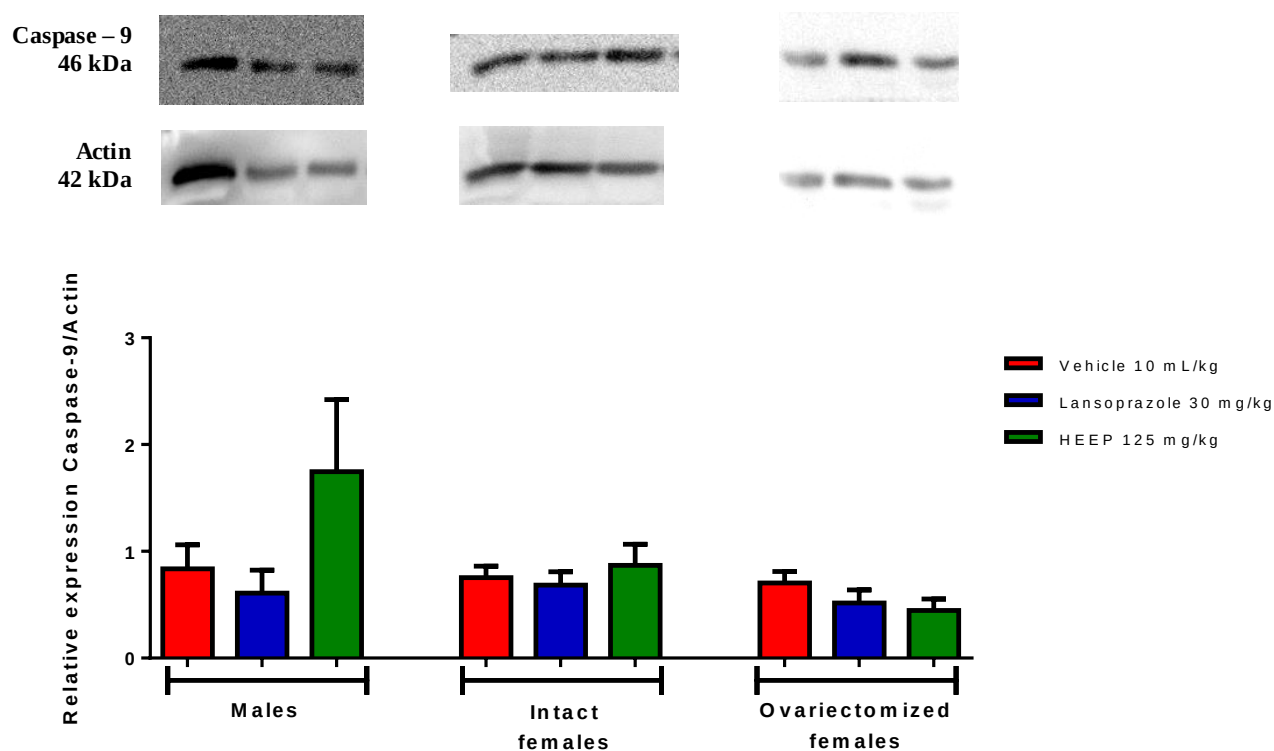


Figure 13 Effects of oral administration during 14 days of HEEP and LZ on Caspase - 9 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test, no statistical differences are found.

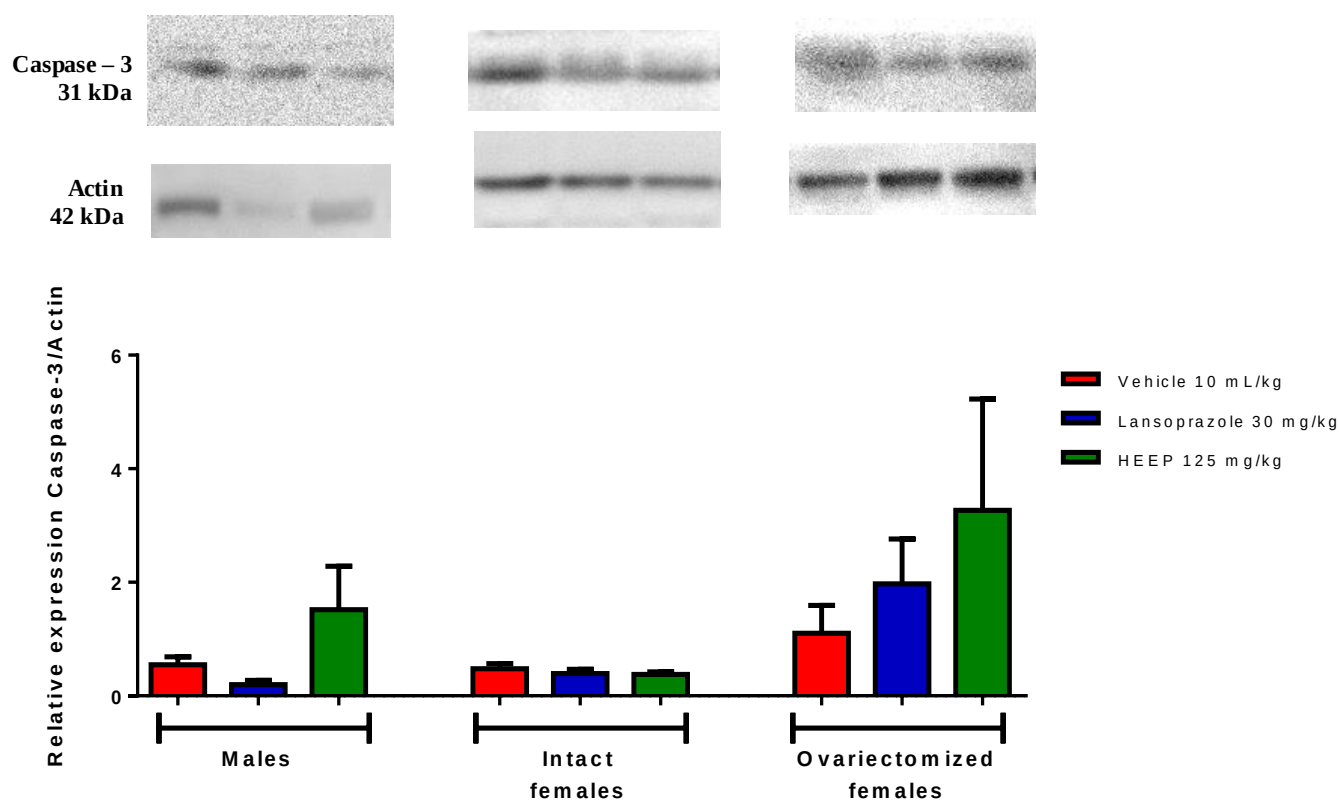


Figure 14 Effects of oral administration during 14 days of HEEP and LZ on Caspase - 3 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test, no statistical differences are found.

