UNIVERSIDADE D
COIMBRA

Jéssica Malheiros

# Vascular Effects of *Agrimonia eupatoria* L., *Fragaria vesca* L. and *Urtica dioica* L.

**Tese no âmbito do Doutoramento em Ciências Farmacêuticas, especialidade de Farmacologia e Farmacoterapia orientada pela Professora Doutora Maria Dulce Ferreira Cotrim, pelo Professor Doutor Artur Manuel Bordalo Machado Figueirinha e pelo Professor Doutor Diogo André Afonso da Fonseca e apresentada à Faculdade de Farmácia da Universidade de Coimbra.**

Agosto 2023

Faculdade de Farmácia
da Universidade de Coimbra

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*"But if you never try, you'll never know"*

Coldplay

# Agradecimentos

A minha orientadora, Professora Doutora Maria Dulce Ferreira Cotrim, principalmente pelo acolhimento oferecido e pela presença permanente ao longo da realização deste trabalho. Os conhecimentos transmitidos, incentivos e conselhos, que levarei comigo para sempre.

Ao meu orientador Professor Doutor Artur Figueirinha, pela disponibilidade, paciência e a sabedoria em transmitir conhecimentos essenciais ao longo da jornada de desenvolvimento desse projeto.

Ao meu orientador Professor Doutor Diogo André da Fonseca, pela constante disponibilidade, trocas científicas e de conhecimentos, suporte e exigência, imprescindíveis para a conclusão desta tese de doutoramento.

À Daniela Simões, pelo companheirismo, pela disponibilidade, suporte e principalmente pela amizade. Esses elementos, em conjunto, foram indispensáveis para a concretização da primeira parte deste projeto.

Ao Laboratório de Farmacologia e Cuidados Farmacêuticos, Faculdade de Farmácia da Universidade de Coimbra, em especial à senhora Rosário pela disponibilidade e cedência de materiais e reagentes necessários para a realização desse trabalho.

Ao Laboratório de Farmacognosia, Faculdade de Farmácia da Universidade de Coimbra, pela disponibilização dos extratos utilizados nesse projeto. Em especial, agradeço a aluna de doutoramento Patrícia Matos pela disponibilidade, suporte e apoio na realização da componente de fitoquímica deste projeto.

A toda equipa do Centro de Cirurgia Cardiotorácica do Centro Hospitalar e Universitário de Coimbra, pela colaboração e disponibilidade na colheita e entrega das amostras de artéria torácica interna, parte crucial para o desenvolvimento desta tese de doutoramento.

Em especial à minha amiga Mariana Zamboli pela sua amizade e lealdade, que em um momento muito delicado ajudou-me a concluir este trabalho. Também agradeço a Célia

Gomes e Thaís Colombo, que me acolheram e me presentearam com a vossa amizade durante esta temporada em Coimbra.

Aos meus fiéis amigos, que mesmo de longe emanaram energias positivas e apoio incondicional e que me incentivaram a realização desse projeto. Em especial, minha amiga Juliana Costa, que sempre se fez presente e sempre acreditou no meu potencial para a conclusão deste trabalho.

E finalmente ao meus pais Iza Amélia e José Antonio Palminha Malheiros, pessoas mais importantes da minha vida, que tenho um amor incondicional. Primeiramente sou grata por sempre acreditarem em mim. E além disso, por todos os ensinamentos, amor, carinho e suporte e confiança no meu potencial, o que me permitiu atravessar o atlântico para que eu pudesse realizar essa tese de doutoramento.

A todos,
A minha mais sincera e profunda gratidão.

# Abbreviations

| | |
|---|---|
| $\lambda$max | Wavelength maxima |
| 3T3-L1 | Murine Pre-adipocyte cell line 3T3-LI |
| **A** | |
| ABTS | 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid) |
| AChE | Acetylcholinesterase |
| AGEs | Advanced glycation end-products |
| ALT | Alanine transaminase |
| a.m. | Ante meridiem |
| ANOVA | Analysis of variance |
| AqFV | Aqueous extract of *Fragaria vesca* L. |
| AST | Aspartate aminotransferase |
| **B** | |
| $BIC_{50}$ | Biofilm inhibition concentration |
| $BK_{Ca}$ | Large conductance $Ca^{2+}$-activated $K^+$ channels |
| BP | Blood pressure |
| BRIN-BD11 | Hybrid cell line formed by electrofusion of immortal RINm5F cell with New England Deaconess Hospital rat pancreatic B-cell |
| BSA | Bovine serum albumin |
| BuChE | Butyrylcholinesterase |
| BV2 | Microglial cells derived from C57/Bl6 murine |
| **C** | |
| $^{13}C$ -NMR | Carbon 13 nuclear magnetic resonance |
| Caco -2 cells | Epithelial cells isolated from human colon tissue |
| CAD | Coronary artery disease |
| cAMP | Cyclic adenosine monophosphate |
| CAT | Catalase |

| | |
|---|---|
| CCRCs | cumulative concentration-response curves |
| CECs | Circulating endothelial cells |
| cGMP | Cyclic guanosine monophosphate |
| COX | Cyclooxygenase |
| CrE AE | Crude extract of *Agrimonia eupatoria* L. |
| CrE UD | Crud extract of *Urtica dioica* L. |
| CRP | C-reactive protein |
| CTB | Subunit B of cholera toxin |
| CTX | Cholera toxin |
| **D** | |
| DM | Diabetes mellitus |
| DNA | Deoxyribonucleic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| **E** | |
| EDHF | Endothelium-derived hyperpolaring factor |
| EDRF | Endothelium-derived relaxing factor |
| EGCG | Epigallocatechin gallate |
| EMA | European medicines agency |
| $E_{max}$ | Maximal effect produced by the agonist |
| EMPs | Endothelial microparticles |
| eNOS | Endothelial nitric oxide synthase |
| EPC | Endothelial progenitor cells |
| ERK1/2 | Extracellular signal regulated kinase ½ |
| EtOAc | Ethyl acetate fraction |
| **F** | |
| FABMS | Fast atom bombardment mass spectrometry |
| FAD | Flavin adenine dinucleotide |

| | |
|---|---|
| FITC | Fluorescein isothiocyanate |
| FMN | Flavin mononucleotide |
| FRAP | Ferric reducing antioxidant power assay |
| **G** | |
| $G^+$ | Gram positive |
| $G^-$ | Gram negative |
| $GM_1$ | Ganglioside $GM_1$ |
| GPx | Glutathione peroxidase |
| **H** | |
| $^1H$-NMR | Proton nuclear magnetic resonance |
| HaE FV | Hydroalcoholic extract of *Fragaria vesca* L. |
| HBV | Hepatitis B virus |
| HbsAg | Hepatitis B surface antigen |
| HDL | High density lipoprotein |
| HeLa | Human cervical cancer cell line |
| HepG2.2.15 | Hepatoma cell line |
| HPLC | High performance liquid chromatography |
| HPLC-DAD | High performance liquid chromatography with a diode array detector |
| HPLC-DAD-MS | High performance liquid chromatography with a diode array and interfaced with a mass spectrometer |
| HPLC/DMACA | High performance liquid chromatography with post column derivatization by *p*-dimethylaminocinnamaldehyde |
| HPLC-PDA | High performance liquid chromatography coupled with photodiode-array detection |
| HPLC-PDA-ESI/$MS^n$ | High performance liquid chromatography equipped with a photodiode detector and interfaced with tandem mass spectrometer detector |
| HPLC UV/MS | High performance liquid chromatography coupled with UV detector and mass spectrometer detector |

| | |
|---|---|
| HT22 | Mouse hippocampal neuronal cell line |
| HT-29 | Human colon cancer cell line |
| **I** | |
| $IC_{50}$ | Half maximal inhibitory concentration |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IL-6 | Interleukin-6 |
| IL-1β | Interleukin 1β |
| IP | Prostacyclin receptor |
| $IP_{50}$ | Inhibition of 50% of induced lipid peroxidation |
| ITA | Internal thoracic artery |
| **K** | |
| $K_{ATP}$ | Adenosine triphosphate sensitive potassium channel |
| $K_V$ | Voltage gated potassium channel |
| **L** | |
| LDL | Low density lipoprotein |
| L-NMMA | $N^G$-Monomethyl-L-arginine |
| LPS | Lipopolysaccharide |
| **M** | |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MEF | Mouse embryo fibroblasts |
| MEK | Mitogen-activated protein kinase kinases |
| MeOH | Methanol |
| MIC | Minimum inhibitory concentration |
| MMP-2 | Matrix metalloproteinase-2 |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **N** | |

| | |
|---|---|
| *n* | Number of experiences |
| NA | Noradrenaline |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa B |
| **P** | |
| PAI-1 | Plasminogen activator inhibitor 1 |
| PDGF | Platelet-derived growth factor |
| PDE5 | Phosphodiesterase |
| $PGH_2$ | Prostaglandin H2 |
| $PGI_2$ | Prostaglandin $I_2$ or Prostacyclin |
| PKC | Protein kinase C |
| p.m. | Post meridiem |
| **R** | |
| RD | Rhabdomyosarcoma cell line |
| $R_{max}$ | Maximal relaxation |
| ROS | Reactive oxygen species |
| **S** | |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of mean |
| SOD | Superoxide dismutase |
| STZ | Streptozotocin |
| sVCAM | Soluble vascular cell adhesion molecule |
| **T** | |
| TBARS | Thiobarbituric acid reactive substances |
| TG | Triglyceride |
| THP-1 | Human leukemia monocytic cell line |
| TLR4 | Toll-like receptor 4 |

| | |
|---|---|
| TNF-α | Tumor necrosis factor-alpha |
| TRP | Endothelial transient receptor potential channels |
| **U** | |
| UPLC-$MS^2$ | Ultra-performance liquid chromatography- mass spectrometer |
| UV | Ultraviolet |
| **V** | |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VEGF | Vascular endothelial growth factor |
| VSMC | Vascular smooth muscle cell |
| vWF | von Willebrand factor |

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# Abstract

Natural products have been used as a source for drug discovery and development in several areas and for cardiovascular diseases. The main objective of this work was to explore natural products as sources of bioactive compounds with therapeutic potential for vascular disease. To this purpose, we primarily focused on studying plant extracts, specifically extracts from *Agrimonia eupatoria* L., *Fragaria vesca* L. and *Urtica dioica* L. First, a screening of the vasoactive properties (contractile effect, modulatory effect on adrenergic contraction and vasorelaxant effect) was performed to identify the best candidates, whilst the phytochemical characterization of the extracts by HPLC-PDA was carried out. The extracts were constituted mainly by phenolic compounds such as phenolic acids, flavonoids and tannins. Vascular activity was studied using distal segments of human internal thoracic arteries harvested from patients undergoing coronary revascularization. *Fragaria vesca* L. leaves extracts did not elicit vasorelaxant effects of human arteries. Also, aerial parts extract of *Urtica dioica* L. did not elicit vasorelaxant effects, but modulated the adrenergic vascular contraction. In contrast, the infusion of aerial parts of *Agrimonia eupatoria* L. exhibited a marked vasoactivity, which lead to multiple assays with the objective to elucidate the mechanisms responsible for its vasoactivity. Our study demonstrated that the infusion of *A. eupatoria* may exhibit a mixed vascular activity, in which some compounds may promote mild vasoconstriction while others elicit pronounced vasorelaxation. The ethyl acetate fraction (EtOAc) elicited a decrease in the contraction to noradrenaline, while maintaining a marked vasorelaxant effect. Moreover, we also showed that this remarkable vasorelaxation involves both the cyclooxygenase (COX) and the nitric oxide endothelial pathways, while the COX pathway exhibits a central role in the vasorelaxant effect of the EtOAc fraction. Isoquercitrin, a major compound found in the infusion and EtOAc fraction, showed a marked COX-mediated vasorelaxation, and this result corroborates the activity observed for the EtOAc fraction. Overall, these findings suggest that the infusion of *A. eupatoria* exhibits vasoprotective properties and warrant further research to clarify the mechanistic basis for this vascular activity (i.e., identify the specific targets and mediators) and to validate the therapeutic potential in several conditions.

**Keywords**: *Agrimonia eupatoria* L.; *Fragaria vesca* L.; *Urtica dioica* L.; HPLC-PDA; vasoactivity; isoquercitrin.

# Resumo

Produtos naturais vem sido utilizados como fonte para a descoberta de novas moléculas e desenvolvimento em diversas áreas, inclusive para doenças cardiovasculares. O objetivo principal deste trabalho foi investigar produtos naturais como fontes de componentes bioativos com potencial terapêutico para doenças vasculares. Para essa finalidade, primariamente o foco foi investigar extratos de plantas, especificamente *Agrimonia eupatoria* L., *Fragaria vesca* L. e *Urtica dioica* L. Em princípio, foi realizado um *screening* das propriedades vasoativas (efeito contrátil, efeito modulador na contração adrenérgica e efeito vasorelaxante) com o objetivo de identificar os melhores candidatos, enquanto a caracterização fitoquímica foi realizada através de HPLC-PDA. A composição dos extratos foi majoritariamente por compostos fenólicos, como ácidos fenólicos, flavonoides e taninos. Os estudos de atividade vascular foram realizados utilizando artérias torácicas internas colhidas de pacientes que foram submetidos à revascularização coronária. Os extratos de folhas da *Fragaria vesca* L. e *Urtica dioica L.* não promoveram vasorelaxamento em artérias humanas. No entanto, o extratro de partes aéreas da *Urtica dioica* L. modulou a contração adrenérgica vascular. Em contraste, a infusão das partes aéreas da *Agrimonia eupatoria* L. demonstrou uma vasoatividade acentuada, o que levou a diversos ensaios que tiveram como finalidade esclarecer os mecanismos responsáveis pela atividade previamente demonstrada. Neste projeto, foi descrito que a infusão da *A. eupatoria* pode promover uma atividade vascular mista, na qual alguns compostos podem promover uma leve vasconstrição enquanto outros promovem uma vasodilatação pronunciada. A fração acetato de etilo provocou um decréscimo na contração à noradrenalina, ao mesmo tempo que manteve um vasorelaxamento acentuado. Além disso, demonstrou-se que esse vasorelaxamento envolve dois mecanismos endoteliais: o da ciclooxigenase (COX) e do oxido nítrico. De acordo com os resultados, a via da COX, representa o papel central no efeito vasorelaxante da fração acetato de etilo. A isoquercitrina, um dos compostos em maior concentração na infusão e na fração acetato de etilo, demonstrou um vasorelaxamento acentuado mediado pela COX, o que corroborou a atividade observada para a fração acetato de etilo. De maneira geral, os resultados sugerem que a infusão da *A. eupatoria* exibiu propriedades de vasoproteção e, portanto, são necessárias novas pesquisas para clarificar o mecanismo para sua atividade vascular (isto é, identificar os alvos e mediadores específicos) e validar o seu potencial terapêutico em diversas condições.

**Palavras-chave**: *Agrimonia eupatoria* L.; *Fragaria vesca* L.; *Urtica dioica* L.; HPLC-PDA; vasoatividade; isoquercitrina.

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# Chapter 1: Background

## 1.1 Plants as sources of new compounds with therapeutic potential

Multiple reports describe the application of natural products in the treatment of diseases due to the absence of patient response to currently available therapeutic options, the cost of medication, side effects, accessibility and availability of drugs (Al Disi et al. 2016).

Herbal remedies and natural substances were the first medicines to be used by humans due to the many pharmacologically active secondary metabolites produced by plants (Buyel, 2018). Hence, natural products have always played a significant role in drug discovery and can also provide lead structures which are starting points for chemical modification and optimization of drugs (Sen and Samanta 2014; Barnes et al. 2016).

The majority of pharmaceutical drugs are developed from medicinal plants based on the knowledge of local communities and subsequent isolation of the main active ingredients (Altemimi et al. 2017; Nyakudya et al. 2020). Also, the plant material used for the preparation of medicinal remedies could provide potential templates for the production of pharmaceutical drugs (Nyakudya et al. 2020).

Drug discovery from natural sources involve multiple phases and a multidisciplinary approach combining several techniques, namely botanical, phytochemical, biological, and molecular. This area still remains important, however sometimes unexplored, where a systematic search may definitely provide important leads against various pharmacological targets (Sen and Samanta 2014).

According to Newman & Cragg, (2012, 2016) and Veeresham (2012) nearly half of all active principles released between 1981 and 2010 were of natural origin or inspired by natural compounds, in addition to 80% of 112 plant-derived drugs were related to their original ethnopharmacological indication.

Sen and Samanta (2014) discussed that among 56% of the currently prescribed synthetic drugs, 24% were derivative of plant species, 9% were synthetic products modeled from natural products and 6% were extracted directly from plant species.

## 1.2 Phytotherapy and cardiovascular diseases

In the last decades, cardiovascular diseases (CVDs) have been considered to be one of the major causes or morbidity and mortality predominantly in middle-aged people

(Nyakudya et al. 2020), taking an estimated 17.9 million lives each year. According to data available from the World Health Organization, more than four out five deaths of CVD are due to heart attacks and strokes, and one third of these deaths occur prematurely in people under 70 years of age.

This condition can be aggravated by a range of risk factors, such as high levels of low-density lipoprotein (LDL), stress, smoking, hypertension, renal disorders, diabetes, endocrine disfunctions, obesity, alcoholism, and a sedentary lifestyle (Sen and Samantha, 2014).

The World Health Organization describes CVDs as disorders of the heart and blood vessels and includes heart, cerebrovascular, rheumatic heart, peripheral arterial and congenital heart diseases besides deep vein thrombosis and pulmonary embolism, among other conditions.

Among the risk factors, arterial hypertension is the major one, affecting more than 25% of the adult population (Landmesser and Drexler 2007). Hypertension can be defined as high or persistently raised blood pressure and is a serious medical condition, increasing the risk of heart, brain, kidney and other disorders. Of note, it is a major cause of premature death worldwide, with up to 1 in 4 men and 1 in 5 women, over a billion people having the condition (World Health Organization 2022).

Hypertension is often associated with abnormal endothelial function in the peripheral, coronary and renal circulations which is suggested to represent an important pathway whereby this condition promotes the development and progression of vascular disease (Landmesser and Drexler 2007). Moreover, hypertension predisposes myocardial infarction, stroke, renal failure, angina pectoris, atherosclerosis, peripheral artery disease and transient ischemic attack (Sen and Samanta 2014; Nyakudya et al. 2020).

The utilization of natural products as a possible alternative for the treatment of hypertension with minimal side effects and low cost has been extensively researched in the last few decades (Andrade et al., 2016). Moreover, according to the World Health Organization, treatments with herbal medicine are practiced by approximately 80% of world population (Soares-Bezerra et al. 2013; Sen and Samanta 2014).

Multiple plants have compounds that have a direct effect on the heart and blood vessel, the most recognized of these compounds are the cardiac glycosides, i.e digoxin that can be found in *Digitallis* spp. is the best known (Sen and Samanta 2014).

Moreover, some plants have been described for its compounds that can possess cardiovascular properties such as *Crataegus oxycantha* L., *Terminalia arjuna* (Roxb. ex DC) Wight & Arn. and combination of *Inula racemosa* Hook.f. and *Commiphora mukul* (Hook.

ex Stocks) Engl., that can be used to treat congestive heart failure and also acts as cardiotonic, antianginal, antihypertensive, lipid-lowering and hypolipidemic (Sen and Samanta 2014).

In South Africa, where CVD has been an emerging situation, traditional healers for instance, have used decoctions of *Helichrysum ceres* S. Moore to treat hypertension. This bioactivity was tentatively associated to the presence of natriuretic and diuretic bioactive compounds. In addition, an ethanolic extract of the same species has been reported for a vasodilatory effect in the vascular smooth muscle, reducing the total peripheral resistance (Nyakudya et al. 2020).

Other plants have been used in traditional medicine in the management of CVD, such as: *Ekebergia capensis* Sparrm, *Opuntia megacantha* Salm-Dyck, *Sclerocarya birrea*and *Allium sativum* L. that are used as hypotensive (Al Disi et al. 2016). *Ficus thonningii* Blume decreases arterial pressure, *Olea europea* var. oleaster acts as diuretic, anti-atherosclerotic and anti-hypertensive and finally, *Tulbaghia violacea* Harv. inhibits the angiotensin- converting enzyme (Nyakudya et al. 2020). All species mentioned above were assayed in scientific models *in vivo* and/or *in vitro* of cardiovascular diseases, corroborating the utilization in the traditional medicine (Nyakudya et al. 2020).

In fact, Sen and Samanta (2014) published a review report that highlights the importance of therapeutical application of different plant products against various pharmacological targets. Regarding the field of CVDs, the authors reported about 23 species of plants that have activity on the cardiovascular system, as well as the plant parts that were used, the active constituents and its mechanism of action.

Furthermore, in reviews published by Al Disi et al. (2016); Kamyab et al. (2021); Verma et al. (2021), a group of plants were described and associated with an antihypertensive potential. These plants were evaluated through *in vivo, ex vivo* or *in vitro* experiments, were associated with the following activities: First, antihypertensive*: Cocus nucifera* L., *Hisian- Tsao*, *Phyllanthus urinaria* L., *Carum copticum* L., *Tropaeolum majus L.*, *Fritillaria Ussuriensis* f. *lutosa* C.F, Fang, *Laelia anceps* Lindl., *Guazuma ulmifolia* Lam. *Clerodendron trichotomum*, *Astragalus complanatus* Bunge, *Achillea millefolium* L., *Erythroxylum gonocladum*, *Cudrania tricuspidata, Antrodia camphorata*, *Solanum torvum* SW, *Jatropha gossypiifolia* L., *Lepidium Sativum* L. and *Melothria maderaspatana*(L.) Cogn, *Allium sativum* L., *Andrographis paniculata* (Burm.f.) Ness*, Apium graveolens* L.*, Camellia sinensis* (L.) Kuntze*, Coptis chinensis* Franch.*, Coriandrum sativum* L.*, Crataegus spp.*, *Crocus sativus* L.*, Panax ginseng* C.A.Mey*, Salviae miltiorrhizae, Zingiber officinale* Roscoe, *Zingiber officinale* var. Rubra and *Hibiscus sabdariffa* L. Indeed, the consumption of an aqueous extract of

*Hibiscus sabdariffa* L. with healthy subjects aiming to evaluate the impact on systemic antioxidant potential was carried out by Frank et al. (2012). As results, the group described the enhancement of the systemic antioxidant potential and reduced the oxidative stress in humans. In addition, *Hibiscus sabdariffa* L. was the highest reported plant in a systematic review with meta-analysis on the antihypertensive efficacy of Nigerian medicinal plants that comprehended 16 preclinical and 3 clinical studies (Abdulazeez et al. 2021).

The second activity associated with these plants was vasorelaxation, that was evaluated either in rats or *ex-vivo*, consisted in: *Allium sativum* L., *Andrographis paniculate* (Burm.f.), *Apium graveolens* L., *Bidens Pilosa* L., *Camelia sinensis* (L.) Kuntze, *Coptis chinensis* Franch, *Crataegus* spp., *Crocus sativus* L., *Cymbopogon citratus* (DC.) Stapf, *Hibiscus sabdariffa* L., *Nigella sativa* L., *Panax ginseng* C.A.Mey, *Salviae miltiorrhizae, Agastache Mexicana* (Kunth) Lint &Epling, *Cocus nucifera* L., *Mammea Africana* Sabine, *Laelia autumnalis* (Lex) Lindl, *Eucommia ulmoides* Oliv., *Lepechinia caulescens* (Ortega) Epling, *Laelia anceps* Lindl., *Tanacetum vulgare* L., *Cirsium japonicum* (Thunb.) Fisch. Ex DC., *Echinodorus grandiflorus* (Cham. & Schltdl.) Michel, *Peganum harmala* L., *Rauwolfia serpentina*, *Pueraria lobata* subsp. *lobata*, *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M.Sm., *Rhus coriaria* L. and *Ocimum basilicum* L. (Al Disi et al. 2016; Kamyab et al. 2021; Verma et al. 2021).

Third, the effect of lowering blood pressure (BP), (hypotensive) were associated with the following species: *Elettaria cardamomum* (L.) Maton, *Aronia mitchurinii, Citrus limetta* Risso, *Polyalthia longifolia* (Sonn.) Thwaites, *Valeriana wallichii* DC, *Salviacinna barina* M. Martens&Galeotti, *Vitex doniana* Sweet, *Echinodorus grandiflorus* (Cham. & Schltdl.) Michel, *Daucus carota* L., *Uncaria rhynchophyllai* (Miq.) Miq. Ex. Havil., *Cassia absusi* L., *Cinnamomum zeylanicum* Blume, *Theobroma cação* L., *Cassia occidentalis* L., *Cynanchum wilfordii* (Maxim.) Hemsl., *Stephania tetrandra* S. Moore, *Cuscuta reflexa* Roxb., *Fuchsia magellanica* Lam., *Plantago ovata* Forssk., *Ulmus macrocarpa* Hance., *Coleus forskohli* (Willd.) Briq., *Pinus pinaster* Aiton, *Agelanthus dodoneifolius* (DC) Polhill & Wiens, *Melothria madesraspatana* (L.) Cogn., *Moringa oleifera* Lam., *Allium cepa* L.., *Gynura procumbens* (Lour.) Merr., *Chunghyul*-dan, *Raphanus sativus* L., *Carthamus tinctorius* L., *Sesamum indicum* L., *Phyllanthus niruri* L., *Ocimum basilicum* L., *Gastro diaelata* Blume, *Lycopersicon esculentum* Mill., *Marrubium vulgare* L., *Acorus Calamus* L., *Viola odorata* Linn., *Musanga crecopioides* R. Apud Tedlie, *Tribulus terrestris* L., *Momordica charantia* L., *Eugenia uniflorai* L., *Geum*

*japonicum* auct, *Averrhoa carambola L.*, *Bursera simaruba* (L.) Sarg and *Embelia ribes* Burm.f. (Al Disi et al. 2016; Kamyab et al. 2021; Verma et al. 2021).

Species such as *Articum lappa* L., *Ocimum basilicum* L. were also related with ROS scavenging; *Theobroma cacao* L., *Apocynum venetum* L., *Cynanchum wilfordii* (Maxim.), *Bursera simaruba* (L.) Sarg. and *Raphanus sativus* L. were described that increased NO production; *Echinodorus grandifloras* (Cham. & Schltdl.) Michel, *Fuchsia magellanica* Lam., and *Viscum articulatum* Burm.f., were associated with a diuretic effect. (Al Disi et al. 2016; Kamyab et al. 2021; Verma et al. 2021).

Other activities were described and associated with the following species: cardiotonic: *Cecropia pachystachya Trécul;* antioxidant: *Raphanus sativus* L. and *Coriandrum sativum* L., *Rhus coriaria* L.; antinflammatory: *Coriandrum sativum* L.; decreases activity of ACE: *Punica granatum* L.; Reduced pheriperal resistance: *Annona muricata* L. and *Dacus carota* L. was described to improve the endothelial function; and *Curcuma longa* L. decreased $AT_1R$. (Al Disi et al. 2016; Kamyab et al. 2021; Verma et al. 2021).

Groups of secondary metabolites that exhibited cardioprotective properties, include: polyphenols such as flavonoids and proanthocyanidins, saponins, xanthones and their glycosylated derivatives. The molecular targets and pathways described included nitric oxide (NO) generation, cGMP pathway; prostacyclin or prostaglandin $I_2$ ($PGI_2$), cAMP pathway; potassium channel activators; inhibitors of voltage-gated calcium channels; phosphodiesterase inhibitors (PDE5); activation of endothelial transient receptor potential (TRP) channels; inhibitors of protein kinase C (PKC); and free radical scavenging (Stoclet et al. 2004; Sajja and Mannam 2015).

In terms of vascular activity, reports suggest the activity of wine polyphenols that induced an endothelium-dependent vasorelaxation in rat aorta (Andriambeloson et al. 1997). Moreover, Andrade et al. (2016) reported an endothelium independent vasodilating effect on rat isolated arteries induced by *Myrciaria cauliflora* (Mart.) O. Berg also known as jabuticaba. Moreover, Chaves et al. (2009) demonstrated the vasoprotective endothelial effect, namely endothelial shear-stress induced vasorelaxation responses of a standardized grape product in humans.

Interestingly, Schini-Kerth et al. (2010) published a review article that summarizes about 18 plant species extracts and their endothelium dependent relaxation *ex vivo* mostly in rat aortae, rabbit aortae, rat mesenteric arteries and porcine coronary arteries. Most pathways of relaxation seemed to be related with nitric oxide.

## 1.3 Internal thoracic artery

Among cardiovascular diseases, coronary artery disease (CAD) has been associated with a major morbidity and mortality rate. The basis of CAD is the development of atherosclerotic lesions in the innermost layer of the artery, the intima. The atheroma plaque contains inflammatory, immune, vascular endothelial and smooth muscle cells. When the atheromatous process limits blood flow through the coronary arteries, an ischemic event may be precipitated with subsequent infarction (Hansson 2005).

Coronary revascularization through coronary artery bypass grafting (CABG) is a strategy that has been accepted for many years and it is performed in cases of ischemic heart disease. Although this surgical procedure still presents some complications (myocardial infarction, heart failure and atrial fibrillation), this procedure is the gold standard (Fonseca et al. 2014).

The internal thoracic artery (ITA) has been long considered the best graft for CABG (Fig. I.1) mainly because according to the literature, is described that this vessel presents some resistance to develop atherosclerosis, better graft patency and lower incidence of vasospasm (Fonseca et al. 2014; Sajja and Mannam 2015).

Patients that undergo coronary revascularization often present multiple risk factors (Fonseca et al. 2014), which may interfere with the regulation of the endothelium function, that will be discussed in the next section of this thesis (vascular endothelium).

**Figure I.1**: Distal segment of ITA (discarded from surgery) from a patient undergoing coronary revascularization.

The internal thoracic artery is derived from the subclavian artery, located on the internal face of the anterior chest wall and it is accompanied by a pair of internal thoracic veins (Sajja and Mannam 2015).

It is the only peripheral artery that presents the features of elasticity. The composition includes an intima that consists of a layer of endothelial cells, the media that contains predominantly smooth muscle cells and elastic fibers and adventitia composed of fibroblasts, collagen fibers and perivascular nerves (Fig. I.1) (Sajja and Mannam 2015; Oak et al. 2018).

According to Sajja and Mannam (2015), the elastic layer separates the arterial intima from the media and can act as a two-way barrier protecting the media from the effect of any noxious luminal stimuli and also protects the intima preventing the inward migration of smooth muscle cells.

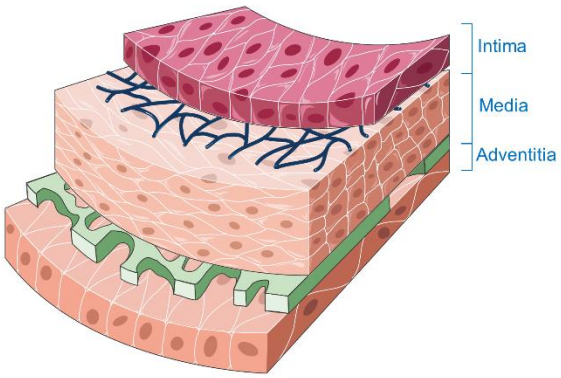



**Figure I.2**: Anatomy of the internal thoracic artery.

In the literature, the elastic layer within the media was suggested to provide some protection against atherosclerosis, preventing the migration of smooth muscle cells into the intima. Further, it was described that the function of medial elastic tunica is to decrease the forceful mechanical stretching of both the internal elastic lamina and medial smooth muscle during systole, and therefore may minimize the penetration of blood components into the vessel wall (Sajja and Mannam 2015).