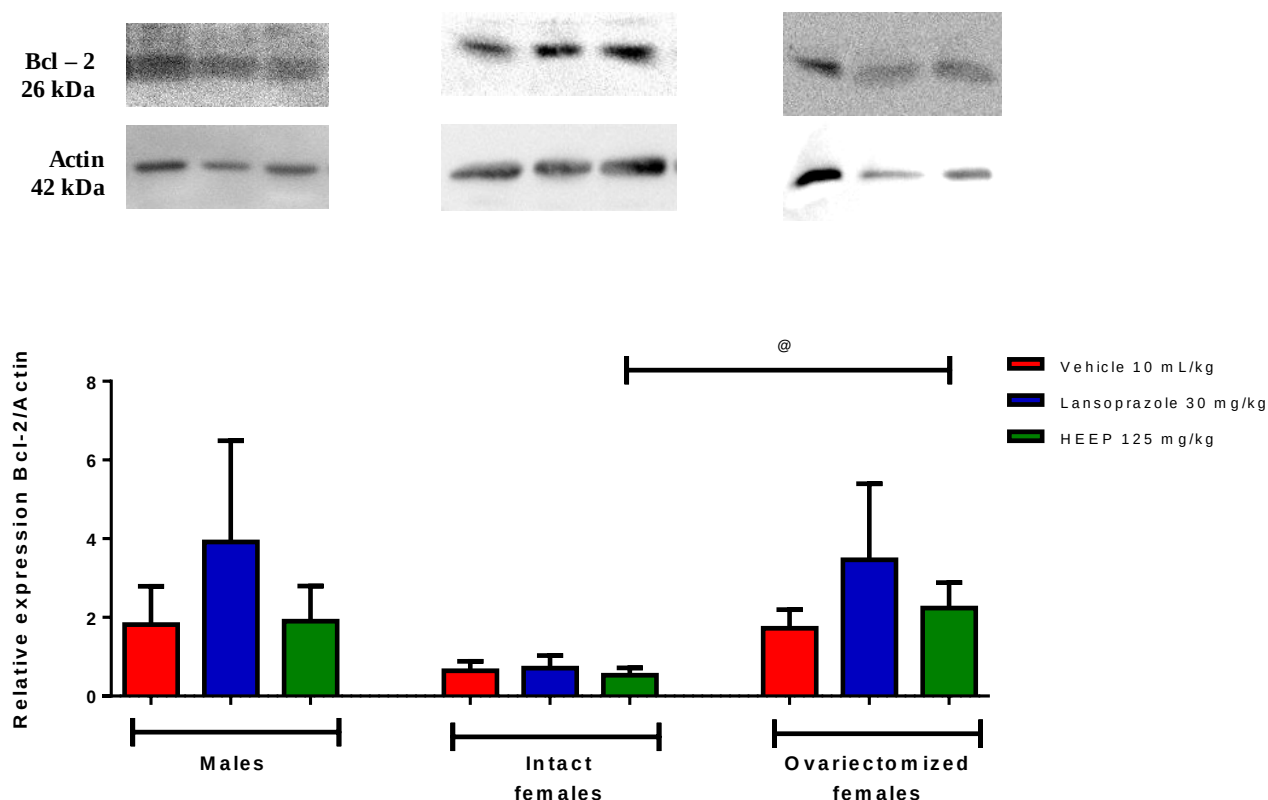


Figure 15 Effects of oral administration during 14 days of HEEP and LZ on Bcl-2 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Fold change was made following normalization with the control (housekeeping gene - actin). The results represent mean $\pm$ S.E.M. Statistical significance are determined by one-way ANOVA followed by Dunnett's test or Student's t-test; @ $P < 0.05$ when compared LZ group of intact and OVZ females.

Toxicological evaluation

After oral administration of HEEP, none of the male rats died and no significant change in daily body weight (fig. 16), organ weight and biochemical parameters (Table 1) was observed when compared to control group (vehicle). When we evaluated the biochemical parameters, we can see that there is a significant increase in glucose levels of LZ group when compared to vehicle (Table 1).

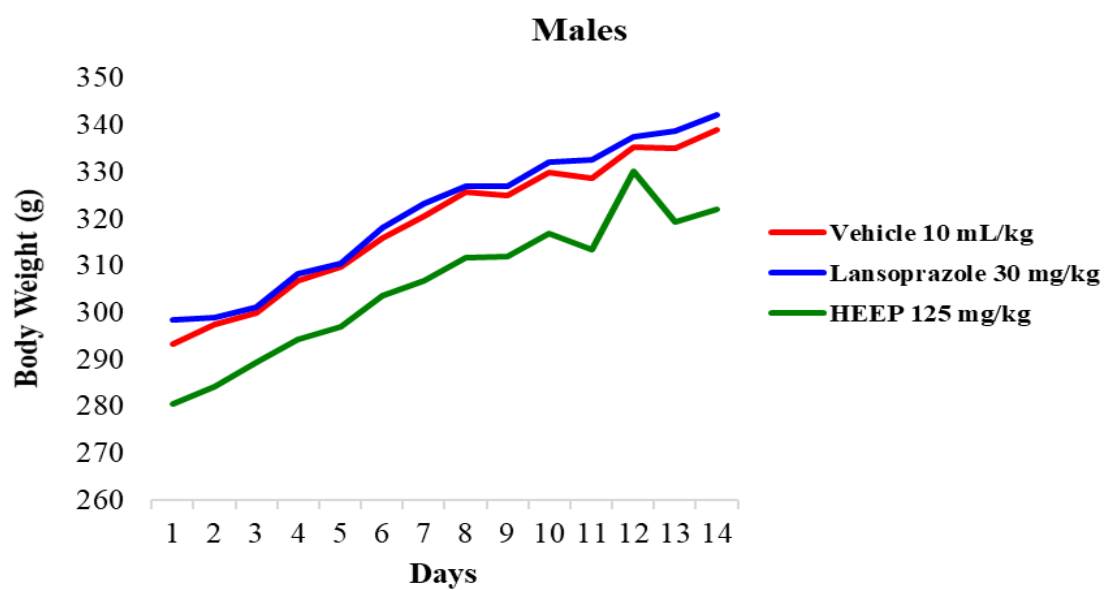


Figure 16 Body weight evolution in male rats after treatment with vehicle, LZ or HEEP administered 14 days after gastric ulcer formation by acetic acid (n=8-10). Results are expressed as the means of body weight(g). Statistical significance was determined by one-way ANOVA followed by Dunnett's test.

Table 1 Effects of HEEP and LZ on selected toxicological parameters in males collected 14 days following treatments.

	Treatments (p.o)		
	Vehicle	Lansoprazole	HEEP
Organ Weights			
Heart	3.15 ± 0.03	3.13 ± 0.03	3.14 ± 0.06
Kidneys	4.81 ± 0.05	4.79 ± 0.04	4.84 ± 0.05
Lung	3.62 ± 0.08	3.73 ± 0.10	3.59 ± 0.06
Liver	11.56 ± 0.18	11.55 ± 0.10	11.26 ± 0.13
Spleen	2.94 ± 0.02	2.98 ± 0.05	2.99 ± 0.04
Biochemical parameters			
Glucose	150.10 ± 6.04	170.90 ± 4.73 *	154.70 ± 3.80
Creatinine	0.34 ± 0.02	0.34 ± 0.02	0.33 ± 0.01
Urea	54.75 ± 3.15	52.13 ± 1.71	54.13 ± 1.94
γ-GT	< 1.0	< 1.0	< 1.0
AST	137.80 ± 18.43	163.60 ± 22.33	144.50 ± 10.76
ALT	42.88 ± 3.55	48.88 ± 3.67	46.25 ± 3.09

Results are expressed as the means ± S.E.M. (n=7-8) obtained from different groups. Statistical significance was determined by one-way ANOVA followed by Dunnett's test; * $P < 0.05$ when compared to the vehicle group. The units for the biochemical parameters of serum are U/L (ALT - alanine aminotransferase, AST - aspartate aminotransferase, γ-GT - gamma-glutamyl transpeptidase) and mg/dL (urea, glucose and creatinine).

It is also important to mention that the oral administration of HEEP did not cause any sign of acute toxicity in intact female rats. Over the 14 days following the oral administration HEEP (125 mg/kg), none of the animals died (fig. 17) and no significant changes in organ weight, hepatic enzymes (γ-GT, AST and ALT), renal functions (creatinine and urea) or glucose were observed at the end of this period (Table 2).