In pharmacological terms, the vasoconstriction mechanisms are mediated through receptors present in the smooth muscle, predominantly adrenergic $\alpha_1$-receptors (Nakatsu et al. 1991; Giessler et al. 2002; Fonseca et al. 2014), endothelin receptors ($ET_A$ and $ET_B$), angiotensin, 5-hydroxytryptamine and thromboxane-prostanoid receptor also seem to be present (Fonseca et al. 2014).

The stimulation of the adrenergic $\alpha_1$-receptors promotes the activation of multiple intracellular pathways involved in the vasoconstriction, specially by changes in the balance of intracellular calcium. Moreover, this vessel has been described by the significantly higher basal production of NO (Sajja and Mannam 2015).

The influence of several risk factors such as: aging, sex, obesity and adipose tissue, hypertension, diabetes mellitus, hypercholesterolemia and smoking, individually or combined seems to interfere with multiple pathways of regulation of vascular function, especially endothelial function (Fonseca et al. 2014).

The utilization of this vessel graft for research purposes includes reports regarding its biological characteristics (Nakatsu et al. 1991; Sajja and Mannam 2015), vasoactivity (Fonseca et al. 2014), vasomotion, that is rhythmic oscillation of the ITA (Fonseca et al. 2018) and also includes the vascular effects of extracts from different plant species such as *Crataegus Almaatensis* Pojark (Soares et al. 2019), *Cymbopogon citratus* (DC) Stapf. (Simões et al. 2020) and *Oxalis pes-caprae* L. (Gaspar et al. 2018; Fonseca et al. 2020).

Besides there are reports that evaluate the vascular effects of natural derived compounds and mechanisms underlying their activity by Novakovic et al. (2015, 2017).

## 1.4 Vascular endothelium

The endothelium is a fundamental cellular layer, responsible for cardiovascular homeostasis and exercise the major regulation of vessel function, influencing the control of blood flow, regulation of vascular resistance and modulation of immune and inflammatory response (Rajendran et al. 2013). Moreover, it produces a wide scale of hormonal substances with vasoconstrictor and vasodilator functions and works as a semi-permeable membrane, regulating the transport of molecules (Rajendran et al. 2013).

Endothelial cells, localized in the tunica intima, lining the luminal surface of all blood vessels, under physiological conditions, have a major role in the control of vascular structure, function mostly via the generation of potent vasoprotective factors including NO, endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (Rajendran et al. 2013). In addition, the endothelial cells, through different anticoagulant and antiplatelet mechanisms, prevent thrombosis and regulate immune response (Rajendran et al. 2013).

For instance, the biochemical activity of the endothelium can refer to the intervention of the metabolism of vasoactive substances such as angiotensin I and angiotensin II, the inactivation of bradykinin, serotonin and noradrenaline, the synthesis of prostaglandins, production of antigens and the intervention at different levels in the coagulation process of thrombomodulin and can also produce several molecules that stimulate angiogenesis and the repair of damaged blood vessels (Stoclet et al. 2004; Schini-Kerth et al. 2010; Rajendran et al. 2013).

The several functions that could be assigned to the endothelium include: formation of a smooth surface that facilitates laminar blood flow and prevents blood cells from adhering; formation of a permeability barrier for the exchange of nutrients between the plasma and the cellular interstitium, at the same time regulating the transport of substances between them; regulation of angiogenesis; contribution to the formation and maintenance of the extracellular matrix; production of growth factors in response to vascular damage, especially influencing vascular smooth muscle cell (VSMC) proliferation; production of substances that regulate platelet aggregation, coagulation, and fibrinolysis; participation in the immune response and release of agents that act in a paracrine manner on adjacent smooth muscle cells, regulating their contraction.

The regulatory function of the endothelium includes the synthesis of potent vasoprotective factors, that will be explored below.

First, regarding the factors derived from the endothelium, EDHF (endothelium - derived hyperpolarizing factor) promotes the activation of the $Na/K^+$ pump or $K^+$ ATP-sensitive channels in the membrane of smooth muscle cells, leading to a hyperpolarization of the cells. This vasodilation cannot be blocked by inhibitors of NO production. Even though a minor role is attributed to EDHF in several types of large arteries, this factor contributes to regulating the vascular tone in the coronary circulation, also in arterioles by hyperpolarizing the vascular smooth muscle (Schini-Kerth et al. 2010).

The NO is known to induce the relaxation of vascular smooth muscle cells, through the activation of guanylyl cyclase. The formed NO diffuses to smooth muscle fibers where it combines with iron from the active position of guanylyl cyclase, activating it to produce cyclic guanosine monophosphate (cGMP), which stimulates smooth muscle fiber relaxation.

The precursor of NO is L-arginine, and is catalyzed by the endothelial nitric oxide synthase (eNOS) (Schini-Kerth et al. 2010). Concerning specifically to eNOS, this enzyme is present in the endothelium and is responsible for the synthesis of NO, that is formed from L-arginine. This phenomenon requires $Ca^{2+}$, flavin adenine dinucleotide

(FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin ($BH_4$) as co-factors (Rajendran et al. 2013).

Considered the most important vasoprotective factor and its diffusion towards the underlying vascular smooth muscle results in the reduction of the vascular tone and prevention of the smooth muscle cell proliferation and migration, thus maintaining the arterial wall in a quiescent stage (Schini-Kerth et al. 2010; Oak et al. 2018).

Moreover, NO has also been shown to prevent the expression of proinflammatory and pro-atherothrombotic mediators namely monocyte chemoattractant protein-1, adhesion molecules, tissue factor and helps in the maintenance of blood flow, preventing the adhesion and aggregation of platelets and the adhesion of monocytes (Schini-Kerth et al. 2010). Futhermore, NO could be able to prevent the the adherence of monocytes to the endothelial surface and inhibit the expression of adhesion molecules, namely vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in addition to prevent the proliferation of vascular smooth muscle cells and the expression of extracellular matrix molecules resulting in the limitation of vascular remodeling and formation of vascular lesions (Oak et al. 2018).

In fact, nitric oxide-mediated vasodilation appears to be the main pathway of relaxation of the internal thoracic artery (Fonseca et al. 2014; Kraler et al. 2021). Sufficient NO bioavailability is positively associated with normal vasodilation and consequently, normal blood pressure. Failure in the generation or enhanced consumption of this factor leads to impairment of endothelial function, specifically of endothelium-dependent vasodilation, and could also cause a shift of the phenotype of the endothelium to a pro-inflammatory, prothrombotic and procoagulant (Landmesser and Drexler 2007) and, as consequence, could result in hypertension. Of note, reactive species of oxygen (ROS), particularly superoxide ($O_2^{\cdot-}$), are effective NO scavengers (Galleano et al. 2010). Moreover, the loss of NO may promote vascular structural changes, such as smooth muscle cell hypertrophy (Landmesser and Drexler 2007).

Bishop-Bailey et al. (1998) described that two isoforms of cyclooxygenase (COX) have been identified in internal thoracic arteries, a constitutive isoform (COX-1) that can be found in platelets and in the vascular endothelium and COX-2, that possesses inflammatory features. The activation of endothelial COX results in the transformation of arachidonic acid into prostaglandin $H_2$ ($PGH_2$), metabolized by several enzymes and leads to different prostaglandins. In vascular endothelial cells, under physiological conditions, the major metabolite is $PGI_2$ (Perez-Vizcaino et al. 2006).

Prostacyclin is synthesized by endothelial cells and acts primarily on IP receptors (Bishop-Bailey et al. 1998). It possesses a marked vasodilatory activity, inhibits vascular smooth cell proliferation and is a natural inhibitor of platelet adhesion and aggregation. It can also act in synergy with NO to perform the actions previously described (Stoclet et al. 2004; Landmesser and Drexler 2007) and in response to specific stimuli such as bradykinin, adenosine phosphate, hypoxia and increased shear forces (Schini-Kerth et al. 2010).

Summarizing all this information, the healthy endothelium displays a vasodilatory phenotype consisting of high levels of vasodilators such as NO and prostacyclin and low levels of ROS. Moreover, it displays an anticoagulant phenotype consisting of low levels of plasminogen activator inhibitor I (PAI-1), von Willebrand factor (vWF) and P-selectin combined with very little inflammation, as indicated by low levels of soluble vascular cell adhesion molecule (sVCAM), E-selectin, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Finally, the population of endothelial progenitor cells (EPCs, indicative of vascular repair capacity) is high; whereas levels of endothelial microparticles (EMPs) and circulating endothelial cells (CECs), indicative of endothelial stress/damage, are low (Landmesser and Drexler 2007).

In a scenario of CVD, the endothelial function is impaired, i.e. endothelial dysfunction, which is characterized by a shift in the actions of the endothelium. Consequently, a reduction of vasodilatory capacity, a proinflammatory and prothrombotic state, due to increased expression of leukocyte adhesion molecules and cytokines such as monocyte chemoattractant protein-1, can be observed (Landmesser and Drexler 2007; Rajendran et al. 2013). Of note, endothelial dysfunction can also be triggered by other conditions such as diabetes or metabolic syndrome, smoking and sedentarism (Rajendran et al. 2013).

The alteration of the endothelium performance frequently involves a reduced bioavailability of NO (that can be caused by free radicals) and EDHF, associated to an increase of NADPH oxidase-dependent oxidative stress and formation of vasoconstrictor factors namely endothelin-I and thromboxane $A_2$ in the arterial wall. This phenomenon has been suggested to be a key event in the initiation and development of CVD (Schini-Kerth et al. 2010; Rajendran et al. 2013).

It is important to highlight that endothelial function impairment can be also associated with the development of other conditions such as angiogenesis in cancer, infectious diseases, and stroke (Rajendran et al. 2013).

In the case of a dysfunctional endothelium, the phenotypic characteristics include impaired vasodilation, increased oxidative stress/uric acid, lipid peroxide radical,

nitrotyrosine and a procoagulant and pro-inflammatory phenotype with decreased vascular repair capacity and increased numbers of EMPs and CECs (Landmesser and Drexler 2007; Rajendran et al. 2013). In addition, the activity of COX-1 and the release of $PGI_2$ are reduced, thereby leaving the vessels susceptible to vasospasm, thrombosis and atherosclerosis (Bishop-Bailey et al. 1998).

## 1.5 Polyphenols: estructure, classification and biological activities

Polyphenols can be characterized as a large group of bioactive phytochemicals, including a wide variety of molecules which have been associated with a full range of health benefits. This type of compounds can be easily found in fruits and vegetables that are present in human diets (Galleano et al. 2010; Tsao 2010; Oak et al. 2018; Fraga et al. 2019).

In general, polyphenols share chemical structures characterized by at least a simple phenol core bearing at least one hydroxyl group that allows them to act as antioxidants, free radical scavengers and/or redox metal chelators (Galleano et al. 2010; Kasote et al. 2015; Oak et al. 2018). The phenolic structures are classified according to the arrangement of the carbon and their substituents in two main classes: flavonoids and non-flavonoids (Oak et al. 2018).

Polyphenols include multiple classes of compounds, including phenolic acids, flavonoids, stilbenes, coumarins and tannins, among others (Fraga et al. 2019).

Phenolic acids can be divided into two main types that are more reported in the literature: benzoic acid (Fig I.3) and cinnamic acid (Fig I.4) derivatives, based on C1-C6 and C3-C6 backbones (Tsao 2010).

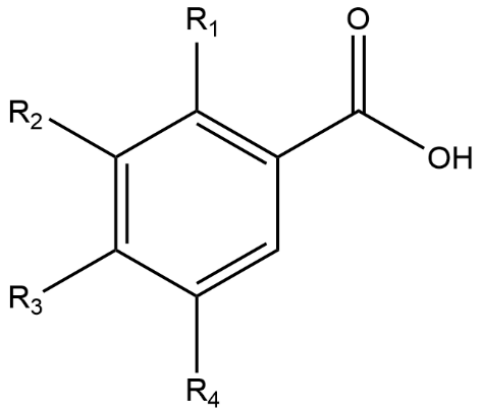



**Figure I.3**: **Basic structure of hydroxybenzoic acid**. For example: Gallic acid, where: $R_1$:H; $R_2$, $R_3$ and $R_4$: OH. Vanillic acid, where: $R_1$ and $R_2$: H, $R_3$: OH, and $R_4$: $OCH_3$. Protocatechuic acid, where $R_1$ and $R_4$: H and $R_2$ and $R_3$: OH.

The bioactivity related to the phenolic acids follows their structural diversity. According to a review report by Araújo et al. (2021), several compounds belonging to the both classes of phenolic acids could act as antidepressant, antihypertensive, anti-inflammatory, neuroprotective, antihyperglycemic, anticancer and anti-diarrheal.

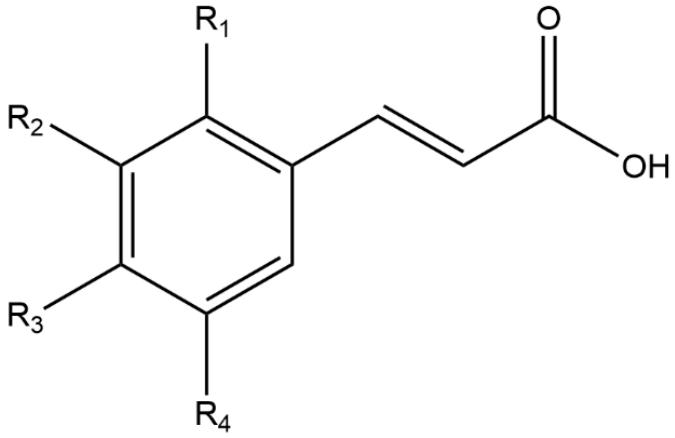



**Figure I.4**: **Basic structure of hydroxycinnamic acid**. For example: *p*-coumaric acid, where: $R_1$, $R_2$, $R_4$: H and $R_3$: OH. Ferulic acid, where: $R_1$ and $R_4$: H, $R_2$: $OCH_3$ and $R_3$: OH. Caffeic acid, where $R_1$ and $R_4$: H and $R_2$ and $R_3$: OH.

Flavonoids are the most common polyphenols found in plants (Simões et al. 2020). They are characterized by the presence of two aromatic rings separated by a three-carbon brigde that in most cases is cyclized, with an exception of the chalcones. (Kumar and Pandey 2013) (Fig. 1.5).

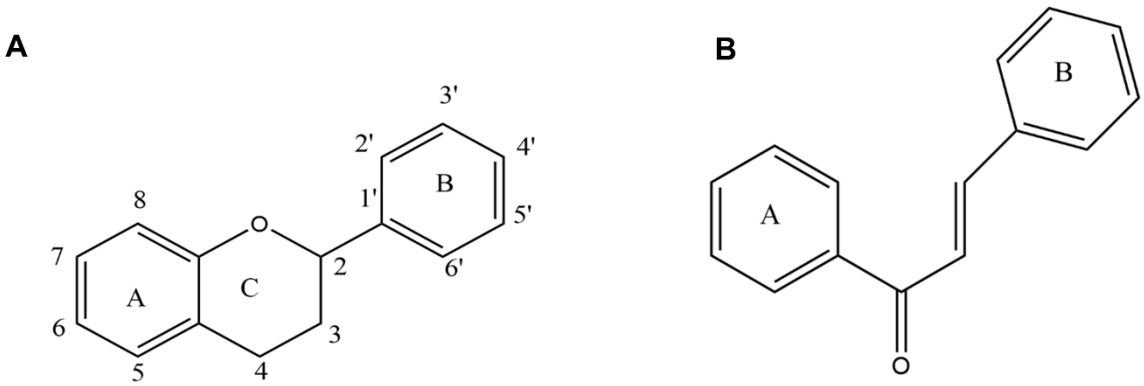



**Figure I.5: Basic structures of flavonoids.** (**A**) Flavonoid. (**B**) Chalcone.

Since this class of compounds have a structural diversity due to hydroxylation pattern and chemical complexity, they are further classified in subclasses, namely: flavonols, flavones, flavanols, flavanones, chalcones, aurones, anthocyanidins and isoflavones (Tsao 2010). Almost all these classes have in common the same basic structure and the difference in groups of flavonoids its due to the structural differences related with the level of oxidation and the pattern of substitution of the heterocyclic

ring in addition to the derivatives of these large groups differentiating among themselves by substitution pattern of the aromatic ring (Fig I.5).

Several flavonoids were often associated with antibacterial, antiviral, anti-inflammatory, antiplatelet, antioxidant, antithrombotic, free radical scavenger and vasorelaxant effects (Woodman et al. 2005; Kumar and Pandey 2013).

The class of compounds with a basic skeleton containing 14 carbons (C6-C2-C6) in which a double-bonded ethylene bridge links the two phenyl rings are denominated stilbenes (Fig1.6). One of the two rings carry two hydroxyl groups, while the other ring carries substituted hydroxyl or methoxy groups in a different position (Teka et al. 2022). Moreover, Teka et al. (2022) reported that the bioactivities often associated with stilbenes include anticancer, antimicrobial, anti-inflammatory and antidegenerative.

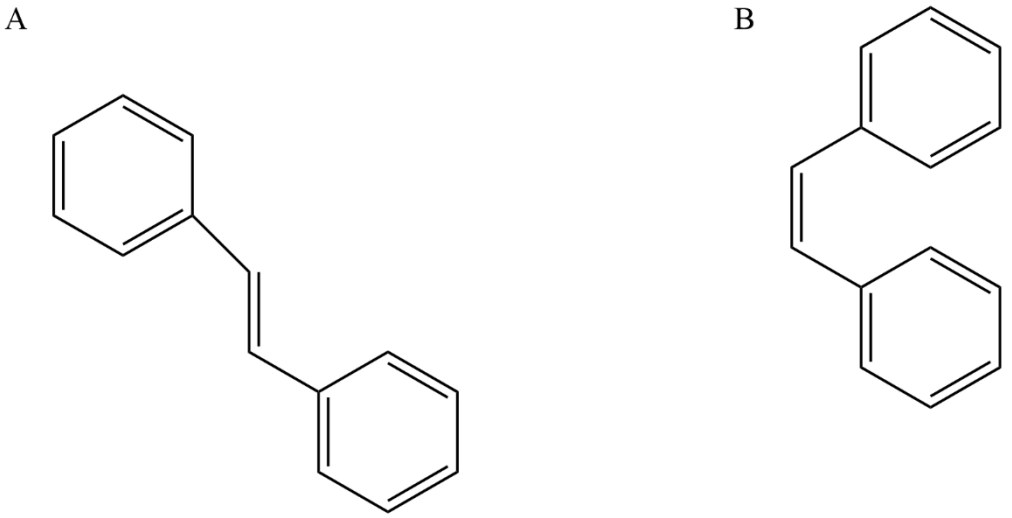



**Figure I.6**: Basic structures of stilbenes. (**A**) *trans* (*E*)-stilbene and (**B**) *cis* (*Z*)-stilbene.

According to Penumathsa and Maulik (2009), *trans*-resveratrol has been reported to have beneficial effects on the cardiovascular system, namely stimulation of eNOS protein expression in cultured endothelial cells (Wallerath et al. 2005; Nicholson et al. 2008) and elicit endothelium-dependent and -independent relaxation in rat aortic rings (Leblais et al. 2008; Schini-Kerth et al. 2010).

Coumarins can be characterized of *cis*-*O*-hydroxycinnamic acid lactones, which contain a $\alpha$-benzopyrone ring system as a basic parent scaffold (Fig I.7) (Pan et al. 2022). This class of compounds are described in the literature as versatile, since they exhibit a wide variety of biological activities, as follows: antimicrobial, antiviral, anticancer, antioxidant, anti-inflammatory, anti-tuberculosis, anti-influenza, anti-Alzheimer and anti-hyperlipidemia (Pan et al. 2022).

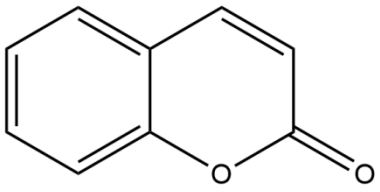


**Figure I.7:** Basic structure of coumarins.

Tannins are polyphenolic compounds of variable molecular size and complexity, often present in plant extracts. These compounds are divided in subclasses based on their resistance, to hydrolysis in the presence of hot water or in the enzymes tannases (Pietta et al. 2003). Hence, tannins can be classified as hydrolysable (Pietta et al. 2003) (Fig I.8) and non-hydrolysable or condensed (Fig I.8).

Hydrolysable tannins contain central glucose molecule linked to gallic acid molecules (gallotannins) or hexahydroxydiphenic acid (ellagitannins) and are readily decomposed in the presence of mineral acids (Pietta et al. 2003).

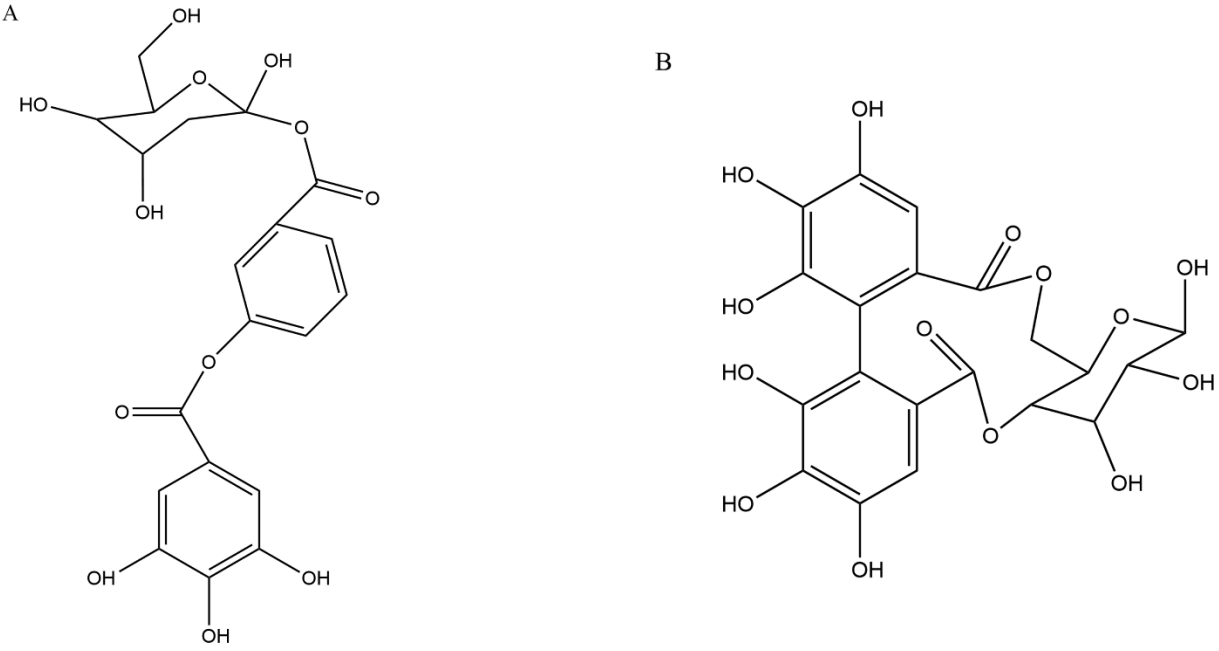



**Figure I.8: Examples of hydrolysable tannins structures**. (**A**) Gallotannin and (**B**) Elagitannin.

In contrast, non-hydrolysable/condensed tannins, also denominated proanthocyanidins, are composed by successive condensation of catechins with a wide range of polymerization (Figure 1.9).

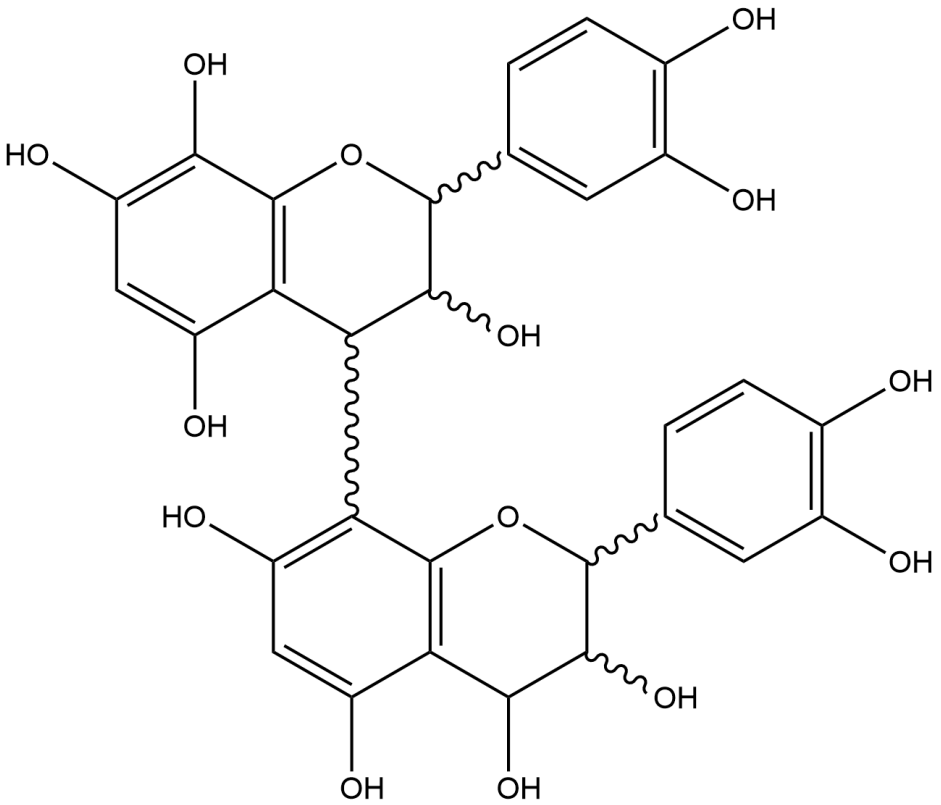



**Figure I.9:** Example of a common structure of a condensed tannin.

According to the literature, tannins can exhibit cardioprotective, antidiabetic, anti-obesity, anti-inflammatory, antioxidant, antimicrobial, antiviral and antimutagenic and anticarcinogenic effects (Pietta et al. 2003).

Anthocyanidins such as cyanidin and cyanidine-3-glucoside, have been shown to induce eNOS protein expression and activation in culture endothelial cells (Xu et al. 2004a; Xu et al. 2004b; Wallerath et al. 2005; Schini-Kerth et al. 2010). In addition, delphinidin induced endothelium-dependent relaxation through NO pathway in isolated arteries (Andriambeloson et al. 1998; Leblais et al. 2008; Schini-Kerth et al. 2010).

## 1.6 Cardiovascular effects of dietary polyphenols

The properties and bioactivity of the polyphenols might be involved in protection against cardiovascular risk, as described by Cheng et al. (2017); Rasines-Perea & Teissedre, (2017), who hypothesized that the antioxidant activity of this group of compounds could protect blood vessels against the consequences of oxidative stress, mechanism highly associated with cardiovascular risk factors.

Ghosh and Scheepens (2009); Schini-Kerth et al. (2010) and Oak et al. (2018) reported that several epidemiological studies suggested that the regular intake of foods such as fruits and vegetables and beverages like red wine and tea, rich in polyphenols i.e flavonoids, was associated with a decrease of the risk of several pathological conditions ranging from hypertension to ischemic heart disease and improvement od the vascular heath, thus reducing the risk of hypertension and CVDs (Cheng et al., 2017; Rasines-Perea and Teissedre, 2017).

Nowadays, the evidence points that the protection against CVD associated with polyphenol-enriched diets may result of a variety of effects on blood vessels, particularly on endothelial cells, and could be produced by multiple mechanisms, and in some cases, different compounds.

Indeed, polyphenols from multiple sources such as tea (green and black), *Vitis vinifera* - grape (purple grape juice, grape marc extract, grape procyanidin extract, grape skin extract and redwines), berries (*Ribes nigrum* L., *Aronia melanocarpa* (Michx.) Elliot and *Vaccinium myrtillus* L. antocyanidin extracts, cranberry juice, *Rubus idaeus* L. extract and fractions and strawberry extracts) and plants (*Crataegus* spp. *Gardenia ternifolia* Schumach & Thonn.), *Hibiscus Sabdariffa* L., *Lysimachia clethroides* Duby, *Parkia biglobosa* (Jacq.) G. Don., *Croton celtidifolius* Baill., *Eucommia ulmoids* Oliv. Bark *and Spondias mombin* L. have been shown to activate endothelial cells to increase the formation of potent vasoprotective factors including NO and EDHF (Schini-Kerth et al. 2010; Oak et al. 2018). Also, almonds (*Prunus dulcis* (Mill.) D.A. Webb) and pistachions (*Pistacia vera* L.) rich in hydroxicinnamic acids and soy products rich in isoflavones, were able to improve blood pressure leves (Grosso et al. 2022).

According to a review by Oak et al. (2018), some authors reported antihypertensive effects after the chronic intake of isolated polyphenols, i.e.: chlorogenic acid acid (Suzuki et al. 2006), epigallocatechin gallate (EGCG) (Potenza et al. 2007), genistein (Vera et al. 2007), quercetin (Duarte et al. 2001; Galisteo et al. 2004; García-Saura et al. 2005; Sánchez et al. 2006; Popiolek-Kalisz and Fornal 2022) and *trans*-resveratrol (DiNatale and Crowe-White, 2022; Rivera et al., 2009) in several models of hypertension.

These antihypertensive effects were associated with an improvement of endothelium dysfunction in response to (-)-epicatechin (Gómez-Guzmán et al. 2012; Galleano et al. 2013; Bernatova 2018) EGCG (Potenza et al. 2007; Sabri et al. 2022), genistein (Vera et al. 2007; Sigowska et al. 2022) and quercetin (Machha and Rais Mustafa 2005; Marunaka et al. 2017). The chronic intake of catechin by normotensive rats (Benito

et al. 2002), and baicalein by hypertensive rats (Machha and Rais Mustafa, 2005) were also associated with an improved endothelial function withoutreduction of blood pressure (Oak et al. 2018).

In addition, the antioxidant effects lead to a decrease of the circulating LDL and membrane lipids oxidation and their consequences in endothelial cells, supporting the evidences that suggest that polyphenols independently from their antioxidant properties, improve the endothelial function (increased NO, EDHF and prostacyclin and decreased endothelin-I formation) and inhibit angiogenesis and migration and proliferation of vascular cells (Stoclet et al. 2004; Torres-Fuentes et al. 2022).

Considering the antioxidant properties of stilbenes, Frombaum, Le Clanche, et al. (2012) discussed that resveratrol was able to decrease the production of intracellular ROS, through direct scavenging and/or activation cellular-enzymatic antioxidant defenses. The direct antioxidant effects of resveratrol could be explained due to presence of the hydroxyl groups that are able to trap ROS in the stilbenic skeleton (Parsamanesh et al. 2021).

Moreover, an analysis of the structure-activity (release of endothelial NO and vasorelaxation of isolated vessels) relationships for polyphenols of different classes has been evaluated (Stoclet et al. 2004). Specifically, in red wines the fractions that showed high activity contained flavanol-3-ols condensed tannins and the anthocyanidin delphinidin were the most active monomer (Andriambeloson et al. 1998; Stoclet et al. 2004).

Also, experimental and clinical studies have been indicating that the chronic intake of several polyphenol-rich natural products is able to ameliorate endothelial dysfunction. Altogether, these observations suggest that natural products, as sources of polyphenols, have the potential to improve vascular functions, hence protect the vascular system (Schini-Kerth et al. 2010).

In fact, a review paper suggested a link between the consumption of polyphenol-rich foods and beverages consumption and reduction in blood pressure, supported by experimental data in animals. The hypothesis that was discussed suggested that by the regulation of NO bioavailability, polyphenols such as quercetin could affect the endothelial function and therefore, blood pressure (Galleano et al. 2010).

Interestingly, Stoclet et al. (2004) suggested that epidemiological studies demonstrate a significant inverse correlation between polyphenol consumption and cardiovascular risk. The improvement of the endothelial function and inhibition of the angiogenesis and cell migration and proliferation in blood vessels have been recently described. According to Stoclet et al. (2004), reports have shown that independently

from their antioxidant effects, extracts enriched in polyphenols, could enhance the production of vasodilating factors such as NO, EDHF and prostacyclin and, in addition, inhibit the synthesis of the vasoconstrictor endothelin-I; inhibit the expression to two pro-angiogenic factors: vascular endothelial growth factor and matrix metalloproteinase-2 (MMP-2) in smooth muscle cells. The mechanistic pathways of these actions involve: in endothelial cells, increased $Ca^{2+}$ levels and of the phosphoinositide 3 (PI3)-kinase/Akt pathway (leading simultaneously to a rapid and sustained activation of eNOS and formation of EDHF) and enhanced expression of eNOS; in smooth muscle cells, both redox-sensitive inhibition of the p38 mitogen activated protein kinase pathway activation (leading to inhibition of platelet-derived growth factor (PDGF)-induced VEGF gene expression) and redox-insensitive mechanisms (leading to inhibition of thrombin-induced MMP-2 formation).

Polyphenols, i.e. procyanidins from grape seeds can induce NO-mediated endothelium-dependent relaxations in isolated arteries (Aldini et al. 2003; Stoclet et al. 2004). The eNOS can be activated by two distinct mechanisms: first, an increase of $Ca^{2+}$ and second the phosphorylation of eNOS by the PI3-kinase/Akt pathway. Polyphenols, can cause EDHF-mediated relaxation of isolated arteries consecutively to a localized and controlled formation of superoxide anions leading to the activation of the PI3-kinase/Aktpathway. Moreover, the participation of EDHF to relaxation induced by polyphenols has also been reported in the rat mesenteric artery by an extract of *Eucommia bark*. Also, resveratrol activated $IK_{ca}$ channels in endothelial cells trough and increase in their open probability (Li et al. 2000).

The increase of the endothelial prostacyclin can also be attributed the polyphenols. Procyanidins from *Vitis vinifera* L. seeds induced a concentration-dependent endothelium-dependent relaxation in the isolated human internal mammary artery. This effect was significantly reduced following the pre-incubation of arterial rings with indomethacin, a COX inhibitor, thus indicating the involvement of prostanoids (Aldini et al. 2003; Stoclet et al. 2004).

The release of this factor inhibits the synthesis and effects of endothelin-I. All these mechanisms might contribute to explain the vasodilatory, vasoprotective and anti-hypertensive effects of polyphenols *in vivo* (Stoclet et al. 2004).

In fact, vasoprotective effects, though multiple pathways, of polyphenols have been discussed. These protective effects could also be due to the ability of polyphenols to decrease the ischemic obstruction event rate and retard the progression of early

atherosclerotic lesions to advanced plaques by catechin, for example (Arts et al. 2001; Stoclet et al. 2004).

The advantageous effects of the dietary polyphenols, in the rate of ischemic obstructions events could be associated with the polyphenol catechin, for example that also may be related with prevention of thrombosis possibly from the inhibition of platelet activation or result from decreased expression of pro-thrombotic and pro-atherosclerotic molecules such as tissue factor and monocyte chemotactic protein-1 (Stoclet et al. 2004).

According to Stoclet et al. (2004), the effects of the dietary polyphenols, such as delphinidin, cyanidin and EGCG on vascular endothelial and smooth muscle cells can be attributed to increased formation of vasodilating, anti-proliferative and antithrombotic factors and decreased formation of vasoconstrictor and proliferative factors produced by endothelial cells, together with inhibition of pro-angiogenic factor namely VEGF and MMP-2 could contribute to the anti-atherogenic and vascular protective mechanisms of polyphenols.

# Chapter 2:
# Aims

The main objective of this work was to explore natural products as sources of bioactive compounds with therapeutic potential for vascular disease. To this purpose, we primarily focused on studying plant extracts, specifically extracts from *Agrimonia eupatoria* L. (infusion of aerial parts), *Fragaria vesca* L. (infusion and hydroalcoholic extract of leaves) and *Urtica dioica* L. (hydroalcoholic extract of aerial parts), and sought to define the mechanisms underlying their vasoactive properties.

First, a screening of the vasoactive properties was performed to identify the best candidates, whilst the phytochemical characterization of the extracts was carried out (Chapter 3). The assessment of the vasoactive properties included three main properties: (a) contractile effect, (b) modulatory effect on adrenergic contraction and (c) vasorelaxant effect.

Thereafter, we planned on carrying out further research with the extract that displayed a marked vascular potential, i.e. absent/minimal contractile effect, evident vasorelaxant effect and/or inhibitory effect on adrenergic contraction.

In our research, the infusion of *A. eupatoria* displayed the best results. As a consequence, an extensive review of the literature on this species, was performed and published (Malheiros, Simões, Figueirinha, et al. 2022). describing the ethnomedicinal use, phenolic profile and bioactivity. This content was included in this thesis as Chapter 4.

In the next step of our work, the objective was to fully understand the mechanisms underlying the vasoactive properties of *A. eupatoria*. For this, a polyphenol-enriched fraction was prepared, characterized and then, we aimed to tentatively identify the vasoactive compounds, through determing the vasoactive properties previously described. The results with *A. eupatoria* have also been published (Malheiros, Simões, Antunes, et al. 2022a) and were included in Chapters 3 and 5.

The results obtained with *F. vesca* have been recently published (Malheiros, Simões, Antunes, et al. 2022b) and were included in Chapter 3. The results with *U. dioica* were included in Chapter 3 of this thesis and are in preparation for submission.

Overall, our study involved 109 experiments with segments of ITA (7 women with a mean age of 72.4 years and 102 men with a mean age of 64.6 years). These grafts were collected at Centro Hospitalar da Universidade de Coimbra, from patients undergoing coronary revascularization, under the approval by the Ethics Committee of Faculty of Medicine of University of Coimbra (CE-118/2018) and of Coimbra University Hospitals (reference PC-388/08), following informed consent.

# Chapter 3: Vascular effects from *Agrimonia eupatoria* L., *Fragaria vesca* L. and *Urtica dioica* L.

Content in part adapted from the following papers:

- Malheiros J, et al. Vascular Effects of Polyphenols from *Agrimonia eupatoria* L. and Role of Isoquercitrin in Its Vasorelaxant Potential in Human Arteries. Pharmaceuticals (Basel) 2022, 15(5):638. doi: 10.3390/ph15050638
- Malheiros J, et al. Vascular effects of *Fragaria vesca* L. in human arteries. Nat Prod Res. 2022. doi: 10.1080/14786419.2022.2152448
- Malheiros J, et al. Vascular effects of *Urtica dioica* L. in human arteries (*in preparation for submission*).

# 3.1 *Agrimonia eupatoria* L.

## 3.1.1 Overview

*A. eupatoria* is a plant that belongs to the Rosaceae family and is native to temperate regions of the Northern Hemisphere. In traditional medicine, this plant has been primarily used for the treatment of inflammatory diseases, due to the high content of polyphenols (Kiselova 2011; Santos et al. 2017). Moreover, the antidiabetic potential of *A. eupatoria* has been shown, involving several mechanisms from glucose formation and absorption to insulin secretion (Gray and Flatt 1998; Kuczmannová et al. 2016). A comprehensive overview of the classification, morphology, distribution, ethnomedicinal use and phytochemistry of *A. eupatoria* (Fig III.1) is available in chapter 4 of this thesis.

Regarding its vascular effects, Kuczmannová et al. (2016) showed improved acetylcholine-induced vasorelaxation in isolated aortic rings from diabetic animals treated with the aqueous extract of *A. eupatoria* from aerial parts, compared to untreated diabetic animals but not to healthy controls. As the high content of polyphenols was suggested to play a role in this vascular protection activity, it remains to be clarified whether the improved endothelium-dependent vasorelaxation was related to the antidiabetic effects of this extract or to a direct effect on the vasculature. To our knowledge, no other studies have reported the direct effects of this extract on the vasculature.

In this context, we aimed to assess the direct vascular effects of the infusion of aerial parts of *A. eupatoria*.


**Figure III.1**: ***Agrimonia eupatoria* L.** Aerial parts containing flowers and leaves. Available from URL: https://flora-on.pt/?q=Agrimonia+eupatoria

### 3.1.2 Material and Methods

Plant material

Aerial parts of *A. eupatoria* were provided by Segredo da Planta, Portugal (batch number 5870 and quality control data identifying no defects), and a voucher specimen (T. Batista 02009) has been deposited at the Herbarium of Medicinal and Aromatic Plants, Faculty of Pharmacy, University of Coimbra. The method used for obtaining the infusion of *A. eupatoria* from the aerial parts has been previously published (Santos et al. 2017). Briefly, 20 g of pulverized plant were infused into 600 mL of water for 15 min. After this procedure, the infusion was vacuum-filtered and washed with *n*-hexane (1:1) to eliminate fat-soluble compounds.

HPLC-PDA

Phytochemical characterization of the infusion was carried out using HPLC coupled with a photodiode array (PDA) detector (Gilson Electronics SA, Villiers le Bel, France). Data were treated with Unipoint® 2.10 (Gilson, Middleton, WI, USA). The sample and standards solutions (100µL) were injected in a Waters® Spherisorb S5 ODS-2 column (250 × 4.6 mm i.d., 5 µm), protected with a guard cartridge C18 (30 × 4 mm i.d., 5 µm) (Nucleosil, Macherey-Nagel, Düren, Germany), and eluted at a flow rate of 1 mL/min and 35 °C. The mobile phase consisted of 5% formic acid (v/v) (eluent A) and methanol (eluent B). The following gradient was utilized: 0–60 min (5–50% B), 60–70 min (50–100% B) and 70–75 min (100–100% B). The concentration for injection was 1 mg/mL. The chromatographic profile was recorded at 280 nm.

The identification of infusion compounds was performed by comparing retention times and their UV spectra with *p*-coumaric acid, isovitexin, isoquercitrin (quercetin-3-*O*-glucoside), ellagic acid and tiliroside. The concentrations of all solutions of commercial standards used for identification were 1 mg/mL.

Vascular activity studies

Experiments were performed on distal segments of ITA harvested from patients undergoing coronary revascularization, as previously mentioned in chapter 2 (Aims).

Vascular tissue preparation was carried out as described in Simões et al. (2020). Briefly, vascular segments were dissected free of fat and connective tissue and cut into 3-mm rings, which were mounted in 10-mL organ bath chambers with Krebs-Henseleit bicarbonate buffer (in mM: NaCl 118.7, KCl 5.4, $CaCl_2.2H_2O$ 1.9, $KH_2PO_4$ 0.9,

$MgSO_4.7H_2O$ 0.6, $NaHCO_3$ 25 and glucose 11.1), previously adjusted to pH=7.4, aerated with 95 $\%O_2$/5 $\%CO_2$ and maintained at 37 °C. After a 2-h stabilization period at a basal tension of 19.6 mN, the isometric tension of vascular rings was recorded using the Powerlab® data acquisition package.

The effect on basal vascular tone was assessed by cumulative concentration-response curves (CCRCs) to the infusion (0.002–0.2 mg/mL) or vehicle, and the results were expressed as absolute contraction (in millinewton, mN).The vasorelaxant effect was tested with CCRCs (0.02–0.2 mg/mL) to the infusion, or vehicle, after sustained pre-contraction with noradrenaline (20 μM), as results were expressed as a percentage of the maximum contraction to noradrenaline (%).The modulatory effect on the noradrenaline-induced contraction was assessed with CCRCs to noradrenaline (0.1–48 μM) before and after 30-min pre-incubations with the infusion (0.02, 0.2 and 2 mg/mL) or vehicle, as the results were expressed as the percentage of the maximal contraction in the control curve, i.e., absence of infusion ($\%E_{max}$).

Tissue viability was tested with potassium chloride (KCl, 60 mM) at the beginning and at the end of each experiment.

Data were generally expressed as mean ± standard error of mean (SEM) unless specified otherwise, and *n* indicates the number of experiments. In assays in which the modulatory effect on the noradrenaline-induced contraction was tested, potency was expressed as the negative logarithm of the effective concentration (in mol/L or M) of noradrenaline able to induce half of the maximum contraction ($pEC_{50}$, -log [M]). In vasorelaxation assays, potency was expressed as the negative logarithm of the effective concentration (mg/mL) of the infusion able to reduce noradrenaline precontraction to half of its maximum, i.e., relaxation of 50% ($pIC_{50}$, -log[mg/mL]). Efficacy was expressed either as $\%E_{max}$ (maximal contraction, %) or $\%R_{max}$ (maximal relaxation, %), respectively, for those assays.

Statistical analysis was performed using GraphPad Prism 7® (GraphPad Inc., La Jolla, CA, USA). First, the normality of data was checked through a Shapiro–Wilk test. Data from CCRCs were analyzed by two-way ANOVA with Tukey's multiple comparisons test to identify differences in specific concentrations throughout the curves. Parameters of potency from CCRCs and data on vascular effects of compounds were analyzed by unpaired one-way ANOVA with Tukey's multiple comparisons. $p< 0.05$ was considered to indicate a statistically significant difference.

Reagents

Chemicals used for Krebs–Henseleit buffer preparation and arterial ring viability testing in the pharmacological studies were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Chemicals used for phytochemical characterization were purchased from Merck® (Darmstadt, Germany) and correspond to the highest grade commercially available. The following reference compounds were used: ellagic acid (Sigma, E2250-5G), *p*-coumaric acid (Sigma, C-9008, Lot: 22H0312), quercetin (G Buchs SG., Buchs, Switzerland, K148-/49/2), quercetin-3-*O*-glucoside (Sigma, 17793-10MG-F), tiliroside (Extrasynthese, Genay, France, 1001 S, Lot: 12080209), vitexin (Extrasynthese, 1232 S) and isovitexin (Extrasynthese, 1235 S).

### 3.1.3 Results and Discussion

Phenolic profile

The phenolic profile of the infusion of *A. eupatoria* was determined through HPLC-PDA (Fig III.2).

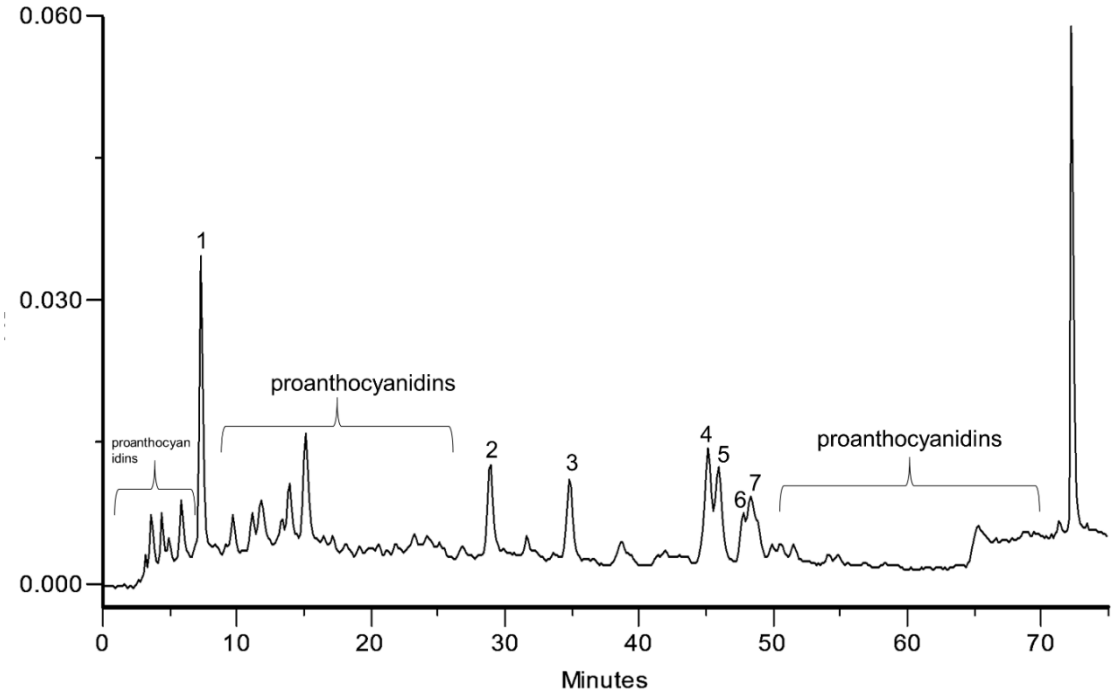



**Figure III.2**: **HPLC-PDA chromatogram of *A. eupatoria* infusion**. Chromatogram recorded at 280 nm.

In the UV spectrum of, the charactheristic of peak 1 suggested the presence of a benzoic acid derivative (wavelength maximum near 313 nm) (Campos and Markhan 2007). Peak 2 with wavelength maxima at 310 nm was identified as *p*-coumaric acid, using the commercial standard compound. Peaks 3, 4 and 5 were 3-*O*-glycosylated quercetin derivatives, as suggested by their UV spectra profile, with band I near 350 nm with absorptivity lower than band II with wavelength maxima near 250 and shoulder approximately at 265 and 295 nm (Mabry 1970). Peak 4 was identified as isoquercitrin (quercetin 3-*O*-glucoside) by comparison of retention time with a commercial standard. Peaks 3 and 5 were previously identified by using HPLC-PDA-ESI/$MS^n$, respectively as quercetin *O*-galloyl-hexoside and quercetin *O*-malonyl-hexoside (Santos et al. 2017). In peak 6, UV spectra, retention time, and the wavelength maxima near 265 and 346 nm with a shoulder near 314 nm, suggested the presence of kaempferol *O*-p-coumaroyl-glucoside (tiliroside) as confirmed with a commercial standard. Peak 7 exhibited a UV spectra profile with wavelength maxima of 248 and 371 nm with a shoulder 322 nm which is characteristic of ellagic acid, also confirmed with the standard compound. Proanthocyanidins were detected due to their characteristic spectral profiles and UV maxima (near 246 and 278 nm) (Couto et al. 2020). The complete characterization of mixture components was previously carried out and published in Santos et al (2017).

Vascular effects

The infusion of *A. eupatoria* induced a mild increase in basal vascular tone ($E_{max}$ = 1.10±0.67 mN; $pEC_{50}$ = 2.72±0.51, $p$<0.05 vs. vehicle). Furthermore, the lower concentration of infusion (0.02 mg/mL) elicited a significant potentiation of the noradrenaline-induced contraction (Fig. III.3 and Table III.1), with an $E_{max}$ increase of 49.18% vs. control ($p$<0.001, Fig. III.3).

This response was also significantly higher compared to the other infusion concentrations (0.2 and 2 mg/mL), which only lead to a significant decrease in potency in comparison with control: 0.2 mg/mL ($p$<0.05) and 2 mg/mL ($p$<0.001).

The ethnomedicinal use of *A. eupatoria* has been reported to derive from several bioactive properties of this plant species, particularly antioxidant, anti-inflammatory and diuretic, among others (Ivanova et al. 2013; Granica et al. 2013; Muruzović et al. 2016; Santos et al. 2017). As the use as anti-diabetic has also been reported, very little information is available on the vascular activity of this plant species.

**Table III.1.** Pharmacological parameters from vascular activity studies with *A. eupatoria* infusion.

| Studies | Concentration | Maximal effect[1] | Potency[2] | *n* |
|---|---|---|---|---|
| *Influence on adrenergic contraction* | Control | 100.00±0.00 | 6.21±0.07 | 15 |
| | 0.02 mg/mL | 149.18±27.76*** | 5.83±0.18 | 5 |
| | 0.2 mg/mL | 84.08±8.55### | 5.69±0.14* | 5 |
| | 2mg/mL | 97.65±10.15+ | 5.44±0.14*** | 4 |
| *Relaxation* | Control | 82.93±9.96 | 2.51±0.13 | 30 |

[1] Maximal effect expressed as maximal contraction (%$E_{max}$ to noradrenaline) for adrenergic contraction studies or maximal relaxation (%$R_{max}$).[2] Potency expressed as $pEC_{50}$ for incubation studies or as $pIC_{50}$ for vasorelaxation studies.*$p$<0.5 vs control, ***$p$< 0.001 vs control, ### $p$<0.01 vs. 0.02 mg/mL, + $p$< 0.05 vs 0.02 mg/mL.

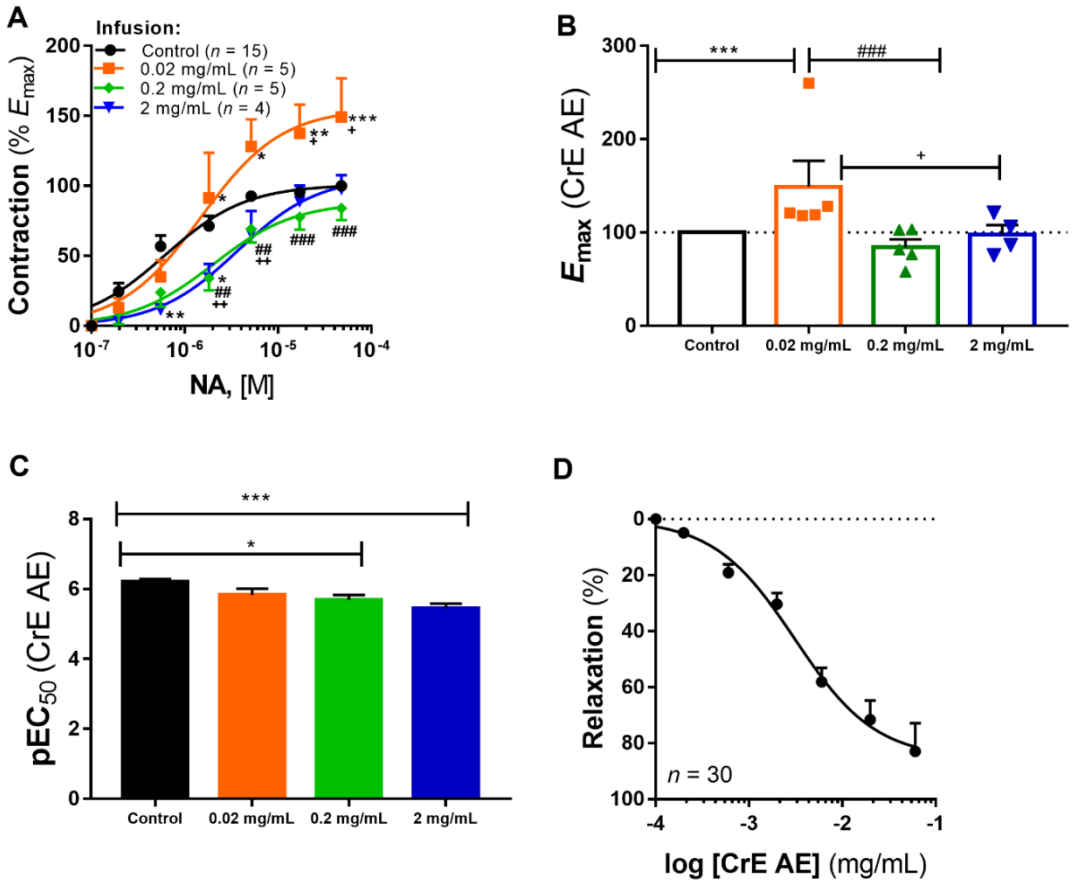



**Figure III.3: Vascular activity of *A. eupatoria* infusion. A**, Effect on the noradrenaline-induced contraction; *$p$<0.05 vs. control, **$p$<0.01 vs. control, ***$p$< 0.001 vs. control, ##$p$< 0.01 vs. 0.2 mg/mL, ####$p$<0.001 vs. 2 mg/mL, +$p$<0.05 vs. 2 mg/mL, ++$p$<0.01 vs. 2 mg/mL.**B-C**, Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$, **C**).*$p$<0.05 vs. control, ***$p$<0.001 vs. control. ###$p$<0.001 vs. 0.2, +$P$<0.05 vs. 0.02 mg/mL. **D**, vasorelaxation elicited by *A. eupatoria* infusion.

To our knowledge, only one report (Kuczmannová et al. 2016) has focused on the vascular activity of *A. eupatoria* showing that the treatment with the aqueous extract improved acetylcholine-induced vasorelaxation in aortic rings isolated from diabetic animals. Importantly, this improvement was observed only in comparison with untreated diabetic animals but not with healthy controls. Whether such effect was a result from the anti-diabetic properties of this extract or from a direct vascular activity remained to be clarified.

Here, we tested the direct vascular activity of *A. eupatoria* and showed that its infusion exhibits potent vasorelaxant activity in human arteries (Fig. III.3D). As a low concentration (0.02 mg/mL) elicited a potentiation of the maximal response to noradrenaline, the infusion displayed a mixed vascular effect thus suggesting some compounds may elicit an increase in vascular tone and others a decrease.

Of note, the magnitude of vasorelaxation could also suggest a direct (endothelium-independent) vascular smooth muscle relaxation effect. In sum, our findings demonstrate the direct vasorelaxation potential and the need for further research to clarify whether this vasorelaxant effect involves a direct vasodilation and also the compounds responsible for such activity.

## 3.2 *Fragaria vesca* L.

### 3.2.1 Overview

*F. vesca* is commonly known as wild strawberry which belongs to the genus *Fragaria* and Rosaceae family (Fig III.4). This species is usually found in Europe, Asia West of the Urals and North America (Liberal et al. 2014) and temperate areas in Chile (Kanodia and Das 2009; European Medicines Agency 2018).

The genus *Fragaria* consists of at least 20 recognized wild species according to the plant list (http://www.theplantlist.org/) and European Medicine Agency. The common *F. vesca* is a light-demanding perennial plant, about 5-20 cm height, which grows in meadows, wood, forest glades, slopes and alongside roads (Dias et al. 2016; European Medicines Agency 2018).

Regarding its botanical features, leaves have the following characteristics: three lobed, feathery, serrated edges and hairy petioles. Moreover, *F. vesca* produces stolons and is flourishing and bearings fruits for a short time, usually in the late spring and

summer. The wild strawberry leaves with petioles or without them, can be collected throughout the growing season (European Medicines Agency 2018).

**Figure III.4**: ***Fragaria vesca* L.** Available from URL: https://upload.wikimedia.org/wikipedia/commons/0/05/Fragaria_vesca_L.jpg

Concerning the ethnomedicinal uses, studies reported a wide spread use of the vegetative parts from wild growing *F. vesca*. Particularly, the leaves are collected during the flowering season in the wild and are commonly prepared as infusion or decoction, being both administrated internally and externally. Preparations of *F. vesca* leaves have been used externally as antiseptic, emollient and dermatologic protector, as well as to treat inflammation of cutaneous and mucosal surfaces (Liberal et al. 2014). The roots are used for diarrhea and the stalks for wounds and the leaves are astringent (Kanodia and Das 2009).

Specifically, fruits of *F. vesca* are reported to contain, for example salicylic acid, responsible for the benefical activity in the treatment of liver and kidney complaints, as well as in the treatment of rheumatism and gout (Kanodia et al. 2011).

*F. vesca* has also been used for respiratory system complaints, such as cough, catarrh and shore throats, cardiovascular diseases including lowering of blood pressure and heart rate of a leaves decoction (Camejo-Rodrigues et al. 2003; Liberal et al. 2014). Also, urinary ailments for the renal stones and a complementary treatment for diabetes were also described (Liberal et al. 2014).

Moreover, *F. vesca* has been used for the treatment of gastrointestinal disorders, especially due to the astringent, antidiarrheic and antidysenteric properties (Kiselova et al. 2006; Kirsch et al. 2020). In addition, the utilization of the leaves of *F. vesca* to treat leukemia, kidney and breast cancers were also reported (Liberal et al. 2014).

Additionally, the employment of *F. vesca* as a diuretic and for the treatment of skin diseases was also described by Kiselova et al. (2006).

In terms of bioactivity, an aqueous extract from leaves, exhibited a potential anti-diabetic, antioxidant, and anti-apoptotic activity in diabetic rats (Ibrahim and Abd El-Maksoud 2015). Furthermore, the anti-*Helicobacter pylori* activity *in vitro* of the leaf extract (Cardoso et al. 2018) and the potential use of the ethanolic extract in the treatment of gastrointestinal inflammatory diseases, such as Crohn's disease, have also been suggested by results obtained in animal models (Kanodia et al. 2011).

According to the European Medicines Agency assessment report concerning *F. vesca*, is described that leaves are collected during the flowering period for use in European traditional medicine and are mainly prepared as an herbal tea for oral use as diuretic and to treat diarrhea. Regarding the posology, for diuretic purposes, 4-8g in 200mL of water as decoction, divided in 2-4 single doses and to treat diarrhea, 1g in 200mL of boiling water 2-3 times daily (European Medicines Agency 2018).

Reports in the literature disclose that *F. vesca* species contain flavonoids, tannins, volatile oils, methyl salicylate, ellagic acid, borneol and also traces of alkaloids (Kanodia and Das 2009; Couto et al. 2020). Moreover, according to the assessment report of *F. vesca,* published by European Medicines Agency, leaves could contain about 5-11% of condensed tannins, flavonoids such as quercetin, rutin, organic acids and glycosides.

Given all the information regarding the composition described of *F. vesca*, the classes of secondary metabolites to the most mentioned to the less mentioned are: tannins (condensed and hydrolysable), flavonoids, phenolic acids, stilbens and furans derivatives. The first three classes of secondary metabolites were found in all parts assayed of *F. vesca*: flowers, leaves, flowers and fruits, whole plant, vegetative parts and roots (Vennat et al. 1988; Mudnic et al. 2009; del Bubba et al. 2012; Bagdonaite et al. 2013; Liberal et al. 2014; Sun et al. 2014; Gasperotti et al. 2015; Dias et al. 2015; Dias et al. 2016; Dias et al. 2017; Couto et al. 2020) and Liberal et al. (2019) reported the composition of an ellagitannin-enriched fraction.

### 3.2.2 Cardiovascular properties

Regarding cardiovascular disorders, *F. vesca* leaves decoction has been reported for lowering blood pressure and heart rate (Camejo-Rodrigues et al. 2003; Manolova 2003; Liberal et al. 2014).

To the best of our knowledge, the only report that assays the vasodilator properties of extracts of *F. vesca* used the rat aorta and showed that the leaves infusion had an endothelium-dependent vasodilator effect, which was mediated by nitric oxide and cyclooxygenase products (Mudnic et al. 2009).

Considering the previously published data and the ethnomedicinal use in cardiovascular disorders, our aim was to assess the vascular effects of leaves extracts of *F. vesca* (an infusion and a hydroalcoholic extract, for purposes of comparison of composition and vasoactivity) in ITA, in order to investigate the clinical application of extracts of this plant species in patients with underlying cardiovascular disease.

### 3.2.3 Material and Methods

Plant material

Wild plants of *F. vesca* were collected from Granja de Figueira do Lorvão, Penacova, Portugal. The plant material was identified by Dr. Célia Cabral, plant taxonomist. A voucher specimen (T. Batista 01011) was deposited at the Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra.

The leaves were selected and dried in the dark, using an oven at 30°C, with air circulation. The hydroalcoholic extract was prepared by extraction with absolute ethanol (2 times) and 50% aqueous ethanol (3 times) (1:10, w/v) at room temperature using Ultra – Turrax homogenizer for 5 min at 8000-9500 rpm. After each extraction, the extracts were filtered under vacuum. Ethanol was removed using a rotary evaporator and the extract was lyophilized and stored at -20°C. A yield of 30% was obtained, expressed in dry plant.

The infusion was prepared according to the recommendations of Physicians' Desk Reference for Herbal Medicines (Fleming, 2000), using 4 g of dried leaves to 150 mL of boiling water in a porcelain laboratory cup for 15 min without additional heating. Then, the infusion was filtered, lyophilized and stored at -20°C. A yield of 23% (expressed in plant) was obtained(Liberal et al. 2014).