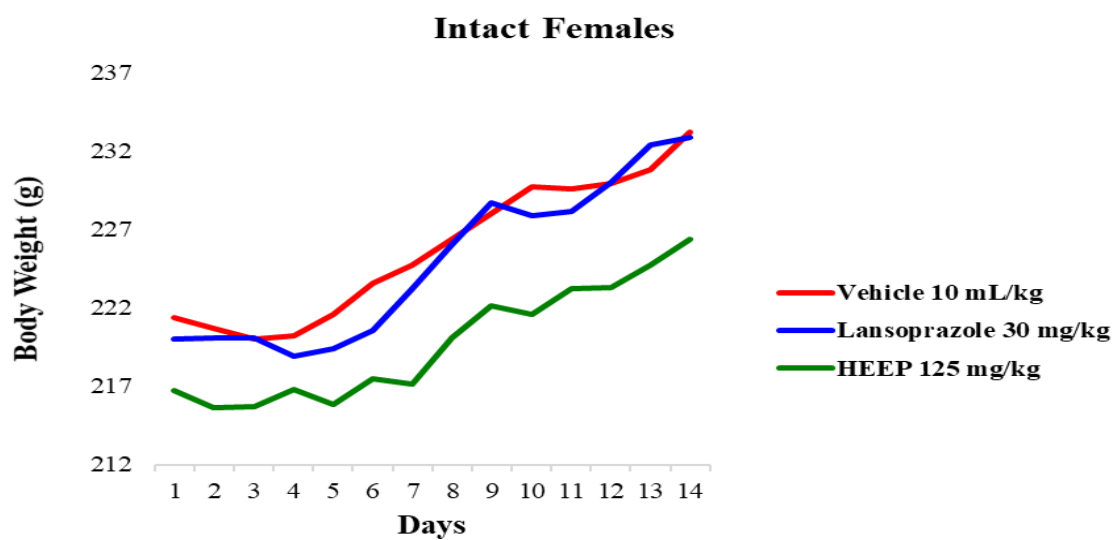


Figure 17 Body weight evolution in intact female rats after treatment with vehicle, LZ or HEEP administered 14 days after gastric ulcer formation by acetic acid (n=8-10). Results are expressed as the means of body weight(g). Statistical significance was determined by one-way ANOVA followed by Dunnett's test.

Table 2 Effects of HEEP and LZ on selected toxicological parameters in intact females collected 14 days following treatments.

	Treatments (p.o)		
	Vehicle	Lansoprazole	HEEP
Organ Weights			
Heart	3.43 ± 0.05	3.50 ± 0.03	3.44 ± 0.06
Kidneys	4.74 ± 0.03	4.81 ± 0.04	4.69 ± 0.06
Lung	4.23 ± 0.07	4.53 ± 0.06	4.01 ± 0.30
Liver	10.67 ± 0.14	10.90 ± 0.10	10.61 ± 0.09
Spleen	2.56 ± 0.06	2.56 ± 0.07	2.48 ± 0.06
Biochemical parameters			
Glucose	119.10 ± 3.27	116.90 ± 3.21	122.30 ± 3.34
Creatinine	0.45 ± 0.02	0.41 ± 0.02	0.45 ± 0.01
Urea	49.50 ± 3.36	53.29 ± 1.11	53.70 ± 3.20
γ-GT	< 1,0	< 1,0	< 1,0
AST	202.70 ± 12.73	198.40 ± 18.84	162.10 ± 7.24
ALT	57.00 ± 3.40	60.57 ± 3.59	65.86 ± 2.79

Results are expressed as the means ± S.E.M. obtained from different groups. Statistical significance was determined by one-way ANOVA followed by Dunnett's test. The units for the biochemical parameters of serum are U/L (ALT, AST and γ-GT) and mg/dL (urea, glucose and creatinine).

None of the animals died and no significant change in daily body weight (fig. 18), organ weight and biochemical parameters (Table 3) were observed at the end of this period in OVZ female rats treated with HEEP for 14 consecutive days.

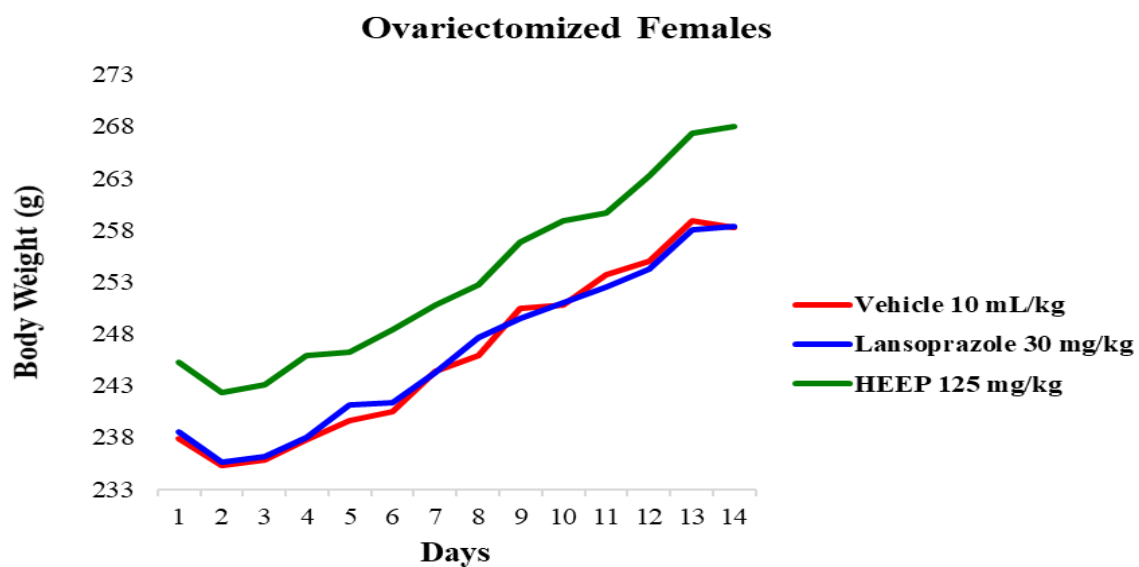


Figure 18 Body weight evolution in OVZ female rats after treatment with vehicle, LZ or HEEP administered 14 days after gastric ulcer formation by acetic acid (n=8-10). Results are expressed as the means of body weight(g). Statistical significance was determined by one-way ANOVA followed by Dunnett's test.

Table 3 Effects of HEEP and LZ on selected toxicological parameters in OVZ females collected 14 days following treatments.

	Treatments (p.o)		
	Vehicle	Lansoprazole	HEEP
Organ Weights			
Heart	3.35 ± 0.04	3.39 ± 0.05	3.49 ± 0.09
Kidneys	4.52 ± 0.07	4.56 ± 0.07	4.61 ± 0.05
Lung	4.28 ± 0.11	4.30 ± 0.10	4.47 ± 0.17
Liver	10.56 ± 0.16	10.61 ± 0.10	10.60 ± 0.11
Spleen	2.59 ± 0.06	2.68 ± 0.08	2.65 ± 0.05
Biochemical parameters			
Glucose	117.30 ± 4.36	126.70 ± 4.48	119.00 ± 5.46
Creatinine	0.42 ± 0.00	0.43 ± 0.01	0.43 ± 0.01
Urea	55.08 ± 2.24	48.97 ± 1.67	55.37 ± 2.92
γ-GT	< 1,0	< 1,0	< 1,0
AST	176.80 ± 15.37	192.70 ± 14.77	191.00 ± 21.04
ALT	62.17 ± 3.68	57.57 ± 2.36	56.43 ± 1.92

Results are expressed as the means ± S.E.M. obtained from different groups. Statistical significance was determined by one-way ANOVA followed by Dunnett's test. The units for the biochemical parameters of serum are U/L (ALT, AST and γ-GT) and mg/dL (urea, glucose and creatinine).

DISCUSSION

The acetic acid model is suitable for chronic gastric ulcers. It is used to evaluate the effect of potential drugs or to test materials on the healing process of chronic ulcers [30, 20]. The model easily and reliably produces round, deep ulcers in the stomach of rats [31, 32]. Due to the model's resemblance to human ulcers, it's been found suitable for use in assessing agents with potential ulcer healing effects in chronic gastric ulcers [31].

One of the least understood aspects of gastric ulcer is the chronicity of the disease that is characterized by repeated episodes of healing and re-exacerbation, a phenomenon which is a challenge to both patients and physicians [31].

The healing process of ulcers can be divided into 3 phases: 0-3 days, stage of ulcer development, with tissue necrosis, ulcer implantation, inflammatory infiltration and ulcer margin formation; 3-10 days, rapid healing phase, involving migration of epithelial cells and contraction of the ulcer base; 10-20 days, slow healing phase, comprising angiogenesis, remodeling of granulation tissues and complete reepithelialization of ulcer [32].