HPLC-PDA

Phytochemical characterization was performed using HPLC coupled with photodiode array (PDA) detector (Gilson Electronics SA, Villiers le Bel, France). Data were treated with Unipoint® 2.10 (Gilson, Middleton, WI, USA). The samples (infusion

and hydroalcoholic extract) (100 µL) were injected in a Waters® Spherisorb S5 ODS-2 column (250 × 4.6 mm i.d., 5 µm), protected with a guard cartridge C18 (30 × 4 mm i.d., 5 µm) (Nucleosil, Macherey-Nagel, Düren, Germany) and eluted at a flow rate of 1mL/min and 24 °C for hydroalcoholic extract and 35°C for the infusion. The mobile phase consisted of 5% formic acid (v/v) (eluent A) and methanol (eluent B). The gradients that were utilized were: for infusion: 0-10 min (0%-5 %B), 10-45 min (5%-80 %B), 45-50 min (80%-100% B) and for hydroalcoholic extract: 0–10 min (5%–15 %B), 10–15 min (15%–25% B), 15–50 min (2%– 50% B), 50–60 min (50%–80% B), 60–70 min (8%–100% B).The concentration of the injected extracts of *F. vesca* was 1.8 mg/mL (Couto et al., 2020).The chromatographic profiles was recorded at 280 nm.

Vascular activity studies

Experiments were carried out as described in section 3.1.2. The effect on basal vascular tone was assessed by CCRCs to the infusion and the hydroalcoholic extract (0.002-0.2 mg/mL) and the results were expressed as absolute contraction (in miliNewton or mN). The vasorelaxant effect was tested with CCRCs after sustained pre-contraction with noradrenaline (20 µM), as results were expressed as percentage of the maximum contraction to noradrenaline ($R_{max}$, %). The modulatory effect on the noradrenaline-induced contraction was assessed with CCRCs to noradrenaline (0.1 - 48 µM) before and after 30-min pre-incubations with the infusion (0.02, 0.2 and 2 mg/mL), as the results were expressed as the percentage of the maximal contraction in the control curve, i.e. absence of infusion (%$E_{max}$).

Data were generally expressed as mean ± SEM unless specified otherwise, as *n* indicates the number of patients (2-4 arterial rings were obtained per segment). In assays with extract incubation, potency was expressed as the negative logarithm of the effective concentration (in mol/L or M) of noradrenaline able to induce half of the maximum contraction ($pEC_{50}$, -log [M]), both in the presence and in the absence of extract. Efficacy was expressed either as %$E_{max}$ (maximal contraction, %) or %$R_{max}$ (maximal relaxation, %), in incubation and vasorelaxation assays, respectively.

Statistical analysis was performed using GraphPad Prism 7® (GraphPad Inc., La Jolla, CA, USA). First, the normality of data was accessed with Shapiro-Wilk test. The analysis of CCRCs was performed generally by two-way ANOVA with Tukey's multiple

comparisons test to identify differences in specific concentrations, including maximal effects.

Reagents

All chemicals used for the *in vitro* pharmacological studies were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA) and for phytochemical characterization were purchased from Merck® (Germany) and correspond to the highest grade commercially available.

### 3.2.4 Results and Discussion

Phenolic profile

The phenolic profile of the infusion and hydroalcoholic of *F. vesca* was carried out by HPLC coupled with PDA detector (Fig. III.5A and III.5B, respectively).

Regarding the infusion (Fig. III.5A), peak 1 is an hydroxicinammic acid derivative due to a wavelength maximum near 313 nm and its spectral profile. Peak 2 presented a UV spectra profile with wavelength maxima near 250 nm and 324 nm and a shoulder at 298 nm which is characteristic of caffeic or ferulic acid derivatives (Liberal et al. 2014). Peak 3 and 4 exhibited the same UV spectra profile with wavelength maxima of 310 nm, suggesting the presence of *p*-coumaric acid derivatives (Liberal et al. 2014). Quercetin derivatives could be identified in peaks 5, 7 and 8, since their UV spectra profile and wavelength maxima were near 250 nm and 350 nm and shoulders approximately of 270 nm and 290 nm, which are characteristic of this type of compounds. Using HPLC-ESI/$MS^n$ data, Liberal et al. (2014) suggested that these compounds could be identified as quercetin hexosyl-glucuronide, quercetin glucuronyl-rhamnoside and quercetin glucuronide, respectively.

Peak 6 was identified a kaempferol derivative due to its UV profile and wavelength maxima at 264 nm, 346 nm and a shoulder of 296 nm. Liberal et al. (2014) identified it as a kaempferol glucuronide based on the results of HPLC-ESI/$MS^n$ analysis. Peak 9 exhibited wavelength maxima of 248 nm, 371 nm and a shoulder 322 nm and these characteristics suggest the presence of an ellagic acid derivative.

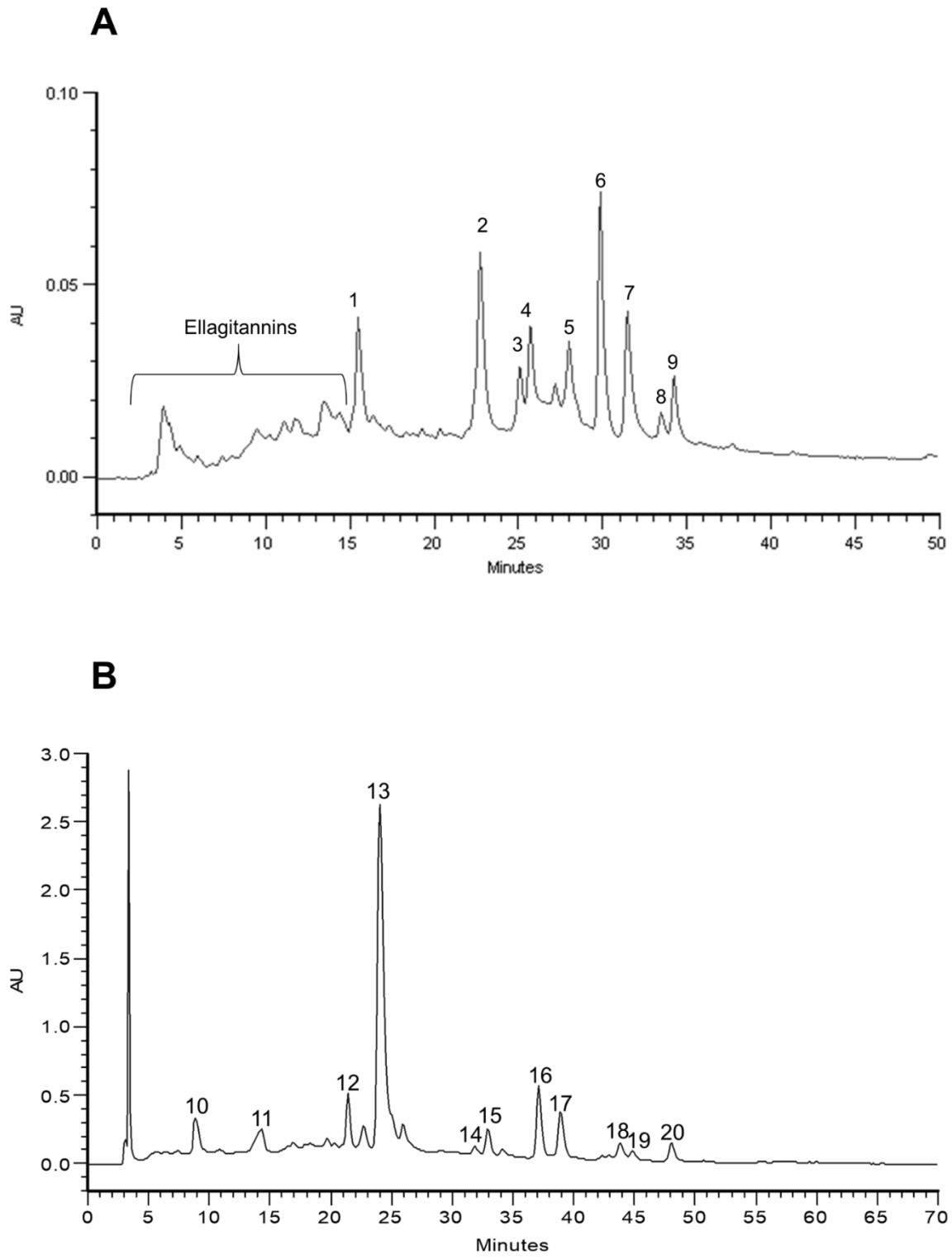



**Figure III.5: HPLC-PDA chromatogram of *F. vesca* leaves extracts:** (**A**) infusion and (**B**) hydroalcoholic extract. Both chromatograms were recorded at 280 nm.

Concerning the chromatogram of the hydroalcoholic extract (Fig. III.5B), the UV spectra of peaks 10 and 11 were identified as proanthocyanidins due to its UV maxima (near 246 and 278 nm) and spectral profile, characteristic of flavan-3-ols, probably

proanthocyanidins. In fact, this assumption was verified by Liberal et al. (2014), that confirmed the presence of proanthocyanidins by HPLC-PDA-ESI/$MS^n$.

The UV spectra profile of peak 12, with wavelength maxima near 250 nm and 324 nm and a shoulder at 298 nm, was chacteristic of a caffeic acid derivative (Campos and Markhan 2007). This assumption was corroborated by Couto et al. (2020) with HPLC-ESI/$MS^n$ which identified 4-*O*-caffeoylquinic acid, at the same retention time, using the same chromatographic conditions. Peak 13 presented wavelength near 240 and 250 and was identified as an ellagitannin (agrimoniin or sanguiin H-6) by Couto et al. (2020) using data obtained from HPLC-ESI/$MS^n$ analysis.

Peaks 14 and 17 presented UV maxima of absorption near 260 nm and 350 nm and spectra profiles suggesting the presence of quercetin derivatives. Our findings confirm the result obtained by Couto et al. (2020), using HPLC-ESI/$MS^n$, who concluded that peak 14 was a quercetin glucoronyl-rhamnoside and peak 17 was a quercetin glucuronide (these peaks correspond to the peaks 7 and 8 from the infusion, respectively).

Peaks 15, 18 and 20 were identified based on their UV spectra, which presented wavelength maxima 254 nm and 360-380 nm that suggests an ellagic acid derivative. This assumption was corroborated by Couto et al. (2020), that reported the presence of ellagic acid and used a commercial compound to confirm their result.

Finally, peaks 16 and 19 showed the same spectra profiles with a maximum at 346 nm, characteristic of kaempferol derivatives. These results were corroborated after HPLC-ESI/$MS^n$ analysis performed by Couto et al. (2020), which suggested the structures of kaempferol glucuronyl-rhamnoside and kaempferol glucuronide, at the same retention time of peaks 16 and 19 respectively. Moreover, kaempferol glucuronide was also identified in the infusion (peak 6).

In summary, the two extracts used in this study are rich in phenolic acids, quercetin and kaempferol derivatives. In contrast, the infusion assayed by Mudnic et al. (2009) contained catechin, epicatechin, epigallocatechin, procyanidin B1 and B2, epicatechin-3-gallate, piceid, astringin and trans-resveratrol, in addition to the presence of quercetin derivatives (quercetin-4'-glucoside).

Vascular effects

The infusion of *F. vesca* did not induce a significant effect on basal vessel tone ($E_{max}$=0.19±0.11mN).

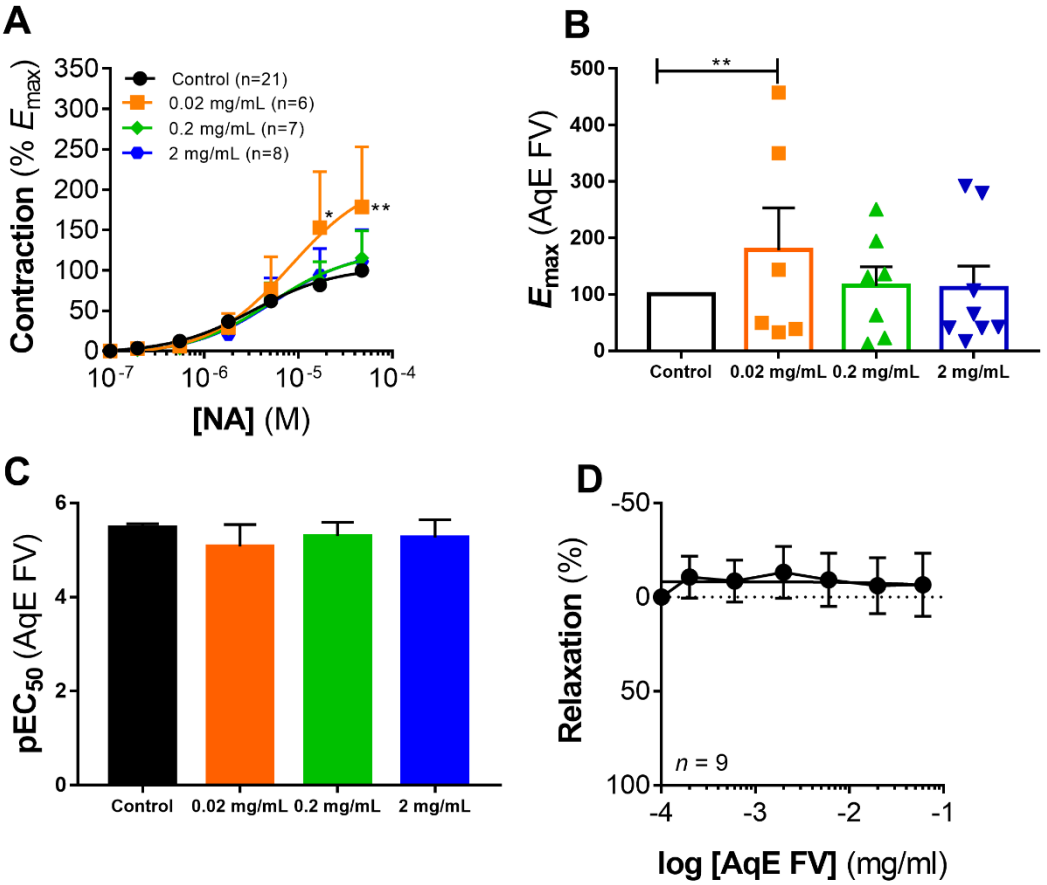



**Figure III.6**: **Vascular activity of *F. vesca* leaves infusion**. **A**, Effect on the noradrenaline-induced contraction. *$p$<0.05 vs control. **$p$<0.01 vs control. **B-C**, Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$, **C**). **$p$<0.01 vs. control. **D**, vasorelaxation elicited by *F. vesca* infusion.

As seen in Fig III.6A, a significant potentiation of the maximal effect of the noradrenaline-induced contraction (about 78.64%, $p$<0.01 vs control) was observed for the lowest concentration of extract, i.e. 0.02 mg/mL. In higher concentrations, no significant effect on noradrenaline-induced contraction was detected. After precontraction with 20 μM noradrenaline, the infusion did not elicit vasorelaxation ($R_{max}$=-6.62±16.52%, Fig III.6D). Overall, the infusion displayed a mixed effect on noradrenaline-induced contraction, by eliciting a potentiation of the maximal effect only in the lower dose, with no changes in intermediate and higher doses. This may result from the fact that plant extracts are mixtures of compounds, some of which may evidence their properties more clearly in lower extract concentrations. Furthermore, no effects on basal vessel tone and vasorelaxation were observed, thus suggesting an absence of clinically-relevant effect in our arterial model.

Similarly, the hydroalcoholic extract of *F. vesca* did not elicit significant changes in basal vessel tone ($E_{max}$=0.62±0.48mN).

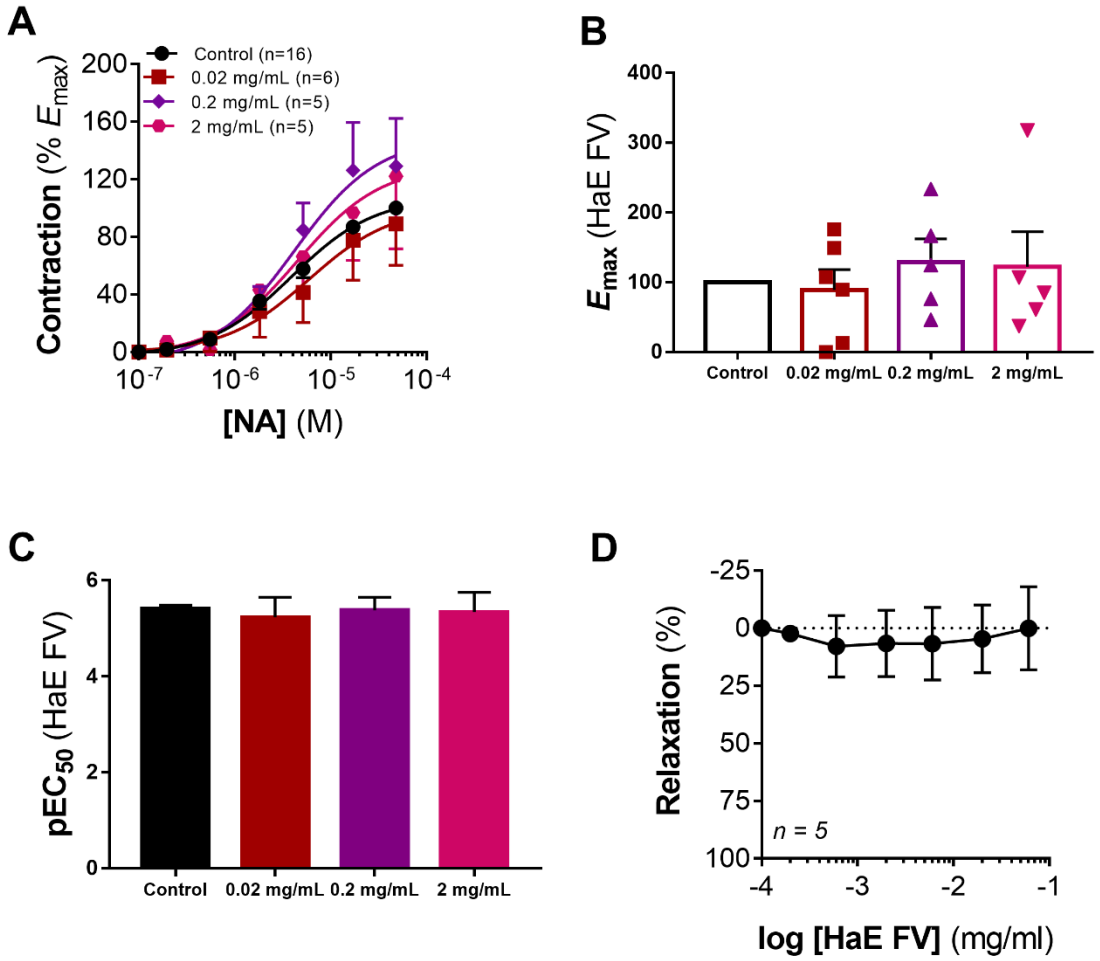



**Figure III.7: Vascular activity of *F. vesca* leaves hydroalcoholic extract**. **A**, Effect on the noradrenaline-induced contraction. **B-C**, Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$, **C**). ***p*<0.01 vs. control. **D**, vasorelaxation elicited by *F. vesca* hydroalcoholic extract.

**Table III.2**. Influence of the infusion and hydroalcoholic extract of *F. vesca* on the parameters of efficacy (%$E_{max}$) and potency ($pEC_{50}$) of the noradrenaline-induced contractile response.

| Extract | Concentration | %$E_{max}$ | $pEC_{50}$ (-log[M]) | *n* |
|---|---|---|---|---|
| *Infusion* | Control | 100.00±0.00 | 5.47±0.08 | 21 |
| | 0.02 mg/mL | 178.64±74.42** | 5.07±0.46 | 6 |
| | 0.2 mg/mL | 115.24±33.50 | 5.29±0.29 | 7 |
| | 2 mg/mL | 111.00±39.39 | 5.26±0.38 | 8 |
| *Hydroalcoholic* | Control | 100.00±0.00 | 5.42±0.07 | 16 |
| | 0.02 mg/mL | 89.12±28.93 | 5.23±0.41 | 6 |
| | 0.2 mg/mL | 129.09±33.18 | 5.37±0.27 | 5 |
| | 2 mg/mL | 122.08±50.39 | 5.34±0.41 | 5 |

Values presented in mean ± SEM and *n* correspond to the number of experiments (n) performed in each experimental setting. ***p*<0.01 vs. control

As can be seen in Fig III.7A, no significant differences were observed between the control curve to noradrenaline and in the presence of different concentrations of the hydroalcoholic extract of *F. vesca*. Furthermore, no significant changes were observed in the potency of the response (Table II.2). Concerning the vasorelaxant properties, the hydroalcoholic extract of *F. vesca* did not show a vasorelaxant effect ($R_{max}$=0.01±17.87%, Fig III.7D).

Our findings differ from the previous study by Mudnic et al. (2009) who showed an endothelium-dependent vasorelaxation mediated by nitric oxide and cyclooxygenase products in the rat aorta with a leaves infusion. A methodological difference should be highlighted compared to our study, i.e. a markedly lower amount of plant material was used in the preparation of our extract (4g/150 mL) compared with the study by Mudnic et al. (2009), who used 15g/150 mL. This difference can directly affect the concentration of bioactive compounds. Moreover, Mudnic et al. (2009) assayed two higher concentrations in their study (6 and 60 mg/mL), also these two concentrations were responsible for the statistically significant effect observed by Mudnic et al. (2009).

In regard to the *F. vesca* extracts assayed in this study, a phytochemical analysis of the hydroalcoholic and the infusion evidenced the presence of phenolic acids, flavonols and ellagic acid and its derivatives (Couto et al., 2020; Liberal et al., 2014). Interestingly, Mudnic et al. (2009) reports the presence of other constituents that could be responsible for the vasodilation that was observed by this author, such as epicatechin and procyanidin B2. In fact, previous studies by A. Novakovic with the ITA have shown the involvement of the vascular smooth muscle in the vasorelaxation elicited by both (-)-epicatechin (Novakovic et al. 2015) and the involvement of the vascular endothelium in the vasorelaxation to procyanidin B2 (Novakovic et al. 2017).

Overall, the differences between our findings and the results from previous studies could also be attributed to the vascular bed that was used. In fact, patients who undergo coronary revascularization commonly present several risk factors that could interfere with several pathways of regulation of vascular function, e.g. endothelial function, particularly the nitric oxide pathway. Therefore, a higher variability in results inherent to the use of human vascular tissue could mask the vascular effects that may be obtained with these extracts (Fonseca et al. 2014; Simões et al. 2020). However, this may also be an advantage from a translational point of view, as it gives a better perspective of the human vascular system, compared to animal-derived tissue. Furthermore, the ITA is a vascular bed with special characteristics as previously

reviewed, namely atherosclerosis resistance (Fonseca et al. 2014; Fonseca et al. 2017). In terms of histomorphology, it has been recognized as a transitional-type artery due to its varying content in elastic and muscular elements, with a muscular pattern being more predominant in the distal end (Borović et al. 2010). Further, its distal portion exhibits a higher pharmacological reactivity (He 2015), which was used in our study, thus making it an interesting model for the investigation of the effects of natural extracts and compounds on blood pressure regulation.

In a clinical scenario, the administration of the *F. vesca* leaves infusion to patients with vascular disorders, who typically exhibit reduced endothelial nitric oxide production, could be debatable, as the main mechanism of action would be compromised, thus limiting the potential benefit of its consumption. However, the available evidence on this topic is scarse. Therefore, we would suggest pursuing further experimental studies with animal models of cardiovascular disease, specifically of atherosclerosis (e.g. ApoE-deficient mice) and hypertension (e.g. spontaneously hypertensive rats). Such studies would allow a clarification of the true cardiovascular benefit of extracts from leaves of *F. vesca*.

## 3.3 *Urtica dioica* L.

### 3.3.1 Overview

*U. dioica* (Fig III.8), also known as nettle, belongs to the *Urticaceae* family. In previous ethnomedicinal reports, the aerial parts of this plant have been recognized as a natural product for hypertension (Ziyyat et al. 1997; Legssyer et al. 2002; Testai et al. 2002; Roschek et al. 2009). This species is one of the most studied medicinal plants worldwide (Esposito et al. 2019).

*U. dioica* is a perennial ubiquitous plant with a wide global distribution and with large agricultural significance (Carvalho et al. 2017) that can be found in different areas of Pakistan like Hazara Division, Gilgit Baltistan, Naran, Kaghan and Balochistan. Moreover, it is widely distributed throughout the USA, Europe and Himalaya (Upton 2013; Qayyum et al. 2016).

*U. dioica* is a temperate herbaceous species that is a common and aggressive weed found in moist soils throughout the USA and Europe, and grows in soils rich in nitrogen between 0 and 1800 m in desert field (Testai et al. 2002; Upton 2013).

This species also has an industrial significance since it is a low-requirement plant and all its parts (the herb, root, and seeds) can be industrially exploited (Vajic et al. 2018).

Regarding the botanical features, *U. dioica* is an annual growing to 0.6 m tall shrub which bears opposite, cordate, deeply serrate, pointed leaves which are downy underneath. Flowering and fruiting time is from May/June to September/October. The stem and leaves of the plant are covered with stinging trichomes (Testai et al. 2002; Upton 2013; Qayyum et al. 2016).

The flowers of this plant are dioecious, thus generally contains either female or male flowers, in separated inflorescences, that occurs as racemes in the axils of the upper leaves (Testai et al. 2002; Roschek et al. 2009). The leaves have characteristics of being oval, long petiolate, elongated with toothed margins (Fig III.8). The fruit is a small oval and greenish-yellow achene. The plant has stinging hairs with a tuft of hair at the apex. The leaves and stems contain abundant non-stinging hairs, with touch sensitive tips. Also, the glandular hairs on the leaves that contain formic acid and histamine, agents known to cause skin irritation after contact (Roschek et al. 2009).

**FigureIII.8:** ***Urtica dioica* L.** Available from URL: https://www.flickr.com/photos/brewbooks/2544110170

*U. dioica* has been used for centuries in traditional medicine, primarily in the treatment of arthritis, rheumatism, muscular paralysis but also gastrointestinal, hepatic disorders and diabetes (Ziyyat et al. 1997; Tahri et al. 2000; Legssyer et al. 2002; Qayyum et al. 2016; Vajic et al. 2018).

Properties such as anti-inflammatory, anti-asthmatic, astringent, nutritive and stimulating have been attributed to *U. dioica* (Esposito et al. 2019). Also, according to Esposito et al. (2019), the leaves extract has been used as anti-hemorrhagic to reduce excessive menstrual flow and nose bleedings.

Also, *U. dioica* has been traditionally used in the management of cardiovascular disorders especially hypertension therapy in northeastern Morocco (Ziyyat et al. 1997; Tahri et al. 2000; Qayyum et al. 2016).

According to Esposito et al. (2019), the roots and herbs from *U.dioica* are used in distinct ways: the roots for benign prostate hyperplasia, the herbs for urinary tract disorders and rheumatic conditions, while fresh freeze-dried leaves are used to treat allergies. Moreover, studies have also reported its analgesic potential and its role as anti-aggregating factor, as well as describing its favorable effects on cardiovascular and smooth-muscle activity as a hypotensive agent (Esposito et al. 2019).

Regarding the phytochemical composition, according to a review published by Esposito et al. (2019), *U. dioica* has been described as a species enriched in phenolic compounds (including flavonoids, coumarins, tannins and lignans). Moreover, the presence of sterols, fatty acids, polysaccharides and isolectins was also reported (Esposito et al. 2019). This report was corroborated by Orcic et al. (2014) that also described the presence of flavonoids, phenolic acids, coumarins and lignans.

Polyphenols such as phenolic acid and flavonoids were also reported by Carvalho et al. (2017), Dhouibi et al. (2018) and Devkota et al. (2022). Moreover, other classes of compounds were also described such as triterpenes (Dhouibi et al. 2018), amino acids, carotenoids, organic acids and fatty acids (Devkota et al. 2022).

### 3.3.2 Cardiovascular properties

In the literature, the bioactivity described for different types of extracts from several parts of *U. dioica* includes anti-hypertensive, antidiabetic activity, among others (Vajic et al. 2018; Samaha et al. 2019). In particular, Vajic et al. (2018) showed that

chronic dietary supplementation with *U. dioica* leaves extract promoted beneficial effects in spontaneously hypertensive rats, namely: reduction of systolic and diastolic blood pressure, without changes in total peripheral vascular resistance, as well as decreased peroxidation and increased antioxidant capacity in the plasma, which could be beneficial from a vascular point of view when considering the ROS-related endothelial dysfunction.

In fact, the anti-hypertensive properties of *U. dioica* were first explored by Tahri et al. (2000). In an *in vivo* study with Wistar rats, the authors showed that the aqueous extract of the aerial parts of *U. dioica* elicits acute diuretic, natriuretic and hypotensive effects. This acute hypotensive effect was attributed to a direct effect on the cardiovascular system, even though a chronic hypotensive effect could be more related to the activation of natriuresis-diuresis mechanism, as discussed by the authors.

Since then, studies have emerged focusing on the potential direct effects on the vasculature. Testai et al. (2002) showed vasorelaxation of rat aortic rings to aqueous and methanolic extracts of the roots. Qayyum et al. (2016) showed vasorelaxation of rat and rabbit aortas to the methanolic extract of rhizomes. In both studies, the vasorelaxant effect was shown to be partly mediated by endothelial release of nitric oxide. Interestingly, Legssyer et al. (2002) showed α1-adrenergic receptor-mediated vasoconstriction of rat aortic rings to an aqueous extract of the aerial parts, thus suggesting differential vascular bioactivity between different parts of the plant.

Here, we aimed to assess the direct vascular effects of an hydroalcoholic extract from aerial parts of *U. dioica* (Carvalho et al. 2017) in ITAs in order to validate previous findings on its vascular bioactivity.

### 3.3.3 Material and Methods

Plant material

The aerial parts were provided by "Confraria da Urtiga, Portugal". The plant material was collected in Serra da Estrela during the flowering season.

The hydroalcoholic extract of *U. dioica* was prepared from 10 g of powdered aerial parts which were macerated in 50% aqueous ethanol (v/v) (200 mL) for 24 h, under magnetic stirring. Ethanol was removed using a rotatory evaporator, and the extract was lyophilized and stored at -20°C, as described in (Carvalho et al. 2017). A voucher specimen (M. Paraíso 02012) has been deposited at the Herbarium of Medicinal and Aromatic Plants, Faculty of Pharmacy, University of Coimbra.

HPLC-PDA

The phytochemical characterization of the extract was performed using HPLC coupled with photodiode array (PDA) detector (Gilson Electronics SA, Villiers le Bel, France). Data were treated with Unipoint® 2.10 (Gilson, Middleton, WI, USA). The samples (100 µL) were injected in a Waters® Spherisorb S5 ODS-2 column (250 × 4.6 mm i.d., 5 µm), protected with a guard cartridge C18 (30 × 4 mm i.d., 5 µm) (Nucleosil, Macherey-Nagel, Düren, Germany) and eluted at a flow rate of 1mL/min and 35°C. The mobile phase consisted of 5% formic acid (v/v) (eluent A) and methanol (eluent B).

The gradients utilized were: 0-10 min (0%-5% B), 10-45 min (5%-80% B), 45-50 min (80%-100% B). The concentration of injected sample was 3 mg/mL and the chromatographic profiles was recorded at 280 nm.

Vascular activity studies

Vascular tissue preparation was carried out as described previously in section 3.1.2. The effect on basal vascular tone was assessed by CCRCs to the crude extract (0.002-0.2 mg/mL) and the results were expressed as absolute contraction (in miliNewton or mN). The vasorelaxant effect was tested with CCRCs after sustained pre-contraction with noradrenaline (20 µM), as results were expressed as percentage of the maximum contraction to noradrenaline (%). The modulatory effect on the noradrenaline-induced contraction was assessed with CCRCs to noradrenaline (0.1 - 48 µM) before and after 30-min pre-incubations with the extract (0.02, 0.2 and 2 mg/mL), as the results were expressed as the percentage of the maximal contraction in the control curve, i.e. absence of extract (%$E_{max}$).

Data were generally expressed as mean ± SEM unless specified otherwise, as n indicates the number of experiments. In assays with extract incubation, potency was expressed as the negative logarithm of the effective concentration (in mol/L or M) of noradrenaline able to induce half of the maximum contraction (pEC50, -log [M]), both in the presence and in the absence of extract. Efficacy was expressed either as %$E_{max}$ (maximal contraction, %) or %$R_{max}$ (maximal relaxation, %), in incubation and vasorelaxation assays, respectively.

Statistical analysis was performed using GraphPad Prism 7® (GraphPad Inc., La Jolla, CA, USA). First, the normality of data was accessed with Shapiro-Wilk test. The analysis of CCRCs was performed generally by two-way ANOVA with Tukey's multiple comparisons test to identify differences in specific concentrations, including maximal effect ($E_{max}$). Pharmacological parameter of potency ($pEC_{50}$) was analyzed by unpaired

one-way ANOVA with Tukey's multiple comparisons test. $P< 0.05$ was considered to indicate a statistically significant difference.

Reagents

All chemicals used for the in vitro pharmacological studies were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA) and for phytochemical characterization were purchased from Merck® (Germany) and correspond to the highest grade commercially available.

### 3.3.4 Results and discussion

Phenolic profile

The phytochemical profile of *U. dioica* is presented in Fig III.9. Peaks 1, 2, 3, 4, 5,7, 8 exhibited the same UV spectra profile with wavelength maxima near 250 and 324 nm and a shoulder at 298 nm with is characteristic of caffeic or ferulic acid derivatives. These compounds were tentatively identified in Carvalho et al. (2017), through HPLC-ESI/$MS^n$ as: 3-*O*-caffeoylquinic acid, caffeoyltartaric acid, hydroxyferulic acid deoxyhexoside, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, feruloylmalic acid and cis-5-*O*-caffeoylquinic acid, respectively.

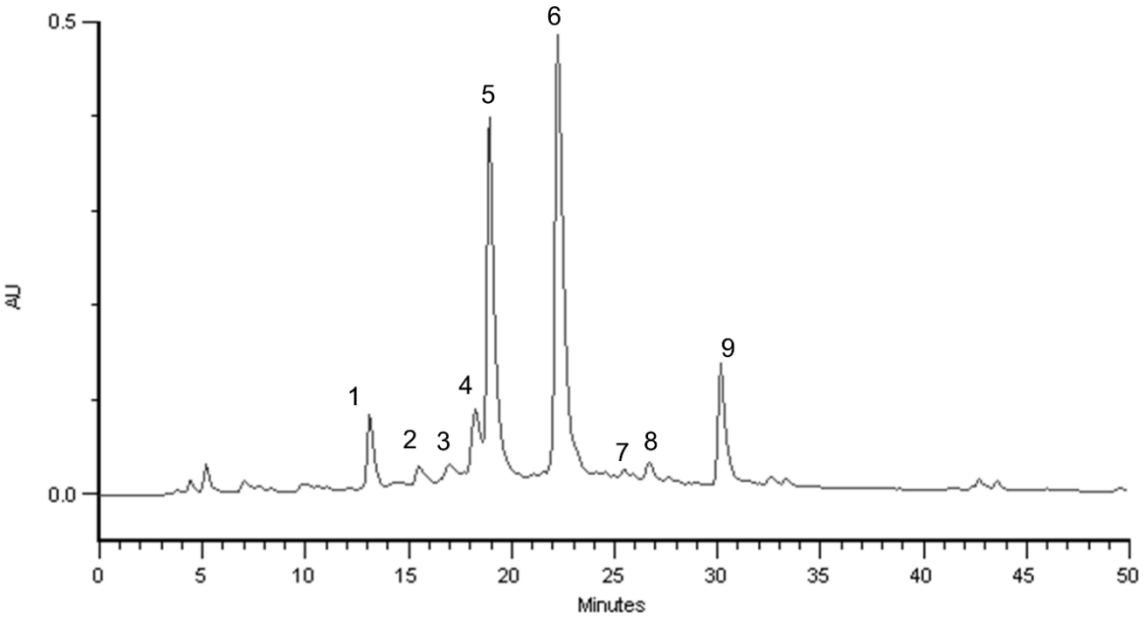



**Figure III.9**: **HPLC-PDA chromatogram of *U. dioica* aerial parts hydroalcoholic extract**. Chromatogram recorded at 280 nm.

The UV spectrum profile and wavelength maximum of 310 nm of Peak 6 suggests a *p*-coumaric acid derivative. This assumption has been confirmed Carvalho et al. (2017) through HPLC-ESI/MS$^n$ that identified *p*-coumaroylquinic acid. Finally, peak 9 was identified as a quercetin derivative due to wavelength maxima at 256 nm, 354 nm (Mabry 1970). Carvalho et al. (2017) also reported a quercetin derivative (quercetin-*O*-rutinoside) through HPLC-ESI/MS$^n$ identification, even though the structure was not fully elucidated.

Vascular effects

Regarding the vascular effects, the hydroalcoholic extract of *U. dioica* induced a mild increase in basal vascular tone ($E_{max}$ = 1.30±0.77 mN). No vasorelaxant effect was observed ($R_{max}$ = -20.69±19.30%, Fig III.10D).

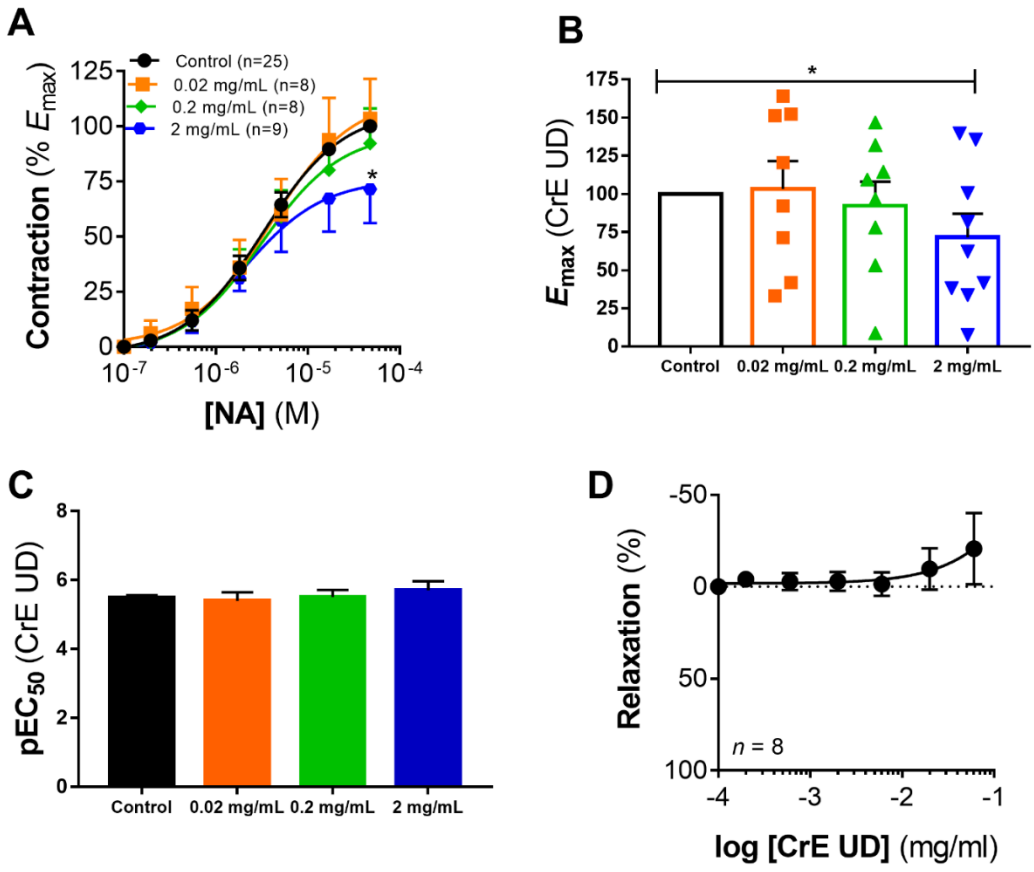



**Figure III.10. Vascular activity of *U. dioica* hydroalcoholic extract of aerial parts**: **A**, Cumulative concentration-response curves to noradrenaline (NA) in the absence or presence of several concentrations of *U. dioica* extract; **B-C**, Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$, **C**). * $p$<0.05 vs. control.**D**, vasorelaxation elicited by *U. dioica*.

**Table III.3** Influence of the hydroalcoholic extract of *U. dioica* on the parameters of efficacy (%$E_{max}$) and potency ($pEC_{50}$) of the noradrenaline-induced contractile response.

| Concentration | %$E_{max}$ | $pEC_{50}$ | *n* |
|---|---|---|---|
| Control | 100.00±0.00 | 5.48±0.07 | 25 |
| 0.02 mg/mL | 103.34±18.19 | 5.39±0.25 | 8 |
| 0.2 mg/mL | 92.19±15.85 | 5.50±0.20 | 8 |
| 2 mg/mL | 71.45±15.50* | 5.69±0.26 | 9 |

Values presented in mean ± SEM and *n* correspond to the number of experiments (*n*) performed in each experimental setting. *$p$<0.05 vs. control.

Conversely, the higher concentration of extract (2 mg/mL) induced a statistically significant decrease in the noradrenaline-induced maximal vascular contraction ($E_{max}$ reduction of 28.55%, $P$<0.05 vs control, Fig III.10A). No significant changes were observed in terms of potency (Fig III.10 B-C, Table III.3). Overall, our results suggest a modulatory effect on the autonomic regulation of vascular tone.

As mentioned above, Testai et al. (2002) and Qayyum et al. (2016) previously showed vasorelaxation of aortic rings to aqueous and methanolic extracts of the plant roots and rhizomes, respectively. However, Legssyer et al. (2002) showed an α1-adrenergic receptor-mediated vasoconstrictor effect for an aqueous extract of the aerial parts. This differential vascular bioactivity is probably a result from the part of the plant that was used in extract preparation. In our study, we observed no vasorelaxant effect and no effects on basal vascular tone with an hydroalcoholic extract of aerial parts, but rather a modulation of adrenergic vasoconstriction. First, this confirms that the vasorelaxant properties of *U. dioica* may be attributable to compounds present in the ground parts rather than in the aerial parts of the plant. Second, our results confirm the existence of compounds with affinity to human vascular adrenergic receptors in the aerial parts of *U. dioica*, as previously suggested by the findings of Legssyer et al. (2002) in rat aortic rings.

Interestingly, Vajic et al. (2018) recently showed that chronic dietary supplementation with *U. dioica* leaves extract promoted a decrease in systolic and diastolic blood pressure in spontaneously hypertensive rats, without changes in total peripheral vascular resistance, suggesting that the anti-hypertensive properties may also be related to cardiac effects. In fact, Testai et al. (2002) initially showed a negative inotropic effect in spontaneously beating guinea-pig atria, in addition to a transient marked hypotensive effect in anaesthetized rats. Furthermore, Legssyer et al. (2002) showed a decrease in heart rate and a positive inotropic effect, with increased left

ventricular pressure, in isolated perfused heart preparations. This activity was not modified by the presence of the muscarinic receptor antagonist atropine or the α1-adrenergic receptor antagonist prazosin. Therefore, the mechanisms behind this cardiac bioactivity remain to be characterized as well as the compounds responsible.

Of note, a randomized clinical trial has been conducted with the administration of *U. dioica* leaves extract (300 mL/day before breakfast) in subjects with mild hypertension for 16 weeks, in which systolic and diastolic blood pressure as well as mean arterial blood pressure were monitored at 4, 8, 12 and 16 weeks (Samaha et al. 2019). At 16 weeks, a duration-dependent significant reduction was observed in all analyzed parameters (Δ systolic blood pressure = -11.89±2.60mmHg, Δ diastolic blood pressure = -7.46±1.16mmHg and Δ mean arterial pressure = -8.94±1.14mmHg), suggesting that the administration of this extract could represent an effective, safe and promising phytotherapeutic approach for the treatment of mild hypertension. However, this study did not attempt the identification of compounds responsible for such activity.

Overall, the compounds responsible for the anti-hypertensive potential of *U. dioica* remain unknown, as well as the underlying molecular mechanisms. From a phytochemical point of view, several compounds have been identified in hydroalcoholic extracts from aerial parts of *U. dioica* as well as other *Urtica* spp. species, namely hydroxycinnamic acid derivatives and flavonoids (Carvalho et al. 2017). The extract assayed by our group displayed a high content of phenolic acids and a low content of quercetin derivatives. As quercetin has been reported in the literature for its vasorelaxant properties (Roghani et al. 2004), phenolic acids do not seem to exhibit such properties, which could explain the lack of vasorelaxant effect in our extract. In exception to Vajic et al. (2018), who reported the presence of chlorogenic acid, 2-*O*-caffeoyl malic acid and rutin in their methanolic leaves extract, none of the other previous reports that assess the vascular or anti-hypertensive activity of *U. dioica* (Tahri et al. 2000; Legssyer et al. 2002; Testai et al. 2002; Qayyum et al. 2016) display the composition of the assayed extract or attempt the identification of compounds that could be responsible for the observed effects.

Some limitations should be recognized in our study. First, the use of human arterial tissue may potentiate variability of results due to differences in baseline characteristics of the patients, which in the majority are hypertensive. Nevertheless, this may also be an advantage, as it provides a closer translation of the findings to the human, particularly when considering patients with cardiovascular disease or hypertension. Second, we did not test the specific role of vascular $\alpha_1$-adrenergic receptors, thus we are not able to confirm the involvement of these receptors in the modulatory effect that

we observed. Third, we did not attempt to identify the compounds responsible for the vascular bioactivity of the hydroalcoholic extract of this plant.

## 3.4 *General discussion*

Cardiovascular diseases are the leading cause of mortality and morbidity worldwide (Novakovic et al. 2017). The use of herbal medicines for the treatment of cardiovascular diseases has become increasingly relevant (Veluswamy et al. 2016). In this context, plants contain a large number of phytochemicals that have been proven beneficial by reducing the risk of various diseases and targeting pathogenic pathways of cardiovascular diseases (Anwar et al. 2016).

In the first phase of this work, we aimed at screening the vasoactive properties (contractile effect, modulatory effect on adrenergic contraction and vasorelaxant properties) of the extracts (*A. eupatoria*, *F. vesca*, *U. dioica*) using ITA as a vascular model, in addition to the chemical composition of the extract.

The first step employed in our protocol was to verify the reactivity of the ITA's rings. For this, we used KCl (60mM), which elicits a vasoconstrictor effect independent of receptor activation. The vascular smooth muscle tonus is highly dependent on the membrane potential, which is mainly determined by the activity of $K^+$ channels. Hence, the addition of KCl to the organ bath promotes the increase of the extracellular $K^+$ and as consequence, a decrease of $K^+$ efflux by the potassium channels from the smooth muscle cells to the cell membrane could be observed. As a result of this phenomenon, the intracellular environment becomes less negative and depolarizes, which leads to the opening of the voltage-sensitive $Ca^{2+}$ channels, present in the smooth muscle cell membrane, leading to the influx of $Ca^{2+}$ and as a result, a contraction occurs. This contraction increases over time until a plateau is reached (maximum contractility). Only the rings that demonstrated this behavior were assayed in the experiences (Fig III.11).

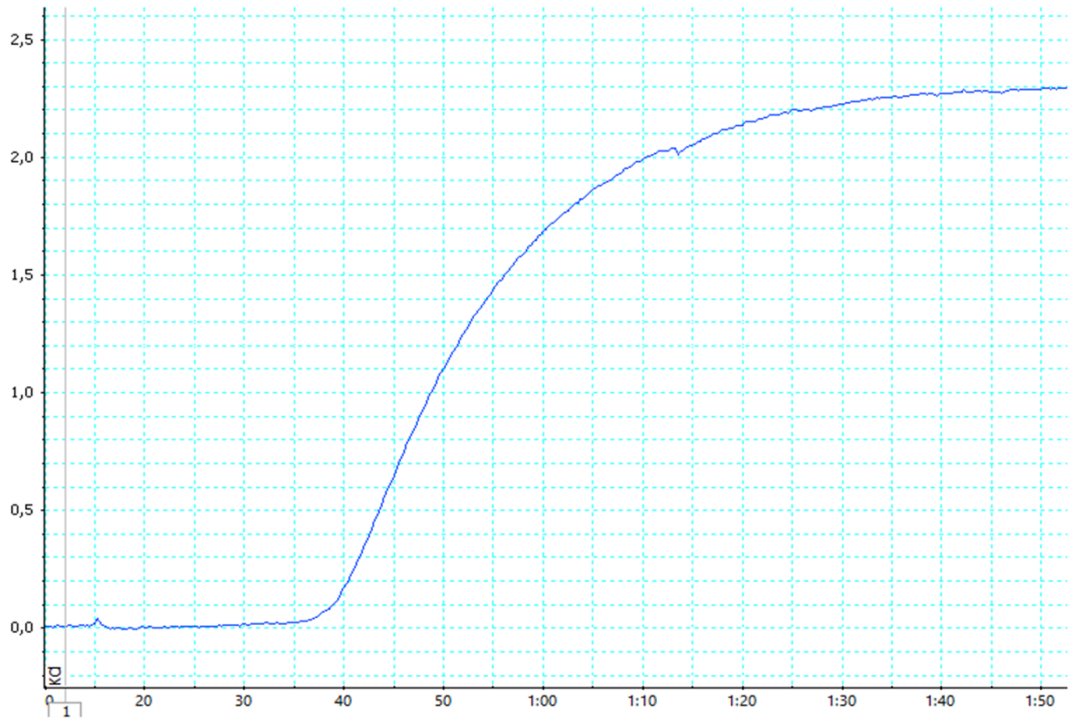



**Figure III.11**: **Type curve of the ITA response to a stimulation of 60 mM KCl**. Vertical scale in grams and horizontal scale in time.

Summarizing, regarding the contractile effects, the infusions of *A. eupatoria* and *U. dioica* induced a mild increase in the basal vascular tone. Both extracts of *F. vesca* did not induce any change on basal vessel tone.

Concerning the modulatory effect on adrenergic contraction, although a potentiation of the noradrenaline maximal contraction was observed for the lower concentration of *A. eupatoria* extract, higher concentrations elicited a significant decrease in noradrenaline contraction, thus confirming the anti-hypertensive potential of this extract. Regarding *F. vesca*, only the lowest concentration (0.02 mg/mL) of the infusion potentiated the noradrenaline-induced contraction. In contrast, the higher concentration of extract of *U. dioica* (2 mg/mL) significantly decreased the noradrenaline-induced maximal contraction. All these results are described in detail in each plant section.

Finally, regarding the vasorelaxant effects of the three plant species evaluated in the screening, a statistically significant vasorelaxant effect was only observed with *A. eupatoria* infusion, thus showing an interesting source of vasodilator compounds. In Fig III.12 and Table III.4, a *pooled analysis* of results is displayed.

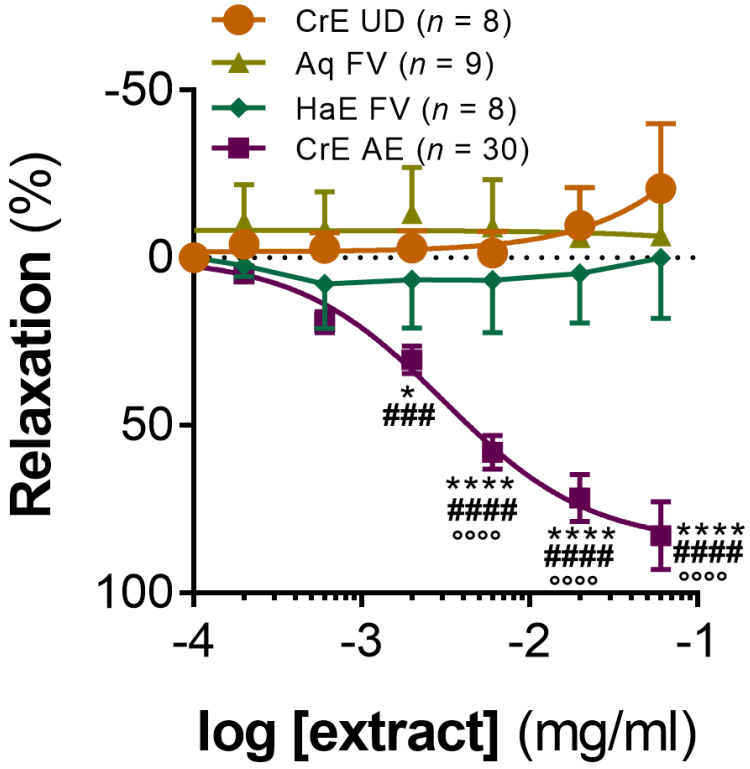



**Figure III.12: Comparison of the maximal relaxation (%$R_{max}$) elicited by the extracts assayed in this work**. *$p$<0.05 vs. CrE UD, ***$p$<0.001 vs. CrE UD, ****$p$<0.0001 vs. CrE UD. ### $p$<0.001 vs Aq FV, ####$p$<0.0001 vs Aq FV, °°°$p$<0.001 vs. HaE FV, °°°° $p$< 0.0001 vs. HaE FV. Significance refers to unpaired two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Abbreviations: **CrE AE**, crude extract (infusion) of *A. eupatoria*; **Aq FV**, infusion of *F. vesca*; **HaE FV**, hydroalcoholic extract of *F. vesca* and **CrE UD,** crude extract (hydroalcoholic) of *U. dioica*.

**Table III.4**: Pharmacological parameters from vasorelaxation studies with *A. eupatoria*, both extracts of *F. vesca* and *U. dioica*.

| Extract | %$R_{max}$ | pEC$_{50}$ | *n* |
|---|---|---|---|
| *A. eupatoria*(CrE AE) | 82.93±9.96 | 2.51±0.13 | 30 |
| *F. vesca*(HaE FV) | 0.01±17.87°°°° | - | 5 |
| *F. vesca* (Aq FV) | -6.62±16.52#### | -0.66±66.17 | 9 |
| *U. dioica* (CrE UD) | -20.69±19.30**** | 0.42±7.05 | 8 |

Results presented as mean ± SEM and *n* corresponds to the number of experiments. ****$p$< 0.0001 vs. CrE AE, #### $p$< 0.0001 vs CrE AE, °°°° $p$<0.0001 vs CrE AE.

Taking into consideration the marked vasoactivity presented by *A. eupatoria* infusion, we proceeded our research into testing the involvement of NO and COX pathways aiming to disclose the possible mechanism underlying the observed activity. These results will be presented and further discussed in chapter 5.

Thereafter, utilizing resources of phytochemistry, we proceeded to the fractioning of this extract. In this next phase, we aimed to determine the compounds present in the fractions and consequently their mechanism of action. These results altogether will also be presented in chapter 5.

Proceeding with the aims of this work, the phytochemical screening and characterization of the extracts was carried out to fully understand the composition of the extracts and the groups of relevant primary and secondary metabolites in them so we could hopefully attribute the activity observed with a specific compound.

In terms of composition, our samples of *A. eupatoria*, *F. vesca* and *U. dioica* corroborate with the literature. For example, for *A. eupatoria*, several studies have been conducted on the phytochemical composition (Granica et al. 2015; Santos et al. 2017) and described a high content of polyphenols such as flavonoids, phenolic acids and procyanidins derivatives. The class of compounds that our group identified was consonant with the previous studies that were published (Correia et al. 2006; Santos et al. 2017).

Regarding the other two plant species presented in this work, *F. vesca* extracts presented a combination of phenolic acids, proanthocyanidins and flavonoids in accordance with (Liberal et al. 2014; Couto et al. 2020).

And finally, *U. dioica* extract presented a high content of phenolic acids and low content of quercetin derivatives, consistent with (Carvalho et al. 2017).

Since the vasorelaxant effect of *F. vesca* and *U. dioica* are described in literature and our results did not demonstrate such activity, this phenomenon could be explained due to many reasons: concentrations utilized in previous assays, ratio of plant and solvent and the use of human arterial tissue that may potentiate variability of results due to differences in baseline characteristics of the patients.

# Chapter 4: Literature review on *Agrimonia eupatoria* L.

Content adapted from:

Malheiros, J. et al. *Agrimonia eupatoria* L.: An integrative perspective on ethnomedicinal use, phenolic composition and pharmacological activity. Journal of Ethnopharmacology. 296 (2022) 115498. doi:10.1016/j.jep.2022.115498

# 4.1 Introduction

Natural products have been used as a source for discovery and development of drugs in several areas, particularly infectious diseases and oncology (Baker et al. 2007). To date, this research continues to provide great value for the discovery of novel chemical structures and bioactive lead molecules for clinical development (Newman and Cragg 2016). Polyphenols are described in the literature as compounds with great potential due to their innumerous bioactivities. In fact, this class of compounds has been extensively researched as a source for the discovery and development of new drugs (Newman and Cragg 2016).

*Agrimonia eupatoria* L. (*A. eupatoria*) is reported for its richness in polyphenols (Granica et al. 2013; Granica et al. 2015; Santos et al. 2017) and, therefore, could be an interesting source of bioactive compounds. As described in its European Union herbal monograph (European Medicines Agency, 2015), this plant is used in traditional medicine for its anti-inflammatory, astringent and diuretic properties, as well as in treating diarrhea. However, there is a clear lack of evidence that can corroborate its therapeutic potential.

In this review, our goal is to provide an overview of the scientific literature on the phenolic composition and ethnopharmacology of *A. eupatoria*. First, we will detail the ethnobotanics of this species, namely its habitat, distribution and botanical features, as well as its ethnomedicinal uses. Secondly, we will explore the phenolic composition of *A. eupatoria* from crude extracts to isolated compounds. Afterwards, we will focus on the bioactivity and pharmacological activity of extracts and compounds from this plant species from experimental and clinical studies, taking into consideration the part of the plant, type of extract and methods of extraction that were used. Also, we will discuss the mechanistic basis for the utilization of this species in traditional medicine. To the best of our knowledge, there are two review articles on *A. eupatoria* (Al-Snafi 2015; Paluch et al. 2020). As these articles provide an overview of the ethnobotanical and bioactivity aspects of this plant, our aim is to provide an integrated and focused perspective on the phenolic composition of this plant species and its correlation with its therapeutic potential.

### 4.1.1 Taxonomy, distribution and botanical description

*A. eupatoria* (Fig IV.1) is an herbaceous plant, also known as agrimony, classified in division Magnoliophyta, class Magnoliopsida and belongs to the Rosaceae family and genus *Agrimonia*, which includes about 20 species according to *The Plant List* (http://www.theplantlist.org/) (Granica et al. 2013; Granica et al. 2015; Lee and Rhee 2016). In terms of geographical distribution, this plant is native in Northern and Central Europe, temperate Asia and North America (Muruzović et al. 2016). Moreover, it is widespread most likely in temperate regions of the northern hemisphere as well as in Africa (Granica et al. 2013; Granica et al. 2015; Muruzović et al. 2016).

**Figure IV.1**. ***A. eupatoria***. Licensed through CC-BY-SA-3.0. Available from URL: https://commons.wikimedia.org/wiki/File:Agrimonia_eupatoria_002.JPG.

The habitat of *A. eupatoria* is meadows, pastures and lowlands as well as mountains up to 1500 m in altitude and it is often found in all types of soil, especially in clay. Growth can be observed in moist and/or moderate dry soils. This species can be spotted along slopes, roadsides, and rocky areas. They are also found in dry grasslands and arid forests (Ivanova et al. 2011b; Muruzović et al. 2016).

As described by Ivanova et al. (2011b), Muruzović et al. (2016) and Santos et al. (2017), *A. eupatoria* has some classic features: it is an erect perennial herbaceous plant, ranging in height between about 50 cm and 150 cm, with a few branches, and with a cylindrical hairy stem; the pinnateleaves are leathery, plumose, serrated and covered with soft hairs; flowers are hermaphrodite with five yellow petals, arranged on

slender,terminal spikes; the flowering stage occurs from June to September; and the fruit grows downwards, surrounded by several rows of soft, hook-shaped bristles.

### 4.1.2 Ethnomedicinal uses

In traditional medicine, *A. eupatoria* has been extensively used as infusion, decoction or tincture from aerial parts (i.e. leaves and flowers), mainly for its antioxidant, anti-inflammatory, astringent, hypotensive and diuretic properties and in gastrointestinal tract diseases, among other applications (Ivanova et al. 2011b; Granica et al. 2013; Granica et al. 2015; Muruzović et al. 2016; Santos et al. 2017; Garcia-Oliveira et al. 2020).

According to Granica et al. (2013), traditional use of the infusion of *A. eupatoria* mainly includes the administration for the treatment of acute diarrhea and inflammation of the oral and pharyngeal mucosa. The infusion can also be used in different herbal combinations, for its anti-inflammatory properties and high content of antioxidants (Ivanova et al. 2011b). Moreover, it is externally applied on skin damage as a mild astringent and anti-inflammatory agent (Granica et al. 2013).

Consistent with this report, Muruzović et al. (2016) and Ivanova et al. (2011b) have also reported its ethnomedicinal use in gastrointestinal tract diseases. In fact, *A. eupatoria* is an important ingredient of various herbal mixtures that are used as dietary supplements for treating increased bile production, stones in the bile duct, and gallbladder and liver pain, and a positive effect on the relief of urinary tract disorders has also been reported (Muruzović et al. 2016). In addition, *A. eupatoria* has also been used for its diuretic and antidiabetic properties in pulmonary and coronary diseases (Kubínová et al. 2012; Lee and Rhee 2016; Muruzović et al. 2016). According to the EMA assessment report (European Medicines Agency, 2015), the only contraindication reported is hypersensitivity, to any components present in the extract. No interactions with other medicinal products were established. Table IV.1 details the use in traditional medicine in European countries.

**Table IV.1**. Traditional uses of *A. eupatoria* reported in the literature.

| Country | Utilization | Indication(s) | Posology | Reference(s) |
|---|---|---|---|---|
| Bulgaria | Internally as infusion<br>Externally as compress or gargle | Rheumatism, hemorrhoids, bleeding gums, varicose ulcers, laryngitis, pulmonary, cutaneous tuberculosis, astringent, antidiarreic, anti-inflammatory agent in urinary diseases and liver | n/s | Cho et al. (2018), Ivanova et al. (2013, 2005) |
| Czech Republic | Herbal preparation as an infusion, compress, gargle or bath additive | **Oral**: as a mild astringent in mild unspecific diarrhea and a as a choleretic in minor gastrointestinal disturbances<br>**Oromucosal**: for treatment of minor inflammations in oral cavity and pharynx<br>**Cutaneous:** minor superficial skin inflammations | **Oral**: 1 tea spoon (1.5 g)/250 ml of boiling water/15 minutes, 2–3 times daily<br>**Oromucosal** or **cutaneous**: 2-3 tea spoons (3–4.5g), 250 mL of boiling water/10 minutes. | European Medicines Agency (2015) |
| Germany | Herbal preparation as an infusion<br>Compress or bath additive | Mild unspecific inflammations on the skin, acute diarrhea, inflammations of the oropharyngeal mucosa | **Oral**: 2 to 4 times daily one cup of tea-infusion<br>**Oromucosal:** gargle with the infusion 1.5g-150 mL boiling water for 10–15 min<br>**Cutaneous:** several compresses per day (10 g–100 mL cold water) | European Medicines Agency (2015) |
| Poland | Infusion | Diarrhea | Concentration range of infusion: 6–16 g/L | Komiazyk et al. (2019) |
| Serbia | n/s | Diarrhea and inflammation of kidney and bladder | n/s | Cho et al. (2018) |
| Turkey | n/s | Whooping cough | n/s | Dulger and Gonuz (2004) |

Abbreviations: n/s, not specified.