For the first time, the results of this work showed that during 14 consecutive days of treatment, HEEP was able to heal the gastric ulcer of males induced by acetic acid in 52.44% (Figure 1). The intact (cycling) and OVZ female rats submitted to gastric acetic acid injury showed a significant reduction of the lesion area in 85.22% and 65.47% when treated for 14 days with HEEP and compared with control group (vehicle). The LZ treated group also significantly decreased the lesion in 84.21% (intact) and 49.40% (OVZ) (Figure 1). In this same group, we can observe that intact females when compared to males submitted to the same procedure of ulceration showed a greater healing effect both in the treatment with HEEP and in LZ. The comparison of the lesion area of OVZ females and male did not present significant difference (figure 1). These results corroborate with the findings of Günal et al. (2003) [33], which demonstrated that the severity of gastric ulcers induced by the application of acetic acid is attenuated by the effect of estrogen (Figure 1) and by the fact that it occurs more frequently in men than in women [33,34].

The antiulcerogenic activity of estrogen was previously explained by its stimulating effect on parietal cell activity and maintenance of mucus integrity [36]. The protective effect of estrogens on endothelial function includes the antioxidant properties, vasodilator, prevention in the formation of platelet

thrombi and angiogenesis promoter [37]. Several studies support the idea that the estrogen-induced vasoprotective effect may be due to the release of nitric oxide (NO) from the vascular endothelium [38].

Gastric ulcer healing is a complex and dynamic genetically programmed process [39]. It is well-established that myofibroblasts are an important component of wound healing [40], responsible for extracellular matrix (ECM) production, morphogenesis and the inflammatory process related to tissue repair [52-54]. In this context, data obtained from previous studies showed that fibroblasts have an important role in the gastric and esophageal ulcer healing in mice and rats [51, 54-55].

Similar to da Silva et al. (2015) [45], the murine fibroblast L929 (NCTC clone 929) cells were used as a complementary *in vitro* model to confirm the cell proliferative effect of HEEP. Fibroblast cell cultures have been proposed as a suitable method for testing wound healing activity *in vitro* [46], and the incubation with the extract was able to cover the scratched wound areas made on the L929 cell monolayer after 24 h (fig. 15) and showed no sign of cytotoxicity (fig. 14) in the MTT assay. Thus, the enhancement of proliferation and migration to cover of the scratched wounds by the extract reinforce the healing potential of HEEP.

Healing of gastric ulcers is an active and complex process that involves reconstruction of the mucosa by formation of granulation tissue at the base of the ulcer, formation of new vessels (angiogenesis), and restoration of the glandular architecture [47]. The integrity of the gastric mucosa is extremely dependent on the continuous generation of PGs by COX-1 and COX-2 [48]. COX-1 is expressively constitutive in the gastric mucosa and mediates the synthesis of PG that regulates blood flow in the mucosa, secretes mucus and bicarbonate. COX-2 also plays an important role in the healing of gastric ulcers, considering that its inhibition slows healing, as it is responsible for mobilizing cell proliferation, promoting angiogenesis and restoring mucosal integrity [40-43].

When we performed the dosage of PGE₂ in the model of gastric ulcer induction by acetic acid, we can see that there is an increase of PGE₂ in both the HEEP and the LZ group of male rats when compared to the vehicle group (fig. 2). This result demonstrates the participation of PGE₂ in the healing process of gastric ulcers of male rats treated with HEEP for 14 consecutive days. The same result did not occur with the female rats.

The injection of acetic acid into gastric mucosa induces the development of deep gastric ulceration and gastric mucosal damage directly associated with extracellular matrix (ECM) degradation, in which the zinc-dependent matrix metalloproteinases (MMPs) play a crucial role. In several animal studies of gastric ulcer, attention has focused on the role of MMPs, mainly MMP-2 and MMP-9 [51]. MMP are divided into different subgroups, including collagenases, gelatinases, stromelysins, membrane type MMP and others [52]. The gelatinase family consists of MMP-2 (72 kDa) and MMP-9 (92 kDa) [53]. Wound formation and the following healing are dynamic processes of ECM remodeling that are mainly influenced by MMP-2 and MMP-9 [54].

Li et al. (2013) described enhanced expression of MMP-9 in the margin of the ulcer. Their study suggested that the presence of MMP-9 at the margin of the ulcer may be indicative of inflammation and poor wound healing. Our results shown that treatment with HEEP and LZ of male (fig. 3), intact (fig. 4) and OVZ (fig. 5) female rats in acetic acid induced chronic ulcers for 14 days inhibited the MMP-9 activity, the bands were not detected.

The activity of MMP-2 in acetic acid induced gastric ulcer in rats was not significantly altered after treatment with HEEP and LZ (fig. 3, 4 and 5) when compared to ulcer control group (vehicle). These results corroborate with the findings of Baragi et al. [55], where the expression of MMP-2 remained constant at ulcer healing phase. Fini and Girard (1990) reported that the expression of MMP-2 was the same in controls and animals injected with acetic acid, and that this MMP may not have a direct role in the formation or healing phase of the

ulcer. It has been speculated that this enzyme may play a surveillance role in maintaining the integrity of the matrix structure by aiding in the proper assembly of new collagen fibrils [56]. Treatment with HEEP for 14 consecutive days in acetic acid induced gastric lesions inhibit the MMP-9 activity and maintain a constant activity of MMP-2.

Ulcer healing requires filling of the defect with cells and connective tissue, which is accomplished by cell migration, proliferation (EGF), cell apoptosis (caspases and Bcl-2), angiogenesis (VEGF) and remodeling (MMPs) ultimately leading to scar formation. All of these processes are controlled by growth factors (PGs – COX-1 and COX-2) – proteins capable of stimulating cell proliferation and division. At the ulcer margin, epithelial cells proliferate and migrate onto the granulation tissue to cover (re-epithelialize) the ulcer and initiate reconstruction of the glands within the ulcer scar [28, 29, 48].

Hence, western blotting analysis of COX-1, COX-2, EGF, VEGF, caspase-8, caspase-9, caspase-3 and Bcl-2 were performed after 14 days treatment with HEEP and LZ in the animals that had gastric ulcer induced by acetic acid (figs. 6, 7, 8, 9, 10, 11, 12 and 13). In the group treated with HEEP none of these proteins were altered when compared with control group (vehicle). Animals that was treated with LZ had an increase in the expression of EGF in male and OVZ females when compared to their respective control group (vehicle) (fig. 8).

Growth factors, such as EGF, activate epithelial cell migration and proliferation and accelerate wound/ulcer healing *in vivo* and *in vitro* [39]. The increase expression of EGF in LZ groups can explain the healing effect of this treatment. The increased effect of EGF in the OVZ females and males group in comparison with the intact females can represent that in this phase the groups are in the process of healing whereas they have increased lesion area than the LZ group of intact females.

Our results of western blotting analysis revealed that the treatment with HEEP did not altered these proteins in this stage of the healing process or after 14

days of treatment these parameters have already been normalized. Furthermore, we can observe that female sex hormones are interfering in the expression of EGF, VEGF, Caspase-8 and Bcl-2 (figs. 8, 9, 10 and 13), once there is a difference. There is a decrease in EGF, Caspase-8 and Bcl-2 and increase in VEGF expression of intact female when compared to males and OVZ females.

In this model used for gastric healing, we also evaluated in all groups treated with HEEP the absence of death, changes in body mass (fig. 16, 17 and 18) and in the weight of the organs in these groups (table 1, 2 and 3). In addition, we evaluated biochemical parameters that are indicative of hepatic integrity (AST, ALT and γ -GT), renal integrity (urea and creatinine), and glycemia (Table 1, 2 and 3).

The liver is the organ in which exogenous substances are metabolized and subsequently excreted and these functions may be impaired due to acute or chronic exposures to toxic substances [58]. The aminotransferases (AST and ALT) are normally found in the hepatocyte cytoplasm and the serum increase of these enzymes indicates liver necrosis [47, 48]. The γ -GT has as its main origin the liver; the increase of this enzyme in the blood occurs through liver diseases regardless of its etiology, since its importance as a hepatic marker [59]. Excretion of urea and creatinine are indicators of renal function. Products of protein metabolism such as creatinine is a strong indicator for measuring glomerular filtration rate, while urea is a less safe indicator when evaluated alone, since fluctuation is affected by variables independent of glomerular filtration rate [49, 50]. Accompanying the serum glucose level is also fundamental because of the possibility of dysfunctions in the maintenance of glycemia, such as hypoglycemia causing serious damage to the central nervous system, since glucose is a primary source of energy [63].

In the results presented in table 1 we can see that HEEP did not alter any of the biochemical parameters analyzed when compared to vehicle, but the LZ group (30 mg/kg) presented a significant increase in glucose levels when compared to

the control group (vehicle). According to the Clinical Laboratory Parameters for Crl:WI (Han) (2008) [64], the reference values for glucose in male rats aged 8 to 16 weeks range from 70-208 mg/dL, which indicates that despite the variation between the groups, the values are within the normal range.

These results show that HEEP when administered for 14 days in males and females (intact and OVZ) has been shown to be safe in relation to subacute toxicity.

In conclusion, the present study demonstrates the ulcer-healing action of the hydroalcoholic extract from the leaves of *Eugenia punicifolia*. The HEEP healed the gastric mucosa of the animals that was injured by acetic acid and this effect is modulated by female sex hormones, since the intact female rats showed a difference in lesion area with male and OVZ female rats. The curative effect of the extract is also mediated by prostaglandin, remodelling of extracellular matrix, proliferation and migration of cells in gastric mucosa. Additionally, the HEEP is safe in relation of subacute toxicity and cytotoxicity.

ARTICLE HIGHLIGHTS

Research background

Gastric ulcer refers to acid injury of the digestive tract, resulting in mucosal break reaching the submucosa layer. Acid gastric disorders are very common in the United States, with 4 million individuals (new cases and recurrences) affected per year. This disease occurs more often in men than in women, but these differences are less pronounced after 45 years old, probably because the incidence of ulcers increases in menopausal women.

Research motivation

The conventional treatment to gastric ulcer is based on inhibition of gastric acid secretion, but this therapy is related to several side effects and a poor quality of ulcer healing. Therefore, alternative therapies are favorable. We investigated

Eugenia punicifolia (HEEP), that is a specie popularly used to treat inflammation and wounds.

Research objectives

We evaluated the effect of HEEP in the healing of gastric ulcers induced by acetic-acid and analyzed the differences in the curative action of the extract between males and female rats.

Research methods

We used the acetic acid-induced gastric ulcer model to evaluate the healing effect of HEEP, measured the prostaglandin levels, analyzed the extracellular matrix by zymography, quality of ulcer healing by western blot and assessed the healing activity by scratch assay. Moreover, the subacute toxicity (*in vivo*) and cytotoxicity (*in vitro*) were also investigated.

Research results

HEEP demonstrated a high healing capacity, with reduction of lesion area in male, intact and ovariectomized female rats. HEEP accelerates the healing gastric lesions and this effect is modulated by female sex hormones. The curative role of HEEP is due to an increase in PGE₂, inhibition (MMP-9) and maintenance (MMP-2) of ECM in gastric mucosa. Furthermore, HEEP healing properties also were confirmed by enhancement of proliferation and coverage of scratched wounds in fibroblast monolayer (*in vitro*). No sign toxicity was observed in this study.

Research conclusions

The healing activity of HEEP in acetic acid model was identified for the first time and this effect is modulated by female sex hormones. The curative effect of the extract is also mediated by prostaglandin, remodelling of extracellular

matrix, proliferation and migration of cells in gastric mucosa. Additionally, the HEEP is safe in relation of subacute toxicity and cytotoxicity.

Research perspectives

The present data validated the popular use of *E. punicifolia* and contributes to the search for new therapies for diseases that affect the GI tract.

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CAPÍTULO 5

Research Article

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Antidiarrheal and antimicrobial activities of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (Kunth) DC *in vivo* and *in vitro*

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Abstract

Background: *Eugenia punicifolia* (Kunth) DC., are popularly used in decoctions or aqueous infusions as a natural therapeutic agent to treat gastritis, inflammation and infectious diseases. Despite its popular use to treat infectious diseases, there is no proven record of the antidiarrheal and antimicrobial activity of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP). Therefore, the present study was undertaken to evaluate the antidiarrheal activity of HEEP in rodent models and its antimicrobial activity *in vitro*.

Methods: The anti-diarrheal effects were studied using the models of diarrhea induced by castor oil, intestinal motility and intestinal fluid accumulation in mice. The antimicrobial activity was investigated by the *in vitro* microdilution methods.

Results: The results indicated that HEEP (250 mg/kg) was effective in reducing the onset of bowel movements induced by cathartic agent, castor oil, as well as reducing the amount of these liquid bowel movements. All doses tested (125, 250 and 500 mg/kg) decreased intestinal motility but did not show a significant reduction in the fluid accumulation. *In vitro*, the extract also displayed evident antimicrobial effects against *Staphylococcus aureus*.

Conclusion: HEEP demonstrates an antidiarrheal and antimicrobial action. This work confirmed that the anti-diarrheal action of this extract is due to decreasing diarrheal faeces and intestinal motility. These effects of the extract are accompanied by an antimicrobial action against the *Staphylococcus aureus*, corroborating, thereby, with the indication of this popular plant for infections.

Key-words: Antidiarrheal, Antimicrobial, *Eugenia punicifolia*, Myrtaceae, *Staphylococcus aureus*.

Background

Worldwide, diarrhea affects about 2.2 million people annually and those most affected are children under five years [1]. Diarrheal disease is the second leading cause of death in children under five years old and is responsible for killing around 525,000 children every year. Diarrhea can last several days and can leave the body without the water and electrolytes that are necessary for survival. In the past, for most people, severe dehydration and fluid loss were the main causes of diarrhea deaths. Now, other causes such as septic bacterial infections are likely to account for an increasing proportion of all diarrhea-associated deaths. Children who are malnourished or have impaired immunity as well as people living with HIV are most at risk of life-threatening diarrhea [2].

Diarrhea is defined as the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual). Diarrhea is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and others parasitic organisms. Infection is spread through contaminated food or drinking-water, or from person-to-person as a result of poor hygiene [2]. Due to unhygienic livelihood conditions, people in developing countries are very prone to several common diseases including diarrhea [3].

Available drugs used in diarrhea pharmacotherapy are related to contraindications and undesirable effects, like bronchospasm, vomiting, fever [4] and most of the current antibiotics have considerable limitations in terms of antimicrobial spectrum and side effects and their widespread overuse has led to increasing clinical resistance of previously sensitive microorganisms and to the occurrence of uncommon infections [5]. In this context, the World Health Organization (WHO) created a Diarrheal Disease Control Program that stimulates studies with natural products, especially traditional medicinal plants, for the management of diarrhea worldwide [6 - 7].

Eugenia punicifolia (Kunth) DC., (Myrtaceae), is a shrub largely distributed in the Amazon region and Savanna biome and is popularly known as “pedra-ume-caá”, “pedra-ume”, “murta” or “muta”. The leaves of this medicinal plant are popularly used in decoctions or aqueous infusions as a natural therapeutic agent to treat fever and flu [5-6], gastritis, inflammation [10], diabetes [11] and in alcoholic infusions for the treatment of wounds and infectious diseases [12].

The phytochemical studies from *E. punicifolia* characterized the presence of flavonoids, tannins, terpenoids, saponins, as described by Oliveira et al. (2006) and also demonstrated the presence of proanthocyanidins, galotannins, quercetin and myricetin derivatives [14]. ESI-MS analyzes also revealed the presence of gallic acid, quercetin-3-O-arabinopyranoside, quercetin galloyhexoside isomer, syringic acid, ellagic acid, monogalloyl-glucose, glycosyringic acid, mudanoxide B, HHDP-glucose isomer and digalloylglucose isomer [15]. Tannins, alkaloids, flavonoids and terpenoids are the major constituents that are primarily responsible for antidiarrheal activity of many medicinal herbs [16]. Despite its popular use to treat infectious diseases, there is no proven record of the antidiarrheal and antimicrobial activity of the hydroalcoholic extract from the leaves of *Eugenia punicifolia* (HEEP). Therefore, the present study was undertaken to evaluate the antidiarrheal activity of HEEP in rodent models and its antimicrobial activity *in vitro*.