### 4.1.3 Monographs

In the *European Pharmacopoeia*, only *A. eupatoria* can be used as source of herbal material in the genus *Agrimonia* (Granica et al. 2015), specifically the dried flowering tops, which is consistent with the EMA report (European Medicines Agency, 2015). However, the *German Commission E Pharmacopoeia* monograph (Wichtl, 2004) considers the use of dried, above-ground parts of both *A. eupatoria* and/or *Agrimonia procera* Wallr. as valid sources of *Agrimonia* (Granica et al. 2015).Moreover, EMA also describes the principal constituents of the herbal substance, preparations and a summary of pharmacopeias and standard reference publications with monographs of agrimony herb.

The European Union herbal monograph on *Agrimonia eupatoria* L. Herba (European Medicines Agency, 2015) describes the traditional uses in herbal preparations and posology (for adolescents, adults and the elderly) and method of administration.

### 4.1.4 Methodology of research

The literature explored in this review was retrieved from several bibliographic sources, namely PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), ScienceDirect (https://www.sciencedirect.com/) and Google Scholar (https://scholar.google.pt/). This article reviewed literature published from the first report on *A. eupatoria* by Bilia et al. (1993) until 2021. As can be seen in Table IV.2, *A. eupatoria* is not a plant which is often described in previous literature. Furthermore, a marked heterogeneity was observed regarding several methodological aspects, particularly the plant material used and the extraction method, which will be further discussed in the following section.

## 4.2 Phenolic composition

A number of phytochemicals have been identified in herbal preparations of *A. eupatoria*, as polyphenolic compounds have been reported as the major constituents, including phenolic acids, flavonoids, tannins, procyanidins and coumarins (Granica et al.

2013; Granica et al. 2015). Other constituents have been identified in *A. eupatoria,* namely terpenoids, steroids and saponins (Tomlinson et al. 2003; Granica et al. 2013; Muruzović et al. 2016; Ginovyan et al. 2020). As these constituents are less commonly reported in the literature, we focused on phenolic compounds in our review. In this context, several studies have quantified the major groups of these secondary metabolites – i.e. phenols (Ivanova et al. 2005; Correia et al. 2006; Kubínová et al. 2016; Muruzović et al. 2016), flavonoid (Correia et al. 2006; Kubínová et al. 2012; Kubínová et al. 2016; Muruzović et al. 2016), tannins (Muruzović et al. 2016) and proanthocyanidins (Muruzović et al. 2016)– according to different extraction methods using the aerial parts (in the flowering stage or not), seeds and stems, as presented in Table IV.2. Moreover, Muruzović et al. (2016) compared four types of extract of the aerial parts (in the flowering stage) and showed that the acetone extract exhibited the highest concentration of these compounds, as the ethanolic and aqueous extracts exhibited about half of those and the diethyl ether extract the lowest concentrations. Interestingly, Kubínová et al. (2012) compared several *Agrimonia* species and showed that the *A. eupatoria* had the highest flavonoid content among the methanolic extracts.

**Table IV.2**. Details on crude extract preparation in previous studies cited in this paper (grouped by plant part and in chronological order) which focused on the characterization of the phytochemistry and/or bioactivity of *A. eupatoria*.

| Plant part | Study | Material origin or Product type | Extraction method | | |
|---|---|---|---|---|---|
| | | | Solvent(s) | Plant material – solvent ratio (g/mL) | Method considerations |
| Aerial parts | Bilia et al. (1993) | n/s | Petrol, $CHCl_3$, CH-$Cl_3$-MeoH | 9:1 | Extracted in Soxhlet apparatus |
| | Kwon et al. (2005) | Collected in Kangwha, Kumsan and Youngdong, Korea (May-August) | Distilled water | n/s | 1 hour at different temperatures (37, 45, 55 and 60°C) |
| | Ivanova et al. (2005) | Commercial product (Thalloderma Pharmaceutical Laboratories) | Boiling water | 0.5:100 | Infusion; Incubation for 10 minutes |
| | Correia et al. (2006) | Commercial product (Segredo da Planta, Portugal) | Aqueous ethanol 45% | 1:15 | 13 days in the dark |
| | Correia et al. (2007) | Commercial product (Segredo da Planta, Portugal) | Aqueous ethanol 45% | 1:15 | 13 days in the dark |
| | Yoon et al. (2012) | Collected in Hamyang, Korea (August 2010) | Distilled water | 1:10 | Extraction made twice at 95°C for 5 hours |
| | Ad'hiah et al. (2013) | Collected in Binqulat Village (Erbil, North Baghdad) in July 2008 | Distilled water/ methanol | 1:5 | Extraction using Soxhlet |
| | Granica et al. (2013) | Commercial products (Flos, Mokrsko, Poland &DaryNatury, Warsaw, Poland) | Boiling deionized water | 1:100 | Infusion; 5 minutes |
| | Ivanova et al. (2013) | Commercial product (Selibum Ltd., Varna, Bulgaria) | Boiled water | 0.5:60 | 10 minutes |

| | | | | | |
|---|---|---|---|---|---|
| | Granica et al. (2015) | Commercial products[a] | Boiling deionized water | 1:100 | 5 minutes |
| | Kubínová et al. (2015) | Collected in Medicinal Herbs Centre of Masaryk University Brno, Czech Republic (July 2008) | Water (at 100°C) | 0.5:60 | Decoction for 10 minutes |
| | Kuczmannová et al. (2015) | Commercial product (Fytopharma, Malacky, Slovakia) | Water | n/s | n/s |
| | Kuczmannová et al. (2016) | Commercial product (Fytopharma, Malacky, Slovakia) | Water | 0.02:100 | Infusion |
| | Lee and Rhee (2016) | Commercial product (Jung Do Herb Co. Ltd, Seoul, Korea) | Ethanol 80% | 1:10 | 48 hours at room temperature |
| | Santos et al. (2017) | Commercial product (Segredo da Planta, Portugal) | Water | 1:30 | Infusion; 15 minutes; then washed with *n*-Hexane (1:1) |
| | Cho et al. (2018) | Collected in Hamyang, South Korea (August 2012) | Distilled water | 1:10 | 3 hours at 95°C |
| | Cardoso et al. (2018) | Commercial product (Segredo da Planta, Portugal) | Ethanol 45% | 1:15 | Maceration during 13 days |
| | Komiazyk et al. (2019) | Dried from natural state | Boiling water | 1:25 | Infusion; pouring the boiling water onto the plan material, was allowed to cool to room temperature and left to stand for over 18 h. After, the extract was decanted and passed by filter paper 0.4 µm cellulose acetate filters. |
| Aerial parts (in flowering stage) | Venskutonis et al. (2008) | Grown in in the experimental field of Kaunas Botanical Garden at Vytautas Magnus University (Lithuania) | Acetone and Methanol | 1:20 | Extraction in automatic extractor; solvents removed with rotary evaporator at 45°C |
| | | | Boiling water | 1:100 | Extraction using three steps, 30 min each, under constant shaking, first with 100 ml of hot water, second and third with 50 ml of |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | hot water; extract was filtered after each step |
| | Kubínová et al. (2012) | Medicinal Herbs Centre of Masaryk University | Aqueous and methanolic | 1:60 | Microwave extraction (10 minutes, 500 W, 15°C) |
| | Muruzović et al. (2016) | Collected in Mount Bukulja, Serbia (summer of 2012) | Ethanol, diethyl ether, water and acetone | 1:5 | Independent extractions; Plant material macerated 3x at room temperature with fresh solvent every 24 hours |
| Leaves | Gray and Flatt (1998) | Commercial Product (The Health Food Centre, Bull Ring Shopping Centre, Birminghan, UK) | Aqueous | 1:40 | Decoction method. The powered material was immersed in cold distilled water, which was brought to the boil, removed from the heat and infused for 15 min. The suspension was filtered (Whatman no. 1). |
| | Gião et al. (2012) | Commercial product (Ervital, Portugal) | Boiling water | 1:110 | Infusion; 5 minutes of infusion; after, the suspension was filtered through 0.45 µm filter |
| Seeds | Copland et al. (2003) | Commercial product (B & T World Seeds sarl, Paguigan, France) | *n*-Hexane, dichloromethane and methanol | n/s | n/s |
| | Tomlinson et al. (2003) | Commercial product (B & T World Seeds sarl, Paguigan, France) | *n*-Hexane, dichloromethane and methanol | 1:10 | Sequential extraction using Soxhlet |
| Stems | Lee et al. (2010) | Commercial product (Biokorea Co., Seoul, Korea) | Methanol 80% | n/s | 2 hours by ultrasonic apparatus |
| Whole plant | Ginovyan et al. (2020) | Collected in Yerevan, Armenia. Voucher specimen deposited to Herbarium of Yerevan State University (ERBC 13207) | Methanol 98% and acetone 99.8% | 1:10 | n/s |
| Whole plant in blooming stage | Pukalskienė et al. (2018) | Commercial product (Kaunas Botanical Garden of Vytauas Magnus University, Liyhuania) | Methanol | 1:20 | 24 hours of constant shaking; filtered using a 0.3 µm filter an the concentrated in a rotatory evaporator |

| | | | | | |
|---|---|---|---|---|---|
| *Agrimoniae Herba* | Bae et al. (2010) | Commercial product (Ten Pharmaceutical Co., Taipei, Taiwan) | Distilled Water | n/s | Distilled for 24 hours at 4°C. The solution was centrifuged at 3000 rev/min for 10 minutes. The supernatant was filtered through Whatman no. 2. |
| Powered dry plant material | Ivanova et al. (2013) | n/s | Ethanol/PBS 40% (v/v) | 150 mg | Extraction by three minutes of continuous vortexing at room temperature |
| Mature plants and their parts | Dulger and Gonuz (2004) | Collected in different cities of Turkey during the months of April-May of 2002 | Ethanol 80% | 1:7.5 | Extraction for 24 h using a Soxhlet. Extract filtered using Whatman filter paper no.1. The filtrates were evaporated under reduced pressure and dried using a rotary evaporator at 55°C. |
| Dried material | Gallagher et al. (2003) | Commercial product (Birmingham (West Midlands, UK) | cold distilled water | 1:40 | Decoction. After removed from the heat source was allowed to infuse for 15 min. Filtered with Whatman no. 1 |
| Herbal extracts | Cwikla et al. (2010) | Commercial product (MediHerb Pty Ltd Brisbane, Eight Miles Plains, Queensland 4113, Australia) | Ethanol 45% | 1:2 | n/s |
| | Bensch et al. (2011) | Commercial product (Integria Healthcare, 8 Clunies Ross Court, Eight Mile Plains, Queensland 4113, Australia) | Ethanol 45% | 1:2 | n/s |

Abbreviations: n/s, non-specified.

[a] *Agrimonia eupatoria* L. Commercial/Ervanario de Augusto Coutinho, Porto, Portugal 11331/20140912 E/DPW
*Agrimonia eupatoria* L. Natural state/Dary Natury, Koryciny, Poland 01112013/20151101 DN/DPW
*Agrimonia eupatoria* L. Commercial/Flos, Mokrsko, Poland 11-1102/20150610 FLOS/DPW
*Agrimonia eupatoria* L. Natural state/near Piduń, warmińsko-mazurskie, Poland 20140912 A/DPW
*Agrimonia eupatoria* L. Cultivated/Exsemine GmbH, Salzatal/Zappendorf, Germany S261.14/127/EX
*Agrimonia eupatoria* L. Natural state/near Ojrzanów, mazowieckie, Poland 20140705 AGR/DPW
*Agrimonia eupatoria* L. Cultivated/Botanical Garden UAM Poznań, Polska 20140915 A/DPW
*Agrimonia* eupatoria L. Cultivated/Botanical Garden PAN Powsin, Polska 0140611 A/DPW

Quercetin glycoside derivatives were identified in all extracts with the exception of Bilia et al. (1993) and Venskutonis et al. (2008), regardless of the solvent that was used (water, methanol, ethanol, acetone, *n*-hexane and dichloromethane). Kaempferol glycoside derivatives were reported in different types of extract, i.e. aqueous, ethanolic and methanolic. Even though Bilia et al. (1993) reported kaempferol derivatives in a methanolic extract, Venskutonis et al. (2008), Kubínová et al. (2012) and Tomlinson et al. (2003) did not.

Phenolic acids were identified mostly in aqueous extracts (Correia et al. 2006; Granica et al. 2013; Granica et al. 2015; Kuczmannová et al. 2015; Santos et al. 2017) but also in the methanolic extract (Pukalskienė et al. 2018). Concerning the tannins, these compounds were reported only in aqueous extracts (Correia et al. 2006; Granica et al. 2013; Granica et al. 2015; Santos et al. 2017; Pukalskienė et al. 2018).

As previously mentioned, a high degree of heterogeneity has been observed in previous studies on the phytochemistry of *A. eupatoria* in terms of methods, e.g. ratio of solvent/plant, time of extraction, gradients in HPLC analysis (Table IV.2 and IV.3). That heterogeneity can influence the composition of the extract thus influencing the findings in bioactivity assays, as further discussed below. As the polyphenolic content of the extract may differ according to the methodologic conditions of extraction (Granica et al. 2015), this section explores the phenolic composition of this plant species by including all types of extraction methods and the methodology utilized in the characterization of the extracts.

**Table IV.3**. Content of phenolic compounds in *A. eupatoria* according to different extraction methods.

| Plant part | Extract/Fraction | Total Phenols [a] | Total Flavonoids [b] | Total Tannins [c] (mg GAE/g extract) | Proanthocyanidins [d] (CchE/g extract) | Reference |
|---|---|---|---|---|---|---|
| Aerial parts | Aqueous | 702.29±6.82 µM | - | - | - | Ivanova et al. (2005) |
| | | 402.5±15.7 mg GAE/g LE | 25.8±0.9 mg QE/ g LE | - | - | Kubínová et al. (2016) |
| | | 0.6-0.9 mg/g[e] | 8.2-10.9 mg/g[e] | 6.3-10.9 mg/g[e] | - | Granica et al. (2013) |
| | EtOH | 15.78±2.93 g% | 1.85±0.00 g% | - | - | Correia et al. (2006) |
| | EtOAc fraction | 27.64±2.06 g% | 2.35±0.00 g% | - | - | Correia et al. (2006) |
| Aerial parts (in flowering stage) | Aqueous | 118.74±0.72 mg GAE/g extract | 20.58±0.92 mg RU/g extract | 107.52±0.16 | 55.85±0.75 | Muruzović et al. (2016) |
| | | - | 72.4±3.8 mg QE/g | - | - | Kubínová et al. (2012) |
| | MeOH | - | 3.5±0.3 mg QE/g | - | - | Kubínová et al. (2012) |
| | EtOH | 123.90±0.47 mg GAE/g extract | 46.50±0.08 mg RU/g extract | 190.33±0.09 | 74.42±0.73 | Muruzović et al. (2016) |
| | Acetone | 220.31±0.00 mg GAE/g extract | 97.06±2.56 mg RU/g extract | 207.27±0.21 | 103.72±0.53 | Muruzović et al. (2016) |
| | Diethyl ether | 19.61±0.10 mg GAE/g extract | 64.90±0.79 mg RU/g extract | 3.06±5.98 | 4.15±0.41 | Muruzović et al. (2016) |
| Leaves | Aqueous | 229.5±33.9 µg epicatechin/mL<br>16.9±2.3 µg gallic acid/mL | 140.9±13.2 µg epicatechin/mL | - | - | Gião et al. (2012) |
| Whole plant | MeOH | 358.9±0.62 mg GAE $g^{-1}$ DW | 26.23±0.85 mg CE $g^{-1}$ DW | 21.54±1.02 CE $g^{-1}$ DW[f] | - | Ginovyan et al. (2020) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Acetone | 348.34±0.97 $g^{-1}$ DW | 83.52±1.485 mg CE $g^{-1}$ DW | 31.90±1.02 CE $g^{-1}$ DW[f] | - | Ginovyan et al. (2020) |
| Whole plant in blooming stage | MeOH | 68.4±0.80 GAE/g DWE | - | - | - | Pukalskienė et al. (2018) |

[a] Total phenols quantification by the Folin-Ciocalteu method. Results expressed as: mg GAE/g LE (i.e. the amount of total phenols was calculated as a gallic acid equivalent (GAE) from the calibration curve of gallic acid standard solutions and expressed as mgs of gallic acid per gram of lyophilized extract), mg GAE/g of extract (i.e. total phenolic and extractable tannin content was expressed as milligram of gallic acid equivalents/gram of extract), g% (i.e. results expressed as grams of gallic acid and rutin equivalents by 100 g freeze-dried samples) mg GAE $g^{-1}$ DW (i.e. results expressed as micrograms of Gallic acid equivalent per mg of dry extract), µg epicatechin in 1 mg/mL of plant extract and µg gallic acid in 1 mg/mL of plant extract.

[b] Total flavonoids quantification by the aluminum chloride method. Results expressed as: mg QE/g LE (i.e. the amount of total flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions and expressed as mgs of quercetin per gram of lyophilized extract), g% (i.e. results expressed as grams of gallic acid and rutin equivalents by 100 g freeze-dried samples), mg QE/g (i.e. the amount of total flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions and expressed as mg quercetin/1 g dry plant material), mg RU/g extract (i.e. concentration of flavonoids were expressed as milligram of rutin equivalents/gram of extract) mg CE $g^{-1}$ DW (i.e. micrograms of quercetin equivalent per mg of dry extract) or µg epicatechin in 1 mg/mL of plant extract

[c] Total tannins quantification by the Polyvinylpolypyrolidone method. Results expressed as mg GAE/g of extract (i.e. total phenolic and extractable tannin content was expressed as milligram of gallic acid equivalents/gram of extract) or Mg CE $g^{-1}$ DW (i.e. micrograms of (+)-catechin equivalent per mg extract)

[d] Proanthocyanidins quantification by the Butanol-HCl method. Results expressed as mg CchE/g of extract (i.e. the proanthocyanidins content was expressed as milligrams of cyanidin chloride equivalents/gram of extracts).

[e] Results obtained with the data of DAD detector.

[f] Total tannins quantification by the Braemer assay.

Abbreviations: EtOH, ethanol; MeOH, methanol.

In Table IV.4, we present the phenolic compounds that have been reported in *A. eupatoria* parts and the methodology of extraction, characterization and the identification. *A. eupatoria* is rich in phenolic acids, flavonoids and tannins. Several of these compounds have been identified by spectral profile analysis rather than comparison with commercial standards (e.g. quercetin and kaempferol derivatives). Full identification is yet to be carried out, which must be complemented by mass spectrometry for complete characterization. Taking into consideration all types of extract and plant parts, the following compounds found more often: astragalin, cynaroside, hyperoside, isoquercitrin, isovitexin, rutin, catechin, procyanidin B3 and agrimoniin.

### 4.2.1 Simple phenolics

Phenolic acids

Bae et al. (2010), Granica et al. (2015), Santos et al. (2017) all using aqueous extracts and Pukalskienė et al. (2018) with a methanolic extract reported the presence of ellagic acid (**1**).Bae et al. (2010) and Pukalskienė et al. (2018) confirmed this compound utilizing the HPLC-DAD technique and UPLC-$MS^2$, respectively.

Hydroxibenzoic acids

Using UPLC-$MS^2$, Pukalskienė et al. (2018) assayed a methanolic extract from the whole plant in the blooming stage and reported a gallic acid (**2**) and carried out a comparison with commercial standard. Correia et al. (2006) reported protocatechuic acid (**3**) in an ethyl acetate fraction of aerial parts that was assayed by HPLC-DAD-MS. However, this compound was not fully characterized.

Hydroxycinnamic acids

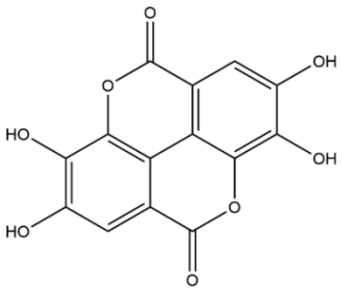
Kuczmannová et al. (2015) using an aqueous extract of aerial parts, in addition to Correia et al. (2006) and Santos et al. (2017), who assayed an ethyl acetate fraction by HPLC-DAD-$MS^3$, reported *p*-coumaric acid (**4**). Kuczmannová et al. (2015) and Santos et al. (2017) confirmed this compound with a commercial standard.

Derivatives of caffeic acid (**5-6**) were also reported in aqueous and methanolic extracts. However, only caffeoyl theronic acid was confirmed by Pukalskienė et al. (2018)

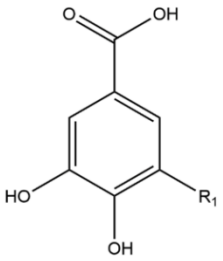
with a commercial standard in the methanolic extract of the whole plant in the blooming stage using ULPC -$MS^2$.

Regarding *p*-coumaric derivatives that are conjugated with quinic acid and isomers (**7-11**), these compounds were reported both in aqueous and methanolic extracts. However, only three authors confirmed the structures: Kuczmannová et al. (2015) utilized commercial standards to confirm 5-*O*-caffeoylquinic acid (**9**), also known as chlorogenic acid. This compound was also compared to a commercial standard by Granica et al. (2015). Both studies assayed an aqueous extract and used HPLC-DAD-MS. Pukalskienė et al. (2018) also reported chlorogenic acid in the methanolic extract that was verified by comparison with a commercial standard, using ULPC-$MS^2$. The compound 3-*O*-*p*-caffeoylquinic acid (**11**), was reported in an aqueous extract of aerial parts by HPLC-DAD-$MS^3$.

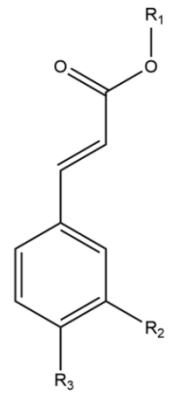
All structures of the identified phenolic acids are displayed in Figure IV.2.



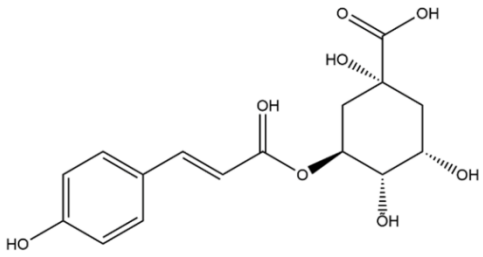
**1**



| *Number* | $R_1$ |
|---|---|
| **2** | OH |
| **3** | H |



| *Number* | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **4** | H | H | H |
| **5** | OH | OH | H |
| **6** | H | H | Treonic acid |



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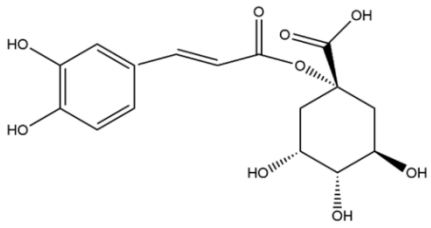

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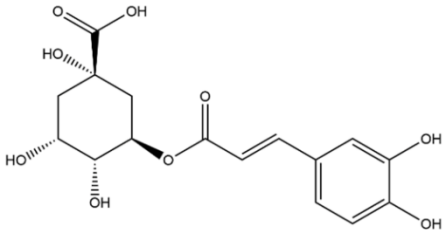
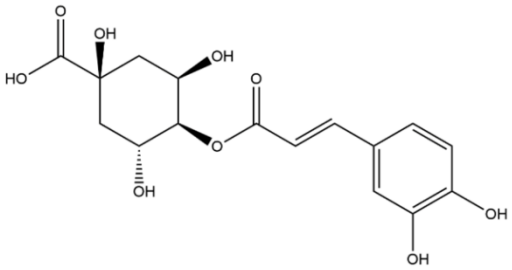


**9**



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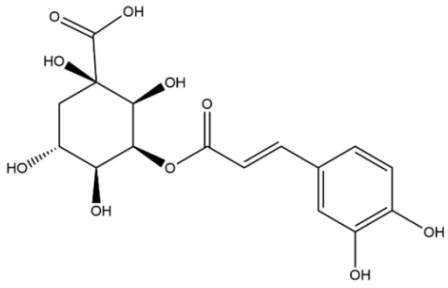


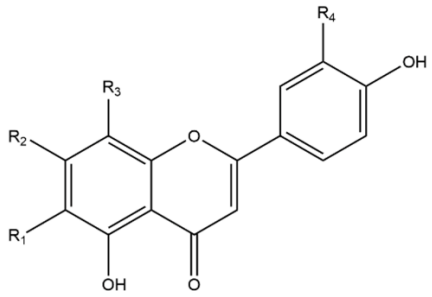
**11**

**Figure IV.2**. **Phenolic acids reported in *A. eupatoria*** (compounds 1–11).

### 4.2.2 Flavonoids

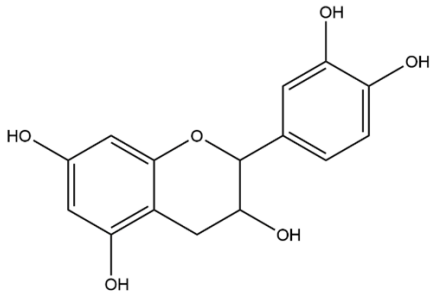
This class of coumpounds (Figure IV.3) was reported in all extracts that are reported in Table IV.4. The aqueous extracts from aerial parts generally showed a varied composition of kaempferol, quercetin, apigenin and luteolin derivatives.

***A***

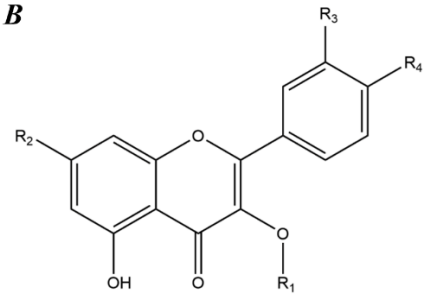



| *Number* | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **12** | C-glucoside | OH | H | H |
| **13** | H | O-glucoside | H | H |
| **14** | H | O-glucoronide | H | H |
| **15** | H | H | C-glucoside | H |
| **16** | H | *O*-*β*-D-glucopyranoside | H | OH |
| **17** | H | *O*-*β*-D-glucoronide | H | OH |
| **18** | H | *O*-glucuronide | H | OH |
| **19** | H | *O*- glucoside | H | OH |

**20**




***B***




| *Number* | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **21** | rhamnoglucoside | OH | OH | OH |
| **22** | galactoside | OH | OH | OH |
| **23** | rhamnoside | OH | OH | OH |
| **24** | glucoside | OH | OH | OH |
| **25** | H | O-rhaminoside | OH | OH |
| **26** | H | OH | *O*-β-D-glucopyranoside | OH |
| **27** | 6''-O- galloyl-3-3-O-glucoside | OH | OH | OH |
| **28** | H | OH | H | $CH_3$ |
| **29** | Rhaminoside | OH | H | $CH_3$ |
| **30** | H | OH | H | OH |
| **31** | Rhaminoside | OH | H | OH |
| **32** | Rutinoside | OH | H | OH |
| **33** | glucoside | OH | H | OH |
| **34** | *β*-D-(2''-*O*-acetyl)glucopyranoside | OH | H | OH |
| **35** | *β*-D (2''-*O*- acetyl'' –I)-*p*-coumaroyl-glucopyranoside (2''-acetyl-tiliroside) | OH | H | OH |
| **36** | 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside | OH | H | OH |

**Figure IV.3**. **Flavonoids reported in *A. eupatoria***: flavones (**A**, compounds 12–19), flavanol catechin (20) and flavonols (**B**, compounds 21–36).

Flavones

Apigenin derivatives (**12–15**) were identified in several reports with the exception of Bilia et al. (1993) and Tomlinson et al. (2003).

Kubínová et al. (2012) assayed both aqueous and methanolic extracts from aerial parts in the blooming stage and analyzed their composition by HPLC-DAD. Using commercial standards, the authors confirmed the presence of isovitexin (**12**) and apigenin-7-*O*-glucoside (**13**). Isovitexin, a recurrent compound, was confirmed in multiple extracts through comparison with commercial standard, namely the aqueous extract of aerial parts (Granica et al. 2013; Granica et al. 2015) and the ethyl acetate fraction also from aerial parts (Correia et al. 2006; Santos et al. 2017). All these studies used the HPLC-DAD-MS technique.

In the matter of apigenin-7-*O*-glucoside (apigetrin, **13**), this compound was also identified by HPLC-DAD-MS using commercial standards in other studies, using aqueous extract (Granica et al. 2015) and ethyl acetate fraction (Santos et al. 2017). Apigenin-7-*O*-glucuronide (**14**) is also mentioned in four reports: Lee et al. (2010), which analyzed the methanolic extract of stems by HPLC UV/MS and reported this compound by confirming data in the literature, Granica et al. (2013) that isolated this compound, and Granica et al. (2015) and Kuczmannová et al. (2015) that confirmed this compound using commercial standards. The latter three reports (Granica et al. 2013; Granica et al. 2015; Kuczmannová et al. 2015) used HPLC-DAD-MS.

Other apigenin derivatives were also mentioned, including apigenin-*O*-hexoside and apigenin-*O*-hexunoride, which were confirmed by Pukalskienė et al. (2018) by comparing with data in the literature, and vitexin (**15**), confirmed with commercial standard by Granica et al. (2015).

Concerning luteolin derivatives (**16–19**), these were identified in several studies (Venskutonis et al. 2008; Lee et al. 2010; Granica et al. 2013; Granica et al. 2015; Kuczmannová et al. 2015; Santos et al. 2017; Pukalskienė et al. 2018). Lee et al. (2010)reported the presence of luteolin-7-*O*-*β*-D-glucopyranoside (**16**) and luteolin-7-*O*-*β*-D-glucoronide (**17**) in the methanolic extract of stems. The method of charactezation was HPLC-UV-MS and $^{1}H$ and $^{13}C$ NMR.

Luteolin-7-*O*-glucoronide (**18**) is the most recurrent compound. This compound was isolated by Granica et al. (2013) and confirmed by commercial standards by Granica et al. (2015) and Kuczmannová et al. (2015). These three reports had in common the

type of extract (aqueous) and the technique for the analysis of the extract (HPLC-DAD-MS). Pukalskienė et al. (2018) also identified this compound in the methanolic extract from the whole plant in the blooming stage, using UPLC-$MS^2$, comparing with data in the literature and by parent ion mass using free chemical databases. The second most reccuring compound is cynaroside (**19**), which was confirmed in the aqueous extract from aerial parts by (Granica et al. 2013, 2015).

Flavanols

Five studies reported the presence of catechin (**20**), which was identified by HPLC-DAD-$MS^3$, both in the aqueous extract of aerial parts (Granica et al. 2013; Granica et al. 2015; Kuczmannová et al. 2015) and in the ethyl acetate fraction from aerial parts (Correia et al. 2006; Santos et al. 2017). These articles report in common the identification of the compound structure through comparison with chemical standards, with the exception of Correia et al. (2006).

Flavonols

Quercetin derivatives (**21–27**) were generally identified except in the report by Bilia et al. (1993).

Rutin (**21**) was a recurrent compound in the compositon of both aqueous (Granica et al. 2013; Granica et al. 2015; Kuczmannová et al. 2015) and methanolic (Kubínová et al. 2012; Pukalskienė et al. 2018) extracts. All these studies used commercial standards for the identification of this compound.

Hyperoside (**22**) was also identified in multiple extracts and confirmed with commercial standards by the following authors: Correia et al. (2006) with an ethyl acetate fraction from aerial parts, Kubínová et al. (2012) with a methanolic extract from aerial parts in the blooming stage, Granica et al. (2013) and (2015) with an aqueous extract from aerial parts and Pukalskienė et al. (2018) with a methanolic extract from the whole plant in the blooming stage using UPLC-$MS^2$.

In a methanolic extract from seeds, Tomlinson et al. (2003) confirmed two compounds: quercetrin (**23**) and quercetin-3"-*O*-β-D-glucopyranoside (**26**) by NMR. Quercetrin was also identified by (Granica et al. 2013, 2015) by comparison with commercial standards.

Five articles reported the use of HPLC-DAD-$MS^3$: Granica et al. (2013) and (2015) and Kuczmannová et al. (2015), with an aqueous extract of aerial parts, and Correia et al. (2006) and Santos et al. (2017), which assayed an ethyl acetate fraction. In addition, one article reported the utilization of HPLC-DAD in a methanolic extract of aerial parts in the flowering stage (Kubínová et al. 2012). These articles report in common the identification of isoquercitrin (**24**), through comparison with chemical standards. Lee et al. (2010) also reported these compounds by analysis with HPLC UV/MS and the identification was carried out with data in the literature.

Other compounds were also identified by comparisons with chemical standards: quercetin-7-*O*-rhamnoside (**25**) from an aqueous extract by HPLC-DAD-MS (Kuczmannová et al. 2015) and quercetin-6''-*O*-galloyl-3-3-*O*-glucoside (**27**) from an aqueous extract from aerial parts also by HPLC-DAD-MS (Granica et al. 2015).

Considering all those studies, it is notable that kaempferol derivatives (**28–36**) were identified in the methanolic extract of stems (Lee et al. 2010) but not in methanolic extracts of aerial parts (Bilia et al. 1993; Venskutonis et al. 2008; Kubínová et al. 2012).

Bilia et al. (1993) identifed kaempferide and derivatives (**28–29**) in a methanolic extract of aerial parts by NMR. In the same report, kaempferol (**30**) and derivatives kaempferol-3-rhaminoside (**31**) and kaempferol-3-rutinoside (**32**) were also identified. Also using NMR in a methanolic extract from stems, Lee et al. (2010) confirmed the presence of kaempferol-3-*O*-β-D-(2''-*O*-acetyl-6''-(E)-*p*-coumaroyl)-glucopyranoside (2''-acetyl-tiliroside) (**35**).

Several authors reported the presence of astragalin (**33**) in extracts from aerial parts, through confirmation with commercial standards (Correia et al. 2006; Granica et al. 2013; Granica et al. 2015; Santos et al. 2017).

Other compounds that were confirmed include: kaempferol-3-*O*-*β*-D-(2''-*O*-acetyl)glucopyranoside (**34**) by Lee et al. (2010)and kaempferol-3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside (**36**) by Correia et al. (2006).

Other quercetin and kaempferol derivatives were reported (summarized in Table IV.4) but the full characterization was not completed.

### 4.2.3 Tannins

Condensed tannins

*Proanthocyanidins*

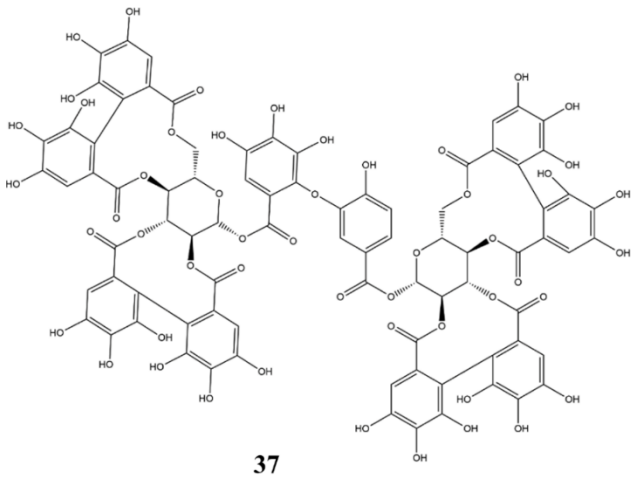
Several procyanidins such as procyanidins B1, B2, B3, B6, B7, C1, C2 (**38–44**) and EEC have been identified, as presented in Table IV.4 and Figure IV.4, particularly in the ethyl acetate fraction from the leaves of *A. eupatoria*. Nevertheless, only procyanidins B1, B2 and B3 were compared with commercial standards while B6 and B7 were characterized by elution characteristics by Correia et al. (2006).

Correia et al. (2006) also used HPLC/DMACA (*p*-dimethylaminocinnamaldehyde) for the characterization of tannins and were able to confirm the presence of procyanidin B1, B2 and B3. Procyanidn B3 was also confirmed by (Granica et al. 2013; 2015).

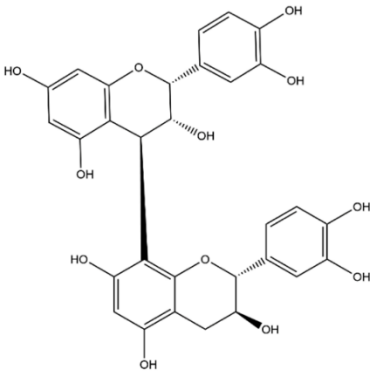
Hydrolyzable tannins

Granica et al. (2013) were able to isolate agrimoniin (**37**) from the plant material that was assayed in their report.

Pedunculagin (**45**) was idenfied based in the UPLC-$MS^2$ results and previous literature and databases by Pukalskienė et al. (2018) on a methanolic extract of the whole plant in the blooming stage (Figure IV.4).



**37**



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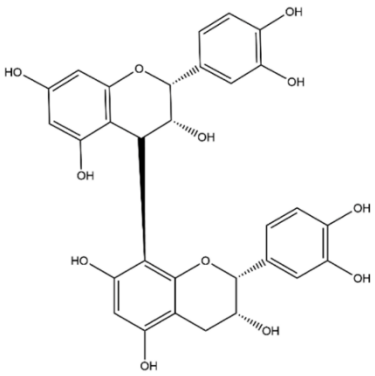

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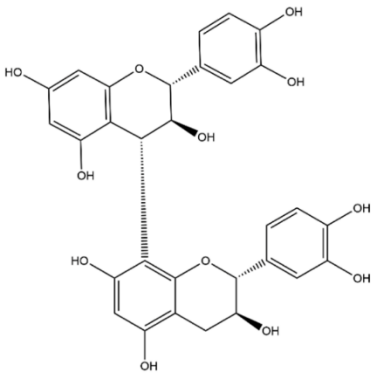

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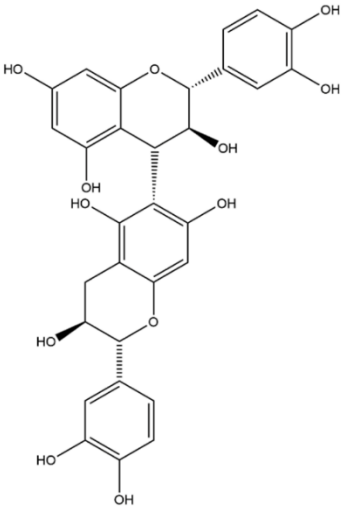

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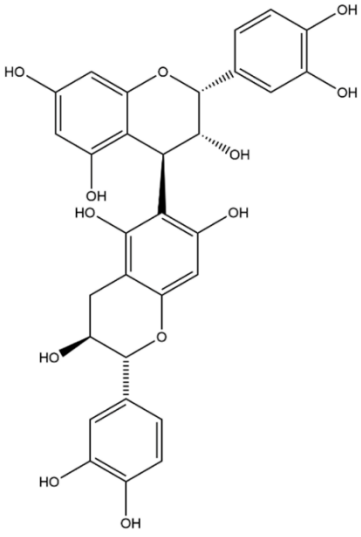

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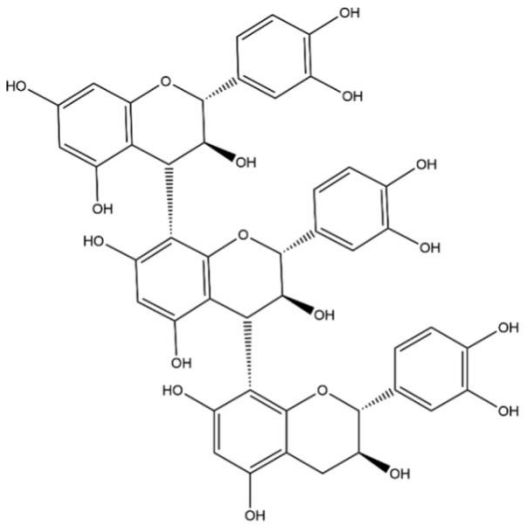

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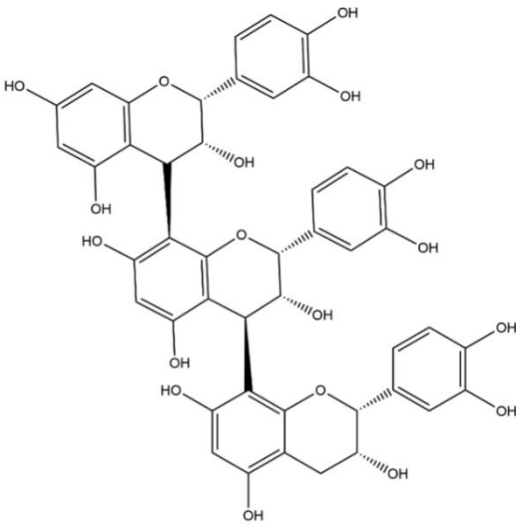

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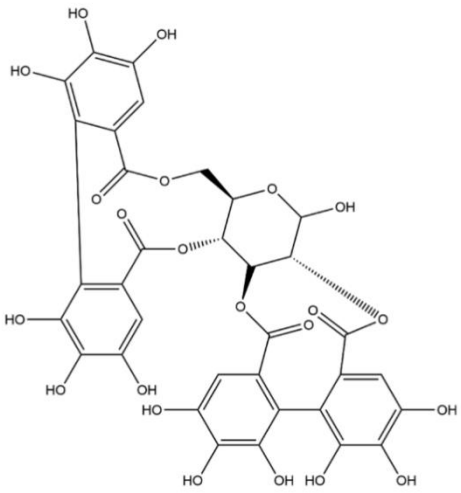

45

**Figure IV.4**. **Tannins reported in *A.* eupatoria** (compounds 37–45).

**Table IV.4**. List of phenolic compounds reported in *A. eupatoria* in previous reports and the methodology utilized to perform the characterization.

| Number | Compound name | Plant part | Extract or fraction | Methodology | λmax (nm) | Reference(s) |
|---|---|---|---|---|---|---|
| | | | ***PHENOLIC ACIDS*** | | | |
| 1 | Ellagic acid | *Agrimoniae Herba* | Aqueous | HPLC-DAD | 254 | (Bae et al., 2010) * |
| | | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) |
| | | | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017)* |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018) * |
| - | Ellagic acid pentose conjugated | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |
| 2 | Gallic acid | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |
| 3 | Protocatechuic acid | Aerial parts | EtOAc fraction | HPLC-DAD-$MS^3$ | 280 and 360 | (Correia et al., 2006) |
| 4 | *p*- Coumaric acid | Aerial parts | EtOAc fraction | HPLC-DAD-$MS^3$ | 280 and 360; 280 | (Correia et al., 2006; Santos et al., 2017*) |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) * |
| | | *Agrimoniae Herba* | Aqueous | HPLC | 254 | (Bae et al., 2010) * |
| 5 | Caffeoyl hexoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) * |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | Caffeoyl threonic acid | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018) * |
| 7 | 3-*O*-*p*-coumaroylquinic acid | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| - | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |
| - | *p*-coumaroyl acid hexoside | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) |
| 8 | 1-*O*-caffeoylquinic acid | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) |
| 9 | 5-*O*-caffeoylquinic acid (chlorogenic acid) | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2015)* |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018) * |
| 10 | 4-*O*-caffeoylquinic acid (Cryptochlorogenic acid) | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| 11 | 3-*O*-caffeoylquinic acid (neochlorogenic acid) | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2013) |
| - | *p*-coumaroyl quinic acid | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| | | | ***FLAVONOIDS*** | | | |
| 12 | Apigenin 6-*C*-glucoside (Isovitexin) | Aerial parts (in flowering stage) | MeOH | HPLC-DAD | 280 | (Kubínová et al., 2012) * |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Aerial parts | Aqueous | HPLC-DAD-MS$^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | | EtOAc fraction | HPLC-DAD-MS; HPLC-PDA-ESI/Ms$^n$ | 280 and 360; 280 | (Correia et al., 2006; Santos et al., 2017) * |
| 13 | Apigenin 7-*O*-glucoside (Apigetrin) | Aerial parts (in flowering stage) | MeOH | HPLC-DAD | 280 | (Kubínová et al., 2012) * |
| | | Aerial parts | Aqueous | HPLC-DAD-MS$^3$ | 280 and 350; 254 and 350 | (Granica et al., 2015) * |
| | | | EtOAc fraction | HPLC-PDA-ESI/Ms$^n$ | 280 | (Santos et al., 2017) * |
| 14 | Apigenin 7-*O*-glucuronide | Aerial parts | Aqueous | HPLC-DAD-MS$^3$ | 280 and 350 | (Granica et al., 2013)$^+$ |
| | | | | | | (Granica et al., 2015) * |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| | | Stems | MeOH | HPLC UV/MS | 256 | (Lee et al., 2010)[a] |
| 15 | Apigenin 8-C glucoside (Vitexin) | Aerial parts | Aqueous | HPLC-DAD-MS$^3$ | 280 and 350; 254 and 350 | (Granica et al., 2015) * |
| | | | EtOAc fraction | HPLC-PDA-ESI/Ms$^n$ | 280 | (Santos et al., 2017) |
| - | Apigenin *O*-glucoronide | Aerial parts | EtOAC fraction | HPLC-PDA-ESI/Ms$^n$ | 280 | (Santos et al., 2017) |
| - | Apigenin glycoside | Aerial parts (in flowering stage) | MeOH | HPLC UV-MS | 280 | (Venskutonis et al., 2008) |
| - | Apigenin-*O*- hexoside | Whole plant in blooming stage | MeOH | ULPC MS$^2$ | 280 | (Pukalskienė et al., 2018)[a] |

| | | | | | | |
|---|---|---|---|---|---|---|
| - | Apigenin-*O*-hexunoride | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a] |
| - | *C*- glycoside apigenin derivate | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) |
| - | Apigenin derivate | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| 16 | Luteolin 7-*O*-*β*-D-glucopyranoside | Aerial parts (in flowering stage); Stems | MeOH | $^1H$ and $^{13}C$ NMR | 256 | (Lee et al., 2010)[a] |
| 17 | Luteolin 7-*O*-*β*-D-glucuronide | Stems | MeOH | $^1H$ and $^{13}C$ NMR | 256 | (Lee et al., 2010)[a] |
| 18 | Luteolin 7-*O*-glucuronide | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350 | (Granica et al., 2013)[+] |
| | | | | | 254 and 350 | (Granica et al., 2015) * |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |
| 19 | Luteolin 7- *O*- glucoside (Cynaroside) | Aerial parts (in flowering stage) | MeOH | HPLC UV/MS; HPLC-DAD | 280 | (Venskutonis et al., 2008) |
| | | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | | EtOAc | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) * |
| - | Luteolin 7-*O*-glucuronide isomer | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| - | Luteolin malonyl hexoside | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350 | (Granica et al., 2013) |
| - | Luteolin-acetyl-hexoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20 | Catechin | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | | EtOAc fraction | HPLC-DAD-MS; HPLC-PDA-ESI/$Ms^n$ | 280 and 360; 280 | (Correia et al., 2006; Santos et al., 2017*) |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| 21 | Quercetin 3-*O*-rhamnoglucoside (Rutin) | | MeOH | HPLC UV/MS; HPLC DAD | 256; 280 | (Kubínová et al., 2012*; Lee et al., 2010[a]) |
| | | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | Lyophilized water infusion | | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018) * |
| 22 | Quercetin 3-*O*-galactoside (Hyperoside) | Aerial parts (in flowering stage) | MeOH | HPLC UV/ MS; HPLC-DAD | 280 | (Kubínová et al., 2012*; Venskutonis et al., 2008) |
| | | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | | EtOAc fraction | HPLC-DAD-MS | 280 and 360 | (Correia et al., 2006) * |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)* |
| 23 | Quercetin 3-*O*-rhamnoside (Quercitrin) | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Stems | MeOH | HPLC UV/MS | 256 | (Lee et al., 2010) |
| | | Seeds | MeOH | UV; FABMS; $^{1}H$ and $^{13}C$ NMR | n/s | (Tomlinson et al., 2003)° |
| 24 | Quercetin 3-*O*-glucoside (Isoquercitrin) | Stems, aerial parts (in flowering stage) | MeOH | HPLC UV/MS; HPLC-DAD | 256; 280 | (Kubínová et al., 2012*; Lee et al., 2010[a]) |
| | | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015)* |
| | | | EtOAc fraction | HPLC-DAD-MS; HPLC-PDA-ESI/$Ms^n$ | 280 and 360; 280 | (Correia et al., 2006; Santos et al., 2017) * |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| 25 | Quercetin 7-*O*-rhamnoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015)* |
| 26 | Quercetin 3'-*O*-β-D-glucopyranoside | Seeds | MeOH | UV; FABMS; $^{1}H$ and $^{13}C$ NMR | n/s | (Tomlinson et al., 2003)° |
| 27 | Quercetin 6"-*O*- galloyl-3-3-*O*-glucoside | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) * |
| - | Quercetin Rhamnoglucoside isomer | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| - | Quercetin-*O*- malonyl hexoside | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350 | (Granica et al., 2013) |
| | | | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| - | Quercetin malonylhexoside isomer | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| - | Quercetin *O*-galloyl-hexoside | Aerial parts | EtOAc fraction | HPLC-PDA-ESI/Ms$^n$ | 280 | (Santos et al., 2017) |
| - | Quercetin acetyl glucoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| - | Quercetin -acetyl-hexoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| 28 | Kaempferide | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) |
| 29 | Kaempferide 3- rhamnoside | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) ° |
| 30 | Kaempferol | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) |
| 31 | Kaempferol 3-rhaminoside | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) |
| 32 | Kaempferol 3-rutinoside | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) |
| 33 | Kaempferol 3-*O*-glucoside | Stems | MeOH | HPLC UV/MS | 256 | (Lee et al., 2010) [a] |
| | (Astragalin) | Aerial parts | MeOH | $^1$H $^{13}$C NMR | 200 MHz | (Bilia et al., 1993) |
| | | | Aqueous | HPLC-DAD-MS$^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) * |
| | | | EtOAc fraction | HPLC-DAD-MS$^3$ | 280 and 360 and 280 | (Correia et al., 2006; Santos et al., 2017) * |
| 34 | Kaempferol 3-*O*-*β*-D-(2''-*O*-acetyl) glucopyranoside | Stems | MeOH | HPLC UV/MS | 256 | (Lee et al., 2010)[a] |
| 35 | Kaempferol 3-*O*-*β*-D (2'' -*O*-acetyl''– E)-*p*-coumaroyl- glucopyranoside (2'' -acetyl-tiliroside) | Stems | MeOH | HMBC; $^1$H $^{13}$C NMR | n/s | (Lee et al., 2010) ° |
| 36 | Kaempferol 3-*O*-(6''-*O*-*p*-coumaroyl)-glucoside | Aerial parts | EtOAc fraction | HPLC-DAD-MS | 280 and 360 | (Correia et al., 2006) * |
| - | Kaempferol *O*-*p*-coumaroyl-glucoside (tiliroside) | Aerial parts | MeOH | HPLC UV/MS | 256 | (Lee et al., 2010)[a] |
| | | | EtOAc fraction | HPLC-PDA-ESI/Ms$^n$ | 280 | (Santos et al., 2017) |

| | | | | | | |
|---|---|---|---|---|---|---|
| - | Kaempferol malonylhexoside | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| - | Kaempferol *O*- malonylhexoside | Aerial parts | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| - | Kaempferide *O*-rhamnoside | Aerial parts | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| - | Kaempferol *O*-acetyl-hexosyl-*O*-rhamnoside | Aerial parts | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| | | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |
| - | Kaempferol *p*-coumaroyl hexoside | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| - | Dimethylated flavonoid | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[b] |
| | | | ***CONDENSED TANNIS*** | | | |
| 38 | Procyanidin B1 | Aerial parts | EtOAc fraction | HPLC/DMACA | 280 and 640 | (Correia et al., 2006) * |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) * |
| 39 | Procyanidin B2 | Aerial parts | EtOAc fraction | HPLC/DMACA | 280 and 640 | (Correia et al., 2006) * |
| 40 | Procyanidin B3 | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) * |
| | | | EtOAc fraction | HPLC-DAD-$MS^3$ | 280 and 360 | (Correia et al., 2006) * |
| | | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| 41 | Procyanidin B6 | Aerial parts | EtOAc fraction | HPLC/DMACA | 280 and 640 | (Correia et al., 2006)# |

| | | | | | | |
|---|---|---|---|---|---|---|
| 42 | Procyanidin B7 | Aerial parts | EtOAc fraction | HPLC/DMACA | 280 and 640 | (Correia et al., 2006)[#] |
| - | Procyanidin dimer | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 254 and 350 | (Granica et al., 2015) |
| 43 | Procyanidin C1 | Aerial parts | EtOAc fraction | HPLC/DMACA/ HPLC-PDA-ESI/$Ms^n$ | 280 and 640; 280 | (Correia et al., 2006; Santos et al., 2017) |
| 44 | Procyanidin C2 | Aerial parts | EtOAc fraction | HPLC/DMACA | 280 and 640 | (Correia et al., 2006) |
| - | Procyanidin trimer | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350; 254 and 350 | (Granica et al., 2013, 2015) |
| | | | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| - | Procyanidin trimer B | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| - | Procyanidin tetramer | Aerial parts | EtOAc fraction | HPLC/DMACA ; HPLC-PDA-ESI/$Ms^n$ | 280 and 640; 280 | (Correia et al., 2006; Santos et al., 2017) |
| - | Procyanidin tetramer B | Lyophilized water infusion | Aqueous | HPLC-DAD-MS | 350 and 330 | (Kuczmannová et al., 2015) |
| | | | ***HYDROLYSABLE TANNINS*** | | | |
| 37 | Agrimoniin | Aerial parts | Aqueous | HPLC-DAD-$MS^3$ | 280 and 350 | (Granica et al., 2013)[+] |
| | | | | | 254 and 350 | (Granica et al., 2015) * |
| | | | EtOAc fraction | HPLC-PDA-ESI/$Ms^n$ | 280 | (Santos et al., 2017) |
| 45 | Pendunculagin | Whole plant in blooming stage | MeOH | ULPC $MS^2$ | 280 | (Pukalskienė et al., 2018)[a,b] |

† Values expressed as nm unless specified otherwise, * confirmed by commercial reference compounds, [#] confirmed by elution characteristics, n/s, non-specified, [°] confirmed by NMR, [+] compound isolated from investigated plant material, [a] confirmed by comparing with data in the literature, [b] confirmed by parent ion mass using free chemical databases (ChemSpider, MetFusion)

## 4.3 Biological and Pharmacological Activity

According to the data collected with the phytochemical screening and assays, it is evident that this plant material contains significant amounts polyphenolic compounds which are represented by a wide variety of compounds and which should be considered as potentially bioactive constituents of *A. eupatoria*.

In Table IV.5, we summarized methodologic details of studies that characterized the antioxidant activity of *A. eupatoria*. Moreover, in Table IV.6 we summarized the methodologic details of studies assessing pharmacologic activity of this plant species.

### 4.3.1 Antioxidant activity

Radical and oxidant species scavenging activity

The antioxidant potential of extracts of *A. eupatoria* has been explored in several studies, with methods that assess the scavenging activity being the most used (Table IV.5).

Over the years, most studies have used methods based on synthetic radicals, namely DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic) acid) radical decolorization assay and ferric reducing antioxidant power assay (FRAP) (Copland et al. 2003; Ivanova et al. 2005; Correia et al. 2006; Venskutonis et al. 2007; Gião et al. 2009; Pukalskienė et al. 2018).

The antioxidant potential was also confirmed through cell-based assays, specifically hydroxyl radical (Correia et al. 2007; Kubínová et al. 2016; Santos et al. 2017) and superoxide anion (Correia et al. 2007; Santos et al. 2017) scavenging assays. Furthermore, Correia et al. (2007) also performed peroxyl radical, peroxynitrite, hydrogen peroxide and hypochlorous acid scavenging assays and showed that the polyphenol-enriched fraction (ethyl acetate fraction) was more efficient in comparison with the hydroalcoholic extract, thus suggesting that the anti-inflammatory activity of *A. eupatoria* may result from the significant scavenging activity of reactive species by polyphenols.

Reducing power and lipid peroxidation inhibition

In addition to scavenging activity assays, reducing power assays which evaluate the electron-donating ability were also used by Muruzović et al. (2016), who showed that extracts from the aerial parts (in the flowering stage) of *A. eupatoria* exhibited a moderate reducing power, compared to the control (ascorbic acid). Furthermore, the authors found that this activity depended on the concentration and varied according to the extract, as the acetone extract was the most active (absorbance from 0.25 to 2.27), followed by the ethanolic (absorbance from 0.09 to 1.62), the aqueous (absorbance from 0.12 to 0.95) and then the diethyl ether (absorbance from 0.05 to 0.51) extracts, in concentrations in the range 7.8–250 μg/mL. Furthermore, the authors found a linear correlation between the phenolic compound content (total phenolic, flavonoids, tannins and proanthocyanidins) and the reducing power.

In addition to the evidence on the ability to scavenge peroxyl radicals (Correia et al. 2007; Kubínová et al. 2016), Correia et al. (2006) has evaluated lipid peroxidation inhibition using the thiobarbituric acid reactive substances (TBARS) method and showed a lower $IP_{50}$ (μg freeze-dried samples required to inhibit 50% of the induced lipid peroxidation) for the ethyl acetate fraction compared to the hydroalcoholic extract (2.12 μg vs 15.08 μg, respectively).

Other assays

The ability of the extract of *A. eupatoria* to induce the endogenous antioxidant system has also been shown in experimental models of metabolic syndrome (Kiselova 2011) and in oxidant-challenged 3T3-L1 pre-adipocyte cells (Ivanova et al. 2011a). In both studies, an anti-inflammatory effect on lipopolysaccharide-induced nitric oxide (NO) and proinflammatory cytokines production was also described, which will be discussed further below.

In addition to the ABTS assay, Kuczmannová et al. (2015), who assayed an aqueous extract of *A. eupatoria*, also conducted assays on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the human monocytic cell line THP-1 as well as on oxidative damage of plasmid DNA. Moreover, the extract was also assayed *in vivo* using the skin flap viability model, followed by the analysis of survived area on the skin flap and histological examination. The results suggested that the extract was able to protect cells from oxidative damage, acting as a hydroxyl radical scavenger.

**Table IV.5**. Antioxidant potential of *A. eupatoria* based on scavenging activity assays.

| Plant part | Extract / fraction | Concentration | Synthetic radicals | | Cellular radicals | | | Oxidant species-based assays | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DPPH | ABTS | $O_2^-$ | $HO^-$ | Peroxyl | $ONOO^-$ | $H_2O_2$ | HOCl | ORAC | |
| Aerial parts | Aqueous | n/s | – | 3.76±0.05 mM [c] | – | – | – | – | – | – | - | (Ivanova et al., 2005) |
| | | n/s | 13.30±0.3 µg/mL [a] | – | – | 30.3±4.3 µg/mL[d] | 30.7±0.7 µg/mL[d] | – | 29.0±0.3 µg/mL[d] | – | - | (Kubínová et al., 2016) |
| | | n/s | 12.80±0.05 µg/mL [a] | – | 13.59±1.03 µg/mL[d] | 126.99±11.97 µg/mL[d] | – | – | – | – | - | (Santos et al., 2017) |
| | Hydroalcoholic | n/s | 18.12 µg/mL [a] | 0.196 µg [c] | – | – | – | – | – | – | - | (Correia et al., 2006) |
| | | DPPH: 25, 50, 75 µg<br>HOCl: 25, 37.5, 50 µg | 20.00 µg/mL [a] | – | 37.14 µg [e] | 24.30 µg [e] | 50 µg | 15.92 µg [f] | 242.76 µg [g] | 72.46±9.06% – 94.34±2.52% [h] | - | (Correia et al., 2007) |
| | EtOAc fraction | n/s | 9.80 µg/mL [a] | 0.608 µg [c] | – | – | – | – | – | – | - | (Correia et al., 2006) |
| | | DPPH & HOCl: 10, 20, 30 µg | 16.00 µg/mL [a] | – | 13.65 µg[e] | 16.00 µg [e] | 10 µg | 3.00 µg [f] | 79.28 µg [g] | 27.81±0.60% – 83.59±5.66% [h] | - | (Correia et al., 2007) |
| | | n/s | 4.60±0.05 µg/mL [a] | – | 3.34±0.20 µg/mL[d] | 90.97±8.29 µg/mL[d] | – | – | – | – | - | (Santos et al., 2017) |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aerial parts (in flowering stage) | Aqueous | 1 mg/mL | 78.0±3.3% | – | – | – | – | – | – | – | - | (Kubínová et al., 2012) |
| | | 7.8-250 µg/mL | 21.9±0.22% – 93.95±0.27% | – | – | – | – | – | – | – | - | (Muruzović et al., 2016) |
| | MeOH | 2 mg/mL | 63.9±0.9% | – | – | – | – | – | – | – | - | (Kubínová et al., 2012) |
| | EtOH | 7.8-250 µg/mL | 19.4±0.43% – 94.88±0.14% | – | – | – | – | – | – | – | - | (Muruzović et al., 2016) |
| | Acetone | | 27.73±0.36% – 97.13±0.26% | – | – | – | – | – | – | – | - | |
| | Diethyl ether | | 3.14±0.50% – 53.1±0.40% | – | – | – | – | – | – | – | - | |
| Leaves | Water | 0.5 mg/mL | 3.9 µg/mL[a] | | | | | | | | | (Gião et al., 2012) |
| Ground parts | MeOH | n/s | 47.2±0.5 [b] | 48.5±2.9 [b] | – | – | – | – | – | – | - | (Venskutonis et al., 2007) |
| | Aqueous | n/s | 65.0±1.4 [b] | 46.4±0.7 [b] | – | – | – | – | – | – | – | |
| | Acetone-hexane | n/s | 9.1±0.8 [b] | 6.7±0.5 [b] | – | – | – | – | – | – | – | |
| | Acetone-t-butylmethylether | n/s | 39.2±2.2 [b] | 67.1±3.2 [b] | – | – | – | – | – | – | – | |
| | Acetone-n-BuOH | n/s | 91.6±0.1 [b] | 58.2±0.4 [b] | – | – | – | – | – | – | – | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acetone-water | n/s | 90.4±0.6 [b] | 79.5±2.8 [b] | – | – | – | – | – | – | – | |
| Seeds | MeOH | n/s | 4.64x10$^{-4}$ mg/mL [i] | – | – | – | – | – | – | – | – | (Copland et al., 2003) |
| | Sep-Pak 30% fraction | n/s | 5.13x10$^{-3}$ mg/mL [i] | – | – | – | – | – | – | – | – | |
| | Sep-Pak 60% fraction | n/s | 4.73x10$^{-4}$ mg/mL [i] | – | – | – | – | – | – | – | – | |
| Whole plant in blooming stage | MeOH | n/s | 0.26±0.01 [j] | 0.48±0.01 [j] | – | – | – | – | – | – | 2.52±0.32 [j] | (Pukalskienė et al., 2018) |
| Lyophilized water infusion material | Aqueous | n/s | - | 0.79 mg/mL | - | - | - | - | - | - | - | (Kuczmannová et al., 2015) |

[a] Results expressed as EC50 (% or µg/mL).
[b] Units and method of calculation not specified.
[c] Results expressed as Trolox equivalent antioxidant capacity (TEAC).
[d] Results expressed as the amount of sample (µg/mL of reaction mixture) that decreased 50% of the absorbance values as compared to control ($EC_{50}$).
[e] Results represent the dry weight necessary to protect 50% of the deoxyribose degradation ($I_{50}$).
[f] Results represent the dry weight necessary to inhibit 50% of the dihydrorhodamine 123 oxidation ($I_{50}$).
[g] Results represent the dry weight necessary to scavenge 50% of the initial hydrogen peroxide concentration ($I_{50}$).
[h] Results represent the percentage of inhibition of elastase activity.
[i] Results expressed as $RC_{50}$.
[j] results expressed as millimoles of Trolox equivalents (TE) per gram of dry weight of extract (mmol/ TE/g DWE).
Abbreviations: $H_2O_2$, hydrogen peroxide; $HO^{\cdot}$, hydroxyl radical; HOCl, hypochlorous acid; MeOH, methanol; n/s, non-specified; $O_2^{\cdot-}$, superoxide anion; $ONOO^-$, peroxynitrite; TBARs, thiobarbituric acid reactive substance; TEAC, Trolox equivalent antioxidant capacity.