Methods

Drugs and Chemicals

According to the experimental protocol, the drugs used were as follows: castor oil, activated charcoal, (Sigma Chemical Co., St.Louis, USA), morphine (Cristália, Itapira, SP, Brazil) and loperamide hydrochloride (Janssen Cilag, São Paulo, SP, Brazil). A saline solution (0.9% NaCl, 10mL/kg) was used as a vehicle to dilute the hydroalcoholic extract from the leaves of *E. punicifolia* (HEEP) and as a negative control.

Plant material

In December 2009, Dr. Catarina dos Santos collected the leaves of *Eugenia punicifolia* at the Forest Institute and Experimental Stations - Assis State Forest at the point 22°33 'to 22°37' Lat. S-50°21 'to 50°24' Long. W, Assis, State of São Paulo, Brazil. Dr. Antônio C. G. Melo identified the species and a voucher (No. 43322) was deposited in the Herbarium D. Bento Pickel, Assis, SP, Brazil.

Preparation of plant extract

The extract was prepared through the dried and crushed leaves (10 g of plant material) and 100 mL of solvent (ethanol: water 70:30 v/v). Dynamic maceration of the solution was performed for 2h at room temperature ($25 \pm 2^\circ\text{C}$). Thereafter, the solution was filtered, and the residue was extracted two more times. For the drying of the solution the rotary evaporator (40°C) was used and the extract yield was 45% (4.49g) of the original plant material.

Animals

The *in vivo* experimental procedures were conducted using male Swiss albino mice (25–30 g) obtained from the company — Anilab Laboratory Animal Creation and Trade Ltda. (Paulínia – SP), divided into (8 individuals/group): negative control, positive control and HEEP. The animals were maintained in metabolic cages to prevent coprophagia under controlled conditions (room temperature of $22 \pm 2^\circ\text{C}$ and 12:12h light:dark cycle). The diet was composed of Presence® feed and water, provided *ad libitum*. During the fasting periods, the animals were deprived of food for a maximum duration of 16h. All the experimental practices agreed with the experimental protocols, which have already been submitted and approved by the Ethics Committee on the Use of Animal of the Institute of Biosciences of UNESP - Botucatu (nº. 806 – CEUA).

Evaluation of antidiarrheal activity *in vivo*

Diarrhea induced by Castor oil

After 16h of fasting, male Swiss mice (n=8) were treated orally with vehicle (saline 0.9% NaCl, 10mL/kg), loperamide (10mg/kg) and HEEP (125, 250, and 500 mg/kg). After 1h, 0.2 mL of castor oil was orally administered as a diarrhea-inducing agent. The mice were placed in individual boxes lined with filter paper and observed for 4h. The initial evacuation time and stool consistency were evaluated Awouters et al., (1978), modified by Mukherjee et al., (1998).

Intestinal motility determined by activated charcoal

After 6h of fasting, male Swiss mice (n=8) were treated with vehicle, HEEP (125, 250, and 500mg/kg, p.o.) and morphine (2.5 mg/kg, s.c.). After 30min of subcutaneous treatment and 1h of oral treatment, activated charcoal (1g/kg) was administered orally. After 30 min, the animals were euthanized. The distance travelled by the charcoal along the small intestine was calculated for each mouse and expressed as the percentage of distance travelled relative to the total length of the small intestine. % Distance covered by marker = $(A/B) \times 100$, where A denotes the mean of the distance traveled by the charcoal meal and B represents the mean total length of the intestinal segment [19].

Castor oil-induced intestinal fluid accumulation (enteropooling)

The enteropooling assay described by Robert and colleagues (1976) was used to measure fluid accumulation, with some modifications. After 6h of fasting, male Swiss mice were assigned to five groups (n = 8) treated with vehicle, loperamide (10 mg/kg, p.o) or HEPP (125, 250, or 500 mg/kg, p.o). After 1 h, the animals received castor oil (0.2 mL/animal, p.o.) and were euthanized 30 min later to collect and weigh the small intestines, between the pyloric and ileocecal junctions. The contents of the intestine were then expelled onto a plate and weighed. The intestines were reweighed, and the difference between the full and empty intestines was calculated.

Antibacterial activity of HEPP *in vitro*

Strains of microorganisms

The strains of bacteria used to test HEPP were *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922) and *Helicobacter pylori* (ATCC43504). We obtained the microorganisms from the American Type Culture Collection. The strains of bacteria were stored frozen at -20 °C in 20% glycerol plus Mueller Hinton broth (MHB). For use, the bacteria were cultured with 2 mL of MHB and incubated for 24h at 37 °C.

Growth conditions and standardization of the microorganism suspensions

After growth of *E. coli* and *S. aureus* on Mueller Hinton Agar (MHA), they were transferred to sterile MHB and diluted to 0.5 on the McFarland scale (10^8 CFU/mL). The concentration of the strains was determined at 620 nm by spectrophotometry and counted in a Neubauer chamber. The suspension was diluted 1/10 to obtain a suspension at 10^7 CFU/mL for the experiments [21]. The *H. pylori* bacteria were cultured in MHA containing 5% of sheep blood and incubated for 72h at 37°C, in a 10% CO₂ atmosphere. In culture medium (50% of fetal bovine serum + MHB) the strain density was standardized to 2.0 according to the McFarland turbidity standard. The concentration of the strain was determined at 620 nm by spectrophotometry and counted in a Neubauer chamber. The suspension was diluted 1/10 to obtain a suspension at 10^7 CFU/mL for the experiments [21].

Determination of the minimal inhibitory concentration (MIC)

To evaluate the antibacterial potential of HEPP, the species *E. coli*, *S. aureus* and *H. pylori* were employed in this study. The MIC values determination were performed using microdilution technique according to the methodology described in the document M7-A6 (for *E. coli* and *S. aureus*) and the supplement M100-S16 (for *H. pylori*) of the Clinical and Laboratory Standards Institute [6-7], with some modifications. Firstly, the 96 wells plates were filled with 80 µL MHB (for *E. coli* and *S. aureus*) and MHB supplemented with fetal bovine serum (for *H. pylori*). Then, 100 µL of HEPP solution was added to the first well of each row and serial 2-fold dilutions were performed to create different concentrations ranging from 1000 to 7.8 µg/mL. In addition, the standardized bacterial suspension (20 µL) was deposited in the corresponding wells.

The microplates were incubated at 37 °C for 24h in the experiments with *E. coli* and *S. aureus* and at 36 °C during 72h in a 10% CO₂ atmosphere in the *H. pylori* experiments. In all experiments, the positive inhibition control was performed using the antibiotics Ampicillin (100 – 0.3 µg/mL) for *E. coli* and *S. aureus*, and amoxicillin (100 – 0.3 µg/mL) for *H. pylori*. Additionally, the bacterial growth controls, sterility controls (medium, sample) and negative controls (solvents) were included. For the visual readings, 30 µL of an aqueous solution (0.01%) of resazurin was added and the microplates were incubated at 37 °C for 2h (*E. coli* and *S. aureus*) and 3h (*H. pylori*). In the absence of bacterial growth, the solution in the wells remained blue, but turned into a pink color in the presence of microorganism growth [5, 8].

Statistical analysis

The results were expressed as the means ± standard error of the mean (S.E.M) of the parameters obtained. The average values obtained were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test for parametric data and Kruskal-Wallis followed by Dunn's test for non-parametric data. The statistical analysis was considered significant with a minimal value of $p < 0.05$.

Results

Evaluation of antidiarrheal activity *in vivo*

Diarrhea induced by Castor oil

HEEP was evaluated in the diarrhea induction model. HEEP and loperamide were effective in reducing the onset of bowel movements induced by cathartic agent, castor oil, as well as reducing the amount of these liquid bowel movements (table 1).

Table 1. Effects of HEEP on diarrhea induced by castor oil

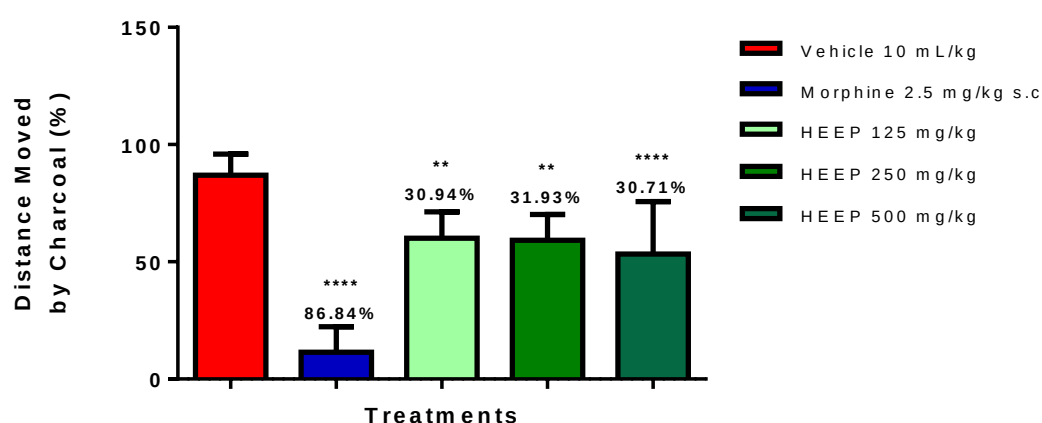
Model	Treatment	Dose (mg/kg)	Time to initial evacuation (min)	Evacuation classification			Inhibition (%) ^a
				Normal	Semi-solid	Liquid	
Diarrhea induced by Castor oil	Vehicle	-	64.57 ± 10.75	1.50 ± 0.26	0.37 ± 0.18	3.62 ± 0.49	-
	Loperamide	10	237.40 ± 2.55 ****	0.22 ± 0.14 *	0.00 ± 0.00	0.00 ± 0.00 ****	100
	HEEP	125	103.00 ± 20.81	0.57 ± 0.36	1.42 ± 0.36	2.85 ± 0.45	21.28
	HEEP	250	170.30 ± 28.07 **	1.00 ± 0.50	1.37 ± 0.26	1.00 ± 0.32 *	72.46
	HEEP	500	159.10 ± 29.25 *	1.00 ± 0.30	1.00 ± 0.30	1.42 ± 0.48	60.78

Results were expressed as the means ± S.E.M and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test (time to initial evacuation) or by the Kruskal–Wallis followed by Dunn's post-hoc test (evacuation classification); * $p < 0.05$; ** $p < 0.01$ and **** $p < 0.0001$ when compared to the vehicle group ^a. The inhibition was related to liquid evacuation compared with the control group treated with vehicle.

Intestinal motility determined by activated charcoal

Figure 1 shows the results of the effect of HEEP on the intestinal motility of mice at different doses. All doses tested decreased intestinal motility ($p < 0.01$ and $p < 0.0001$) when compared to the vehicle group, as well as animals treated with morphine ($p < 0.0001$).

Figure 1. Evaluation of the effect of HEEP on intestinal motility determined by activated charcoal in male Swiss mice.

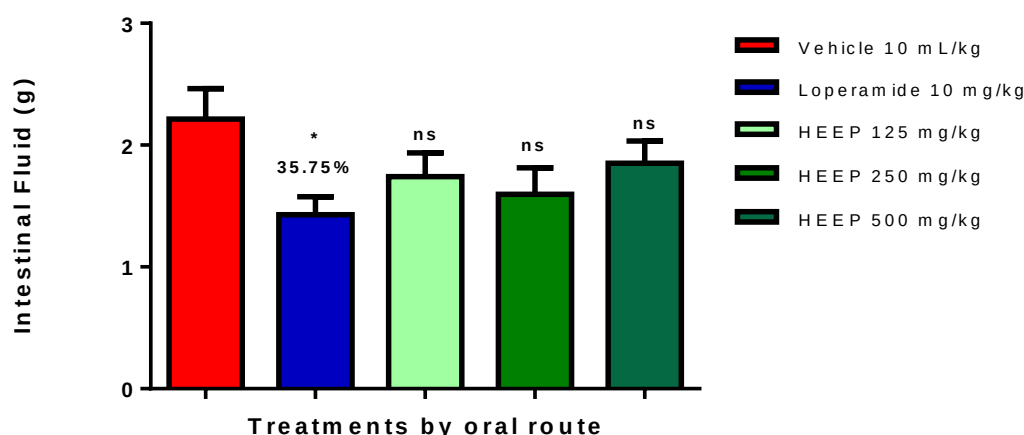


Results were expressed as the means $\pm$ S.E.M and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test; *** $p < 0.001$ and **** $p < 0.0001$. The percentage indicates the reduction in motility relative to the vehicle group.

Castor oil-induced intestinal fluid accumulation (enteropooling)

Oral treatment with HEEP did not show a significant reduction in the accumulation of intestinal fluid when compared to the control group (Figure 2). Treatment with loperamide (positive control) inhibited the accumulation of intestinal fluid induced by castor oil ($p < 0.05$).

Figure 2. Evaluation of the effect of HEEP on the castor oil-induced fluid accumulation in male Swiss mice.



Results were expressed as the means $\pm$ S.E.M and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test; * $p < 0.05$. The percentage indicates the reduction in fluid accumulation relative to the vehicle group. ns = non-significant when compared to the vehicle group.

Antibacterial activity of HEEP *in vitro*

Table 2 shows the effect of HEEP on the growth of *Staphylococcus aureus*, *Escherichia coli* and *Helicobacter pylori*. The results show that HEEP had a minimum inhibitory concentration (MIC) $> 1000 \mu\text{g/mL}$ with respect to *E. coli* and *H. pylori* bacteria. HEEP when tested against *S. aureus* obtained a MIC of $500 \mu\text{g/mL}$.

Table 2. Biological evaluation of the effect of HEEP against *S. aureus*, *E. coli* and *H. pylori*. The numbers indicate the MIC of the extract in the different evaluations.

Bacteria	HEEP ($\mu\text{g/mL}$)
<i>Staphylococcus aureus</i>	500
<i>Escherichia coli</i>	>1000
<i>Helicobacter pylori</i>	>1000

Discussion

Diarrhea is a gastrointestinal disorder characterized by rapid transport of gastric contents through the intestine, leading to an increase in the number of evacuation three or more times a day, with an increase in stool flow rate and/or the presence of blood and mucus. It is also accompanied by an increase in water secretion and decreased liquid uptake, leading to water loss and electrolytes [14, 15]. Non-potable water consumption, lack of basic sanitation and hygiene are some of the common causes of diarrhea [16, 17]. For many decades, diarrhea has been recognized as the leading cause of death,

especially among socioeconomically disadvantaged populations in developing countries [29].

In our models of diarrhea induction, castor oil is used as an effective laxative that decreases fluid absorption, increases electrolyte secretion and alters intestinal motility [30]. It is a triglyceride characterized by having a high content (about 90%) of unsaturated hydroxylated fatty acid, which is the active metabolite responsible for producing diarrhea. After oral ingestion of castor oil, ricinoleic acid is released by lipases into the intestinal lumen and considerable amounts of ricinoleic acid are absorbed into the intestine. The presence of this active metabolite in the small intestine induces a response that stimulates peristaltic osmotic activity, leading to changes in the permeability of the intestinal mucosal electrolyte. In addition, ricinoleic acid also stimulates the secretion of endogenous prostaglandins, nitric oxide, platelet-activating factor, tachykinins, and cAMP [20-24]. The results obtained with the HEEP indicated that there was no loss of liquid and electrolytes due to the treatment (table 1). The dose of 250 mg/kg decreased the number of liquid evacuations (72.46%) and did not affect normal and semi-solid bowel movements, as well as delaying the time of onset of bowel movements.

Intestinal motility is a continuous state of contraction, relaxation and secretion [33]. These functions are controlled by the neuromotor system [34], which in turn regulates several receptor systems, such as cholinergic [35], adrenergic [36], serotonergic [29, 30], opioidergic [31, 32] and the calcium channels [33, 34]. A large number of factors such as chemical agents, stressful life, drugs, emotional state, as well as physiological and pathological conditions can alter intestinal motility and transit time [43]. Extracts that alter the intestinal transit interfere in the absorption and can modify its therapeutic action and of other substances. The results obtained with HEEP on intestinal motility show a reduction in this parameter (figure 1; $p < 0.0001$). Reduced motility allows longer contact between gastric contents and gastrointestinal tract and increases reabsorption of water and electrolytes through the intestine. The alteration in motility interferes in the absorption process, modifying the plasma levels of several substances, which can cause damage to the body.

In addition to intestinal motility and antidiarrheal activity, we verified possible effects of HEEP on the model of intestinal fluid accumulation induced by the cathartic agent castor oil. The intestinal fluid accumulation assay described by Robert et al. (1976) was used to evaluate the amount of intraluminal fluid generated by the diarrheal process in the small intestine. Previous studies have shown that a reduction in the volume and fluidity of feces is usually achieved by reducing both the intraluminal accumulation of water and the loss of electrolytes, such as sodium, potassium and chlorides [32]. Our results showed that HEEP did not alter the accumulation of intestinal fluid (figure 2; $p > 0.05$), therefore, it was not possible to avoid the loss of water and electrolytes induced by the cathartic agent castor oil.

Considering the significant antidiarrheal effect observed by the oral administration of the extract and considering that some pathogenic bacteria are directly involved with this symptom, our next step was to evaluate the antimicrobial effect of HEEP against the bacterium *Escherichia coli*, *Staphylococcus aureus* and *Helicobacter pylori*.

The enteropathogenic bacterium *Escherichia coli* is important in the cause of diarrhea and gastrointestinal problems, both in animals and in humans [9- 11]. While many strains occur as commensal members of the microbiota in the intestinal tract of animals and humans, some strains are nevertheless important pathogens that cause a broad spectrum of diseases to range from self-limiting to life-threatening [11-14]. According to Webster et al. (2008) an extract is satisfactory when it has MIC values equal to or less than 1000 µg/mL. HEEP showed no activity on the growth of *E. coli* bacteria, since its MIC was higher than 1000 µg/mL (table 2).

Helicobacter pylori colonizes the stomach of more than a half of the world's population and its acquisition occurs predominantly in early childhood, especially in preschool age [49]. This bacterium is classified as gram-negative, in a spiral and microaerophilic form that colonizes the gastric mucosa specifically [50]. The first line of treatment against *H. pylori* consists of a triple therapy, proton pump inhibitors, such as omeprazole, combined with two antibiotics, usually amoxicillin and clarithromycin. Triple therapy has a 75% efficacy but is expensive and associated with drug resistance and numerous side effects as noted. Therefore, the search for new therapeutic sources with anti-*Helicobacter pylori* action is extremely important. Currently, plants are seen as the main source for the discovery of new compounds [51]. In 2001, Aligiannis and coworkers reported that MIC values above 1600 µg/mL are weak inhibitors, MICs between 600 and 1500 µg/mL are moderate inhibitors and MICs with values equal to or less than 500 µg/mL are potent inhibitors. Our results showed that HEEP does not show activity on the growth of *H. pylori*, since our MIC ranged from 7.8-1000 µg/mL, and the result was greater than 1000 µg/mL (table 2).

Staphylococcus aureus, a pathogenic Gram-positive bacterium is a ubiquitous commensal bacterium on human skin and mucous membranes [53]. Being present in up to 80% of healthy individuals, it is responsible for various infections in hospital and community settings [54]. *S. aureus* is generally considered a virulent and pathogenic strain amongst staphylococci. The infections caused by *S. aureus* is related to a consortium of virulence factors that allow it to adhere to the surface, invade or avoid the immune system and cause harmful effects to the host [55-57]. It is a cause of various uncomplicated skin infections, abscesses, septicaemia/bacteraemia, gastroenteritis, endocarditis, toxic shock syndrome and food intoxications [54]. Clinically, it is characterized by a short incubation after ingestion. Clinical symptoms include nausea and vomiting followed by abdominal pain and diarrhea [58]. *S. aureus* was implicated as a major cause of antibiotic associated enterocolitis and antibiotic-associated diarrhea [59-60].

WHO's first release of surveillance data on antibiotic resistance reveals high levels of resistance to a number of serious bacterial infections in both high- and low-income countries. The most commonly reported resistant bacteria were *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*, followed by *Salmonella* spp [61]. *S. aureus* has been described to present resistance to beta-lactam antibacterial drugs (methicillin, methicillin-resistant *S. aureus* [MRSA] [62]. Antimicrobial resistance is a complex problem that affects all of society and is driven by many interconnected factors. Greater innovation and investment are required in research and development of new antimicrobial medicines, vaccines, and diagnostic tools [63]. One of the alternatives is the use of medicinal plants with antimicrobial actions [64]. We evaluated HEEP against the *S. aureus*. HEEP was able to inhibit the in

vitro growth of *S. aureus* (MIC = 500 µg/mL; table 2), an important indicator of the potential bactericidal action of this plant species.

Conclusion

The present study demonstrates the antidiarrheal and antimicrobial action of the hydroalcoholic extract from the leaves of *Eugenia punicifolia*. This work confirmed that the anti-diarrheal action of this extract is due to decreasing diarrheal faeces and intestinal motility. These effects of the extract are accompanied by an antimicrobial action against the *Staphylococcus aureus*.

Abbreviations

E. coli: *Escherichia coli*; *H. pylori*: *Helicobacter pylori*; HEEP: hydroalcoholic extract from the leaves of *Eugenia punicifolia*; MHA: Mueller Hinton Agar; MHB: Mueller Hinton Broth; MIC: minimal inhibitory concentration; NaCl: sodium chloride; *S. aureus*: *Staphylococcus aureus*; WHO: World Health Organization.

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Authors' contributions

All authors contributed to the conception and design of the study, performed most of the experiments, coordinated the study, participation in writing the manuscript and approved the final version of the article to be published.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All study protocols involving animals in this manuscript were approved by the Ethics Committee on the Use of Animal of the Institute of Biosciences of UNESP - Botucatu (nº. 806 – CEUA).

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CONSIDERAÇÕES FINAIS

O presente estudo demonstrou que o extrato hidroalcoólico das folhas de *Eugenia punicifolia* contribui efetivamente para a cicatrização da lesão gástrica e duodenal promovidas por uma grande diversidade de agentes ulcerogênicos e que os mecanismos envolvidos nesta ação se mostram diversificados e abrangentes de acordo com o grau de severidade da lesão e do tempo de tratamento com o extrato. Nossos resultados demonstram que existe uma clara interferência do hormônio sexual feminino no processo de cicatrização das úlceras pépticas. Aliado a ação cicatrizante, tem-se que o extrato se mostrou seguro em relação à toxicidade subaguda. Por fim, o extrato também exerce uma ação antidiarreica e antimicrobiana. Desta forma, conclui-se que a *Eugenia punicifolia* mostra-se promissora na cicatrização de úlceras pépticas.



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



Certificado

Certificamos que o Protocolo nº **675-CEUA**, sobre "Avaliação dos mecanismos de ação do extrato hidroalcoólico das folhas de *Eugenia punicifolia* frente a modelos experimentais crônicos de úlcera péptica", sob a responsabilidade de **Clélia Akiko Hiruma-Lima**, está de acordo com a legislação vigente (Lei 11.794/2008 e Decreto 6.899/2009), com as resoluções normativas aplicáveis à luz dos Princípios Éticos na Experimentação Animal elaborados pela Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL/COBEA), e foi aprovado "Ad referendum" da **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA), nesta data.

Botucatu, 18 de novembro de 2014.


Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

Certificado

Certificamos que o projeto intitulado "Avaliação do efeito antidiarreico do extrato hidroalcoólico das folhas de *Eugenia punicifolia* (Kunth) DC em modelos animais", Protocolo nº 806-CEUA, sob a responsabilidade de **Lúcia Regina Machado da Rocha**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)**, nesta data.

Vigência do Projeto:	Início: 14/12/2015	Término: 31/08/2016
Espécie/linhagem:	Camundongo Swiss	
Nº de animais:	75	
Peso:	35g	Idade: 45 dias
Sexo:	Masculino	
Origem:	Biotério Central da Unesp – Câmpus de Botucatu/SP	

Botucatu, 11 de dezembro de 2015.

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