### 4.3.2 Antimicrobial activity

Several studies have shown an antimicrobial activity with different *A. eupatoria* extracts, including antibacterial (Copland et al. 2003; Muruzović et al. 2016; Cardoso et al. 2018), antifungal (Muruzović et al. 2016), antibiofilm (Muruzović et al. 2016) antiviral (Kwon et al. 2005).

Antibacterial

Regarding the bacteriostatic activity, Copland et al. (2003) assayed crude seed extracts of *A. eupatoria* using 96-well microplate-based broth dilution assay and showed that among the tested extracts, the *n*-hexane extract showed inhibitory effect (minimum inhibitory concentration or MIC of 0.75mg/mL) on *Bacillus cereus* and *Bacillus subtilis*. Moreover, the authors also assayed four Sep-Pak fractions eluted with 30, 60, 80 and 100% methanol with the same strains. The 30% methanol fraction inhibited *Bacillus subtilis* with a MIC of 0.50 mg/mL and *Staphylococcus aureus* with a MIC of 0.75 mg/mL. The 60% methanol fraction inhibited *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* with a MIC of 0.50, 0.38 and 0.38 mg/mL respectively. Moreover, the 80% methanol fraction inhibited *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* with a MIC of 0.38, 0.50 and 0.38 mg/mL, respectively.

Dulger and Gonuz (2004) prepared a methanolic extract of mature plants and their parts and assayed the antimicrobial activity with an antibiogram which included nine species (Table IV.6). Although the extract showed great activity with all species having 8–16 mm of zone of inhibition, a higher susceptibility was observed for *Staphylococcus aureus* in comparison to standard antimicrobials: penicillin, ampicillin, cefotaxime, vancomycin, ofloxacin, tetracycline and nystatin.

Cwikla et al. (2010) tested an ethanolic extract of *A. eupatoria in vitro* for its activity against *Helicobacter pylori* through the micro-dilution assay and showed an anti-*Helicobacter pylori* effect of 97±5% of inhibition. Recently, Cardoso et al. (2018) confirmed this finding with a hydroalcoholic extract of leaves and stems by showing a bacteriostatic activity against *Helicobacter pylori* independently of the pattern of virulence and susceptibility for the two highest concentrations of extract, 50 and 75 mg/mL.

An antimicrobial effect against *Campylobacter jejuni* has also been investigated, even though the results were not consistent. On one hand, Cwikla et al. (2010) showed

a 96±30% of inhibition with the crude extract of *A. eupatoria*. On the other hand, Bensch et al. (2011) showed no antibacterial activity against this species.

In a study with 18 bacterial strains (including probiotic strains, standard strains and clinical isolates), Muruzović et al. (2016) compared four types of extracts of the aerial parts in the flowering station (ethanolic, diethyl ether, aqueous and acetone) with ampicillin (a β-lactam antibiotic that targets $G^+$ species and some $G^-$ bacteria) and tetracycline (a broad-spectrum antibiotic effective against aerobic and anaerobic $G^+$ and $G^-$ bacteria). The intensity of the antibacterial activity depended on the type of extract (acetone > aqueous > ethanolic > diethyl ether) and bacterial species (a stronger activity was detected on $G^+$ bacteria and probiotics in comparison with $G^-$ bacteria). Furthermore, *Escherichia coli*, *Salmonella enterica* and *Salmonella typhimurium* showed resistance to the extracts (> 20 mg/mL), whereas *Enterococcus faecalis* showed resistance to all extracts except for the acetone extract (10 mg/mL MIC and MMC). Muruzović et al. (2016) also showed an inhibitory effect of the acetone extract on *Bacillus cereus* and *Bacillus subtilis* (MIC of 0.315 mg/mL), which confirms previous findings from Copland et al. (2003) with the *n*-hexane seed extract (MIC of 0.75 mg/mL).

Komiazyk et al. (2019) assayed the antibacterial activity against the cholera toxin with a microdilution assay. A modest bacteriostatic activity was found, but *A. eupatoria* extract suppressed the binding of subunit B of cholera toxin to the surface of the cell and immobilized ganglioside $GM_1$.

According to Correia et al. (2006) and Cardoso et al. (2018) the antibacterial activity may be explained by the high polyphenolic content. An attempt to identify the specific compounds has been carried out by Ginovyan et al. (2020), who suggested that complex compounds may be responsible for this bioactivity. Interestingly, kaempferol and its glycosides, which have been identified in *A. eupatoria* (Bilia et al. 1993; Correia et al. 2006; Lee et al. 2010; Granica et al. 2013; Granica et al. 2015; Santos et al. 2017; Pukalskienė et al. 2018), display antibacterial activity and may act synergistically with antibiotics such as rifampicin, vancomycin, methicillin, erythromycin and clindamycin against antibiotic-resistant bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *Bacilus subtilis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Helicobacter pylori*, *Clostridium perfringens*, *Vibrio cholerae* and *Propionibacterium acnes* (Calderón-Montaño et al. 2011).

Moreover, *p*-coumaric acid may also play a role, as this compound exhibits slight antimicrobial activity against some species of $G^+$ and $G^-$ bacteria (Pei et al. 2016).

Antifungal

Regarding antifungal activity, the acetone extract of aerial parts in the flowering stage (tested in the range of 0.156 mg/mL to 20 mg/mL) displayed the highest activity in comparison with other extracts (i.e. aqueous, ethanolic, diethyl ether). As a low sensitivity was evidenced by the examined fungal species (Table IV.6), higher activity was observed on yeasts compared to filamentous fungi (Muruzović et al. 2016).

These findings on antifungal activity confirmed previous findings from Dulger and Gonuz (2004), who assayed a methanolic extract of the mature plants and its parts against three fungal species: *Candida albicans*, *Rhodotorula rubra* and *Kluyveromyces fragilis*. The extract showed activity against the three species, displaying an inhibition zone of 10–14 mm.

Of note, both reports assayed a particular species of fungi: *Candida albicans*. While Muruzović et al. (2016) showed no activity, Dulger and Gonuz (2004) showed a potential effect. The difference in results can be attributed to the doses that were used (0.156–20 mg/mL vs 200 mg/mL, respectively) but also to the solvents and parts of the plants used to extract the compounds of the plant.

Antibiofilm

As shown by Muruzović et al. (2016), the extract of aerial parts of *A. eupatoria* inhibited biofilm formation, as the acetone extract showed higher activity compared of *Proteus mirabilis* (the concentration required to reduce biofilm coverage by 50% or $BIC_{50}$ of 4.32 mg/mL) and *Pseudomonas aeruginosa* ($BIC_{50}$ of 4.47 mg/mL) compared to the aqueous extract, similarly to the antibacterial and antifungal activity that was discussed above.

Antiviral

The antiviral activity of an infusion of aerial parts of *A. eupatoria* against hepatitis B virus (HBV) has been tested by measuring the inhibition of HBV surface antigen (HbsAg) release in HepG2.2.15 cells in a range of concentration of 44–132 µg/mL (Kwon et al. 2005). In this study, the authors reported an inhibition of HBsAg release, which

varied according to the growing season (highest in mid-July) and was higher at a temperature of extract preparation of 60°C (compared to 37°C, 45°C or 55°C).

### 4.3.3 Antidiabetic activity

One of the major ethnomedicinal applications of *A. eupatoria* is its use as antidiabetic. This is supported by several studies using a variety of assays that measure multiple parameters, from glucose formation and absorption, to insulin secretion and also diabetes-related complications.

In regard to glucose formation and absorption, inhibitors of *α*-glucosidase (enzyme involved in the breakdown of oligo- and polysaccharides into glucose) have been developed as oral antihyperglycemic drugs aiming at decreasing postprandial hyperglycemia (Hedrington and Davis 2019). In fact, aqueous and methanolic extracts of *A. eupatoria* have been shown to inhibit α-glucosidase activity (Kubínová et al. 2012; Kuczmannová et al. 2016). Among extracts from five *Agrimonia* species, Kubínová et al. (2012) showed that the methanolic extract of *A. eupatoria* displays the highest inhibition of *α*-glucosidase, 94.2±1.7%. An inhibition of *α*-glucosidase activity ($IC_{50}$ of 46.31±8.76 µg/mL) has also been demonstrated by Kuczmannová et al. (2016), who also showed that an aqueous extract of *A. eupatoria* elicited an inhibition of advanced glycation end-products (AGEs) formation ($IC_{50}$ of 156.48±70.75 µg/mL). Also, *A. eupatoria* has been shown to decrease glucose diffusion across the gastrointestinal tract *in vitro* by more than 50% (Gallagher et al. 2003).

Using streptozotocin-induced diabetes mellitus rat models, studies have shown that a decoction of *A. eupatoria* leaves reduces diabetes-associated weight loss, polydipsia and hyperphagia (Swanston-Flatt et al. 1990; Gray and Flatt 1998). Moreover, Gray and Flatt (1998) also showed that the aqueous extract of *A. eupatoria* may display an "insulin-like" activity through three main mechanisms, i.e. glucose oxidation, stimulation of 2-deoxy-glucose transport and incorporation of glucose into glycogen, as well as promote glucose-independent insulin secretion in BRIN-BD11 pancreatic *β*-cells *in vitro*. Interestingly, the ability to evoke insulin secretion was dependent on the use of heat during extraction (Gray and Flatt 1998).

In contrast, Kuczmannová et al. (2016) showed that the daily administration of *A. eupatoria* infusion of aerial parts did not elicit an anti-hyperglycemic effect, as no

significant effect was observed on serum glucose levels and body weight. However, the authors attributed these findings to a lower concentration of infusion (0.2 mg/mL).

Two aqueous extracts (a decoction and an infusion, respectively) of aerial parts from *A. eupatoria* have also been investigated for their anticholinesterase activity (Kubínová et al. 2016; Kuczmannová et al. 2016), as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors can be useful in early-onset type 2 diabetes mellitus (Rao et al. 2007). Of note is that such activity may also hold potential in the dementia phase of Alzheimer's disease (Shah et al. 2017; Dos Santos et al. 2018). Kubínová et al. (2016) showed that extracts of *A. eupatoria* inhibited AChE and BuChE activity at a concentration of 100 μg/mL, even though to a lesser extent compared to galantamine control (AChE inhibitor in clinical use). The modulation of BuChE activity by an infusion of aerial parts of *A. eupatoria* has also been tested by Kuczmannová et al. (2016), who showed that the animals treated with plant extracts displayed higher BuChE activity compared to untreated diabetic animals and even lower compared to healthy (control) animals.

In general, these findings support an antidiabetic activity of *A. eupatoria*. So far, the specific compounds responsible for this activity are unknown, even though the contribution of flavonoids has been suggested (Kubínová et al. 2012).

### 4.3.4 Anti-tumor activity

The anti-tumor activity has been evaluated by Ad'hiah et al. (2013) using two cell lines: human cervical cancer (HeLa) and rhabdomyosarcoma (RD cell line). A primary cell culture of mouse embryo fibroblasts (MEF) was also used as control. The methanolic and aqueous extracts showed anti-tumor properties in a concentration-dependent manner, even though the methanolic extract displayed better activity in terms of percentage of growth inhibition compared to the aqueous extract in the three incubations periods (24, 48 and 72 h). A lower level of activity was found in MEF cells. Concerning the concentrations, 96.0 μg/ml of the extract was the most effective in producing a percentage of growth inhibition.

### 4.3.5 Vascular activity

The vascular activity of the aqueous extract of aerial parts has been studied by Kuczmannová et al. (2016), who showed improved acetylcholine-induced vasorelaxation in isolated aortic rings from diabetic animals treated with *A. eupatoria*, compared with untreated diabetic animals but not with healthy controls. Furthermore, it was suggested that the high content of polyphenols plays a role in this activity.

Whether the improved vasorelaxation to acetylcholine was related to the antidiabetic effects of *A. eupatoria* or related to a direct effect on the vasculature remains to be confirmed. As the compounds responsible for the vascular effects of this plant species are still not known, several compounds found in *A. eupatoria* (either isolated from other plant species or as standards) have been reported as exerting direct vascular effects.

For instance, (-)-epicatechin has been shown to elicit endothelium-dependent vasorelaxation of rat mesenteric arteries (Huang et al. 1999), primarily through NO and partially by NO-dependent activation of iberiotoxin-sensitive $K^+$ channels, in addition to endothelium-independent vasorelaxation. In human internal mammary arteries, (-)-epicatechin elicits a strong endothelium-independent vasorelaxation which may be mediated by smooth muscle $K_V$, $BK_{Ca}$ and $K_{ATP}$ channels (Novakovic et al. 2015). Moreover, this compound could inhibit the influx of extracellular $Ca^{2+}$ and interfere with intracellular $Ca^{2+}$ release and uptake to the sarcoplasmic reticulum (Novakovic et al. 2015).

Procyanidins have also been reported as eliciting endothelium-dependent vasorelaxation, namely procyanidin B2 (Novakovic et al. 2017) on human internal mammary arteries (mediated primarily by NO production and partially by prostacyclin and $K^+$ channels) and procyanidin C1 (Byun et al. 2014) on rat aortas (mediated by NO production and partially by $K^+$ channel activation).

Vitexin and isovitexin have been reported to elicit several protective effects in the cardiovascular system (He et al. 2016). In the vasculature, vitexin elicited vasorelaxation following phorbol ester-induced vascular contraction (but not thromboxane A2 or fluoride-induced contraction), through MEK inhibition and subsequent ERK1/2 phosphorylation (Je et al. 2014).

### 4.3.6 Antinociceptive activity

Previous studies have tested the antinociceptive potential of *A. eupatoria* extracts. Lee and Rhee (2016) evaluated the anti-nociceptive effect of a hydroalcoholic extract of the aerial parts of *A. eupatoria* (200 mg/kg) on a cisplatin-induced neuropathic pain rat model, using pin prick, plantar, paw-pressure and chemical tests. Compared to gabapentin (positive control), the extract of *A. eupatoria* elicited a lower paw withdrawal duration time and threshold in mechanical hyperalgesia tests. Also, the extract displayed a higher potential to prevent thermal hyperalgesia and to repair neuropathic pain due to cisplatin.

Santos et al. (2017) showed no analgesic activity for the extract in the hot plate test (which evaluates central analgesia). However, the authors found an inhibition of acetic acid-induced abdominal writhing (test of peripheral analgesia) for both the infusion and the ethyl acetate fraction. Furthermore, a decrease in time spent licking on the late phase of a formalin test was observed with the ethyl acetate fraction, suggesting an antinociceptive effect on inflammatory pain. Based on these findings, the fact that this analgesic effect could be related to an anti-inflammatory activity may be hypothesized, as further discussed below.

### 4.3.7 Anti-inflammatory activity

The anti-inflammatory potential of the infusion of aerial parts of *A. eupatoria* has been previously demonstrated (Santos et al. 2017; Kiselova et al. 2011). Using *in vitro* testing with lipopolysaccharide (LPS)-stimulated macrophages, Santos et al. (2017) showed that both the infusion of aerial parts (193–770 μg/mL) and the polyphenolic fraction (69–276 μg/mL) decreased NO production compared to LPS control (11.34% vs 22.46%, respectively) in noncytotoxic concentrations (382 μg/mL and 138 μg/mL, respectively). In the same study, the authors confirmed these findings *in vivo*, as a significant decrease in carrageenan-induced paw oedema was observed 4 h after infusion (43.2% to 52.2% decrease) and polyphenolic fraction administration (34.6% to 35.4% decrease).

An *in vivo* anti-inflammatory effect was previously reported by (Kiselova et al. 2011), who showed a decreased monocyte chemoattractant protein-1 (MCP-1) expression in extract-treated animals, in addition to the antioxidant effect that has been discussed above. *In vitro*, (Bae et al. 2010) also showed in LPS-induced BV2 microglial cells that *Agrimonia herba* inhibited the release of NO and proinflammatory cytokines (TNF-α, IL-1β and IL-6) and decreased inducible NO synthase expression.

Together, these findings suggest an anti-inflammatory effect of *A. eupatoria* which may be useful in metabolic syndrome (Kiselova et al. 2011) or brain inflammation (Bae et al. 2010). Also, the polyphenolic compounds found in this plant species may be responsible for such activity (Santos et al. 2017), even though this assumption remains to be confirmed.

### 4.3.8 Hepatoprotective properties

Due to the anti-inflammatory and antioxidant properties of *A. eupatoria*, (Yoon et al. (2012) investigated the hepatoprotective potential of this plant species, assaying an aerial parts extract on chronic ethanol-induced liver injury in rats, utilizing the concentrations (10, 30, 100 and 300 mg/kg/day). In this study, the authors showed an attenuation of the several effects of chronic ethanol consumption, namely: increased serum aminotransferase activity, increased proinflammatory cytokines, increased activity of cytochrome P450 2E1, increased lipid peroxidation, decreased concentration of reduced glutathione, increased levels of toll-like receptor 4 (TLR4), increased expression of myeloid differentiation factor 88 and inducible NO synthase and cyclooxygenase-2, as well as nuclear translocation of NF-κB (Yoon et al. 2012). In general, the authors suggested that the hepatoprotective effect is likely to result from antioxidant activity and from TLR-mediated inflammatory signaling.

### 4.3.9 Neuroprotective properties

The neuroprotective potential of *A. eupatoria* has been assayed on glutamate-induced oxidative injury to HT22 hippocampal cells *in vitro* (Lee et al. 2010). In a study that tested the methanolic extract of stems, four fractions (*n*-hexane, ethyl acetate, *n*-

butanol and water) and 10 isolated flavonoids, Lee et al. (2010) showed that the methanolic extract, the ethyl acetate and the *n*-butanol fractions significantly attenuated glutamate-induced oxidative stress (76.2%, 64.5% and 37.1% of reduction, respectively at 75 μg/mL). The authors then proceeded to isolate flavonoids from the extract and test their neuroprotective activity and showed that four isolated compounds – astragalin, isoquercitrin, quercitrin and luteolin 7-*O*-*β*-D-glucuronide – exhibited significant neuroprotective activity, partly due to an antioxidant effect. Further, the authors found that neuroprotective ability differed according to the structural variation of hydroxyl groups and sugar moieties.

### 4.3.10 Cytotoxicity

In this matter, Bensch et al. (2011) showed that the hydroalcoholic extract of *A. eupatoria* (5 mg/mL) displayed activity below 10%, determined by the MTT assay using HT-29 cells.Kuczmannová et al. (2015) evaluated an aqueous extract of *A. eupatoria* on THP-1 cell lines in two concentrations (0.05 and 0.1 mg/mL, after 24 h) and reported that no cytotoxicity was observed. Komiazyk et al. (2019) recently reported an $IC_{50}$ of 5 mg/mL in the MTT assay in primary human fibroblast line cells (C688) for the assayed commercial product. Moreover, Pukalskienė et al. (2018) demonstrated *in vitro* that a methanolic extract from the whole plant in the blooming stage did not induce gene mutations in bacteria and cytogenic damage in mammalian cells.

## 4.4 Clinical evidence

On the basis of the preclinical evidence, two clinical trials have been conducted on the consumption of an infusion of *A. eupatoria* aerial parts.

The first, conducted by Ivanova et al. (2013), included 19 healthy volunteers from Bulgaria, aged 18 to 55 years, and assessed the consumption of the *A. eupatoria* tea (200 mL, prepared from commercial product, Selibum Ltd., Varna, Bulgaria) twice a day at the same day time (9 a.m. and 2 p.m.) for 30 days. Focusing on lipid metabolism, oxidative stress and inflammation in healthy adults, the authors found the following: (a) an increase

in plasmatic total antioxidant capacity; (b) a decrease in IL-6 serum levels, with no significant changes in C-reactive protein or TNF-α; and (c) an increase in total cholesterol and HDL (high density lipoprotein) cholesterol levels, with no significant changes in triglycerides (TGs), LDL (low density lipoprotein) cholesterol or HDL/LDL cholesterol ratio. Based on these findings, the authors proposed that moderate *A. eupatoria* tea consumption may have a protective role in cardiovascular disease, metabolic disorders and diabetes. Some limitations must be highlighted, such as the low number of participants (*N* = 19) and the fact that the participants were all healthy, thus limiting the extrapolation of findings for disease settings.

Recently, Cho et al. (2018) carried out a randomized, double-blind, placebo-controlled trial in subjects with mildly to moderately elevated ALT (alanine transaminase) levels, which focused more on the hepatoprotective potential of the infusion of *A. eupatoria* (administered as two capsules containing either powdered extract (40 mg of extract and 60 mg maltodextrin) or placebo (100 mg maltodextrin), twice a day. After eight weeks, the *A. eupatoria* treated group displayed a significant reduction in ALT, aspartate aminotransferase (AST) and TG serum levels. No changes were observed in alkaline phosphatase, total bilirubin, γ-glutamyltransferase and vital signs (i.e. blood pressure, pulse, body weight and body temperature), as serum levels of total protein, albumin, blood urea nitrogen, creatinine and total cholesterol were normal for both groups. Despite five adverse events being reported (mild hives, common cold, cough or sore throat), no causal relationship was established. In general, this study confirmed the safety of *A. eupatoria* consumption and also its hepatoprotective potential.

An important limitation of the current clinical evidence on the consumption of *A. eupatoria* is the very limited pharmacokinetic data that has been reported, which is recognized as a major constraint in the therapeutic application of natural products. In this context, Gião et al. (2012) have explored the stability and bioavailability of phenolic compounds in an infusion of leaves of *A. eupatoria* and three other plant species, using an *in vitro* digestion Caco-2 cell model which simulated the digestive tract, from mouth to stomach and gut. According to the authors, several antioxidant compounds, particularly flavonoid glycosides, are not affected by the simulated digestive process. However, others (i.e. catechins) display a decrease in their activity throughout the digestive process. Also, rutin showed the ability to be transported across the Caco-2 cell barrier. Of note is that Pei et al. (2016) reported that the bioavailability of *p*-

coumaric acid is higher than chlorogenic acid, caffeic acid and ferulic acid, as this is the only report on the bioavailability of a compound found in *A. eupatoria* (*p*-coumaric acid).

**Table IV.6**. Methodologic details of previous studies that assessed the pharmacological activities of *A. eupatoria* (by plant part and chronological order of publication).

| Plant part | Study | Extract type or fractions | Tested concentrations or doses | Method | Activity |
|---|---|---|---|---|---|
| Aerial parts | (Kwon et al., 2005) | Aqueous | 44–132 µg/mL | Inhibition of hepatitis B virus (HBV) surface antigen (HbsAg) release in HepG2.2.15 cells | Antiviral |
| | (Yoon et al., 2012) | Aqueous | 10,30,100 and 300 mg/kg/ day | Chronic ethanol-induced liver injury in rats | Hepatoprotective |
| | (Ad'hiah et al., 2013) | Aqueous and MeOH | 6, 12, 24, 48 and 96 µg/mL | Incubation with HeLa, RD and MEF cell lines | Anti-tumor |
| | (Kubínová et al., 2016) | Aqueous | 100 µg/mL | Inhibition of acetylcholinesterase and butyrylcholinesterase | Anticholinesterase |
| | (Kuczmannová et al., 2016) | Aqueous | 0.5-1000µg/mL | Inhibition of α-glucosidase activity, inhibition of AGE formation (BSA-glucose assay) | Antidiabetic |
| | | | Infusion of 0.2 mg/mL/day for 5 weeks | Serum glucose levels and body weight (streptozotocin-induced DM rat model) | |
| | | | | Inhibition of butyrylcholinesterase (Ellman method) | Anticholinesterase |
| | | | | Modulatory effect on acetylcholine-induced vasorelaxation in isolated aortic rings | Vascular |

| | | | | | |
|---|---|---|---|---|---|
| | (Lee and Rhee, 2016) | Hydroalcoholic | 200 mg/kg | Pin-prick, plantar, paw-pressure and chemical tests in cisplatin-induced neuropathic pain model | Anti-nociceptive |
| | (Santos et al., 2017) | Aqueous and EtOAc fraction | Infusion: 193–770 µg/mL<br>Fraction: 69–276 µg/mL | Cell viability by MTT assay; Nitrite production LPS-stimulated macrophages | Cytotoxicity, Anti-inflammatory (*in vitro*) |
| | | | Infusion: 99.59 and 199.18 mg/kg<br>Fraction: 18.12 and 36.24 mg/kg | Carrageenan-induced paw-edema | Ant-inflammatory (*in vivo*) |
| | | | Infusion: 199.18 and 398.26 mg/kg<br>Fraction: 36.24 and 72.48 mg/kg | Peripheral analgesic potential by the acetic acid-induced writhing assay; central analgesic potential by hot-plate test; Formalin assay | Anti-nociceptive |
| | (Cardoso et al., 2018) | Hydroalcoholic | 75, 50, 25 and 5 mg/mL | Agar diffusion method with 12 clinical isolates of *Helicobacter pylori* | Antibacterial |
| | (Komiazyk et al., 2019) | Aqueous | 2.5-0.078 mg/mL; 2.5 mg/mL; 2.5-0.075 mg/mL; 25 mg; 5.0, 2.5 or 1.25 mg/mL and 1-0.0015 mg. | Microdilution [a]; cAMP; ganglioside $GM_1$-CTX biding; $GM_1$ and CTB-FICT binding; fluorescent activated cell sorting. Fluorescent microscopy and SDS-page assay | Antibacterial |
| Leaves | (Gray and Flatt, 1998) | Aqueous | 0.25, 0.5, 1, 5, 10 mg/mL | Dietary administration of agrimony on (STZ)-diabetic mice | Anti-diabetic |
| | (Gião et al., 2012) | Aqueous | 100 ml | Simulated digestive tract, mouth digestion, stomach digestion, gut digestion | Simulated digestion |

| | | | | | |
|---|---|---|---|---|---|
| Aerial parts (in flowering stage) | (Kubínová et al., 2012) | Aqueous, MeOH | n/s | Inhibition of α-glucosidase activity | Antidiabetic |
| | (Muruzović et al., 2016) | EtOH, diethyl ether, aqueous and acetone | 0.156 mg/mL to 20 mg/mL | Microdilution method with 18 bacterial strains [b] and 6 fungal strains [c] | Antibacterial & Antifungal |
| | | Aqueous and acetone | Serial dilutions from 5 mg/mL to 39.06 μg/mL | Crystal violet assay with *Proteus mirabilis* and *Pseudomonas aeruginosa* | Antibiofilm |
| Stems | (Lee et al., 2010) | MeOH, 4 fractions (*n*-hexane, EtOAc, *n*-BuOH, aqueous) and 10 isolated compounds | MeOH extract and fractions: 75 μg/mL<br>Isolated compounds: 10 and 100 μM | MTT assay with glutamate-induced oxidative stress in HT22 hippocampal cells | Neuroprotective |
| Seeds | (Copland et al., 2003) | *n*-Hexane, dichloromethane, MeOH and Sep-Pak Fractions (30, 60, 80 and 100%) | n/s | Broth dilution assay with 6 bacterial species [d] | Antibacterial |
| Lyophilized water infusion material | (Kuczmannová et al., 2015) | Aqueous | 0.1 mg/mL; 0.1 or 0.05 mg/mL | CAT and SOD activity and expression; oxidative damage of plasmid DNA; skin flap viability model (*in vivo*) | Antioxidant |

| | | | | | |
|---|---|---|---|---|---|
| Mature plants and their parts | (Dulger and Gonuz, 2004) | MeOH | 200 mg/mL | Strains of bacteriaand fungi [e] | Antimicrobial |
| Dried material | (Gallagher et al., 2003) | Aqueous | 50g/L | Sealed dialysis tube | Anti-diabetic |
| | (Ivanova et al., 2011a) | Ethanol/PBS 40% | 20,50, 100, 150, 200, 250 and 300 µl | Cytotoxic assay with 3T3-L1 pre-adipocytes | Expression of glutamate-cysteine ligase and glutathione peroxidase |
| *Agrimoniae Herba* | (Bae et al., 2010) | Aqueous | 0.01, 0.1 and 1 mg/mL | BV2 microglial cells pre-treated with *Agrimoniae Herba* for 30 minutes before the addition of 1µg liposaccharide for 24 hours | Anti-inflammatory (*in vitro*) |
| Herbal extract | (Cwikla et al., 2010) | Ethanol | 1:25-1:2000 | Microdilution test with 2 bacterial strains [f] | Antibacterial |
| | (Bensch et al., 2011) | Ethanol | 1:50 | Antiadhesive and cytotoxic (HT-29 cells) and bacterial assays [g] | Antibacterial |
| Whole plant | (Ginovyan et al., 2020) | MeOH and acetone | 200 mg/mL | Bioautographic assay with *S. aureus* [h] | Antibacterial |
| Whole plant in blooming stage | (Pukalskienė et al., 2018) | MeOH | 50-250 µg/mL | Comet assay; cytokinesis-block micronucleus assay; Ames test [i] | Genotoxicity and antioxidant |

[a]*Escherichia coli* ATCC25922, *Escherichia coli* O44 (834/04, collection of National Medicine Institute, Warsaw, Poland), *Vibrio cholerae* O395-tacCTB strain (Chiron Srl./Novartis) and *Lactobacillus rhamnosus* (ATCC 53103).

[b] Bacterial strains: probiotic strains (*Lactobacillus rhamnosus*, *Bifidobacterium animalis* subsp. *lactis* and *Bacillus subtilis* IP 5832), standard strains (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) and clinical isolates (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella enterica*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*).

[c] Fungal species: *Aspergillus flavus*, *Aspergillus niger*, *Penicillium italicum*, *Penicillium chrysogenum*, *Candida albicans*, and *C. albicans* ATCC 10231.

[d] Bacterial species: *Bacillus cereus* (NCTC 9689), *Bacillus subtilis* (NCTC 7801), *Escherichia coli* (NCTC8110), *Pseudomonas aeruginosa* (NCTC6750), *Salmonella goldcoast* (NCTC 13175) and *Staphylococcus aureus* (NCTC 10788).

Abbreviations: AGE, advanced glycation end-products; HBV, hepatitis B virus; n/s: non-specified.

[e] Bacterial species: *Escherichia coli* ATCC 11230, *Staphylococcus aureus* ATCC 6538P, *Klebsiella* pneumoniae ATCCUC57, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 8427, *Bacillus cereus* ATCC 7064, *Mycobacterium smegmatis* CCM 2067, *Listeria monocytogenes* ATCC 15313 and *Micrococcus luteus* CCM 169Fungal species: *Candida albicans* ATCC 10231, *Rhodotorula rubra* DSM 70403 and *Kluyveromyces fragilis* ATCC 8608.

[f] *Helicobacter pylori* bacterial strain: NCTC 11639 and *Campylobacter jejuni strain*: NCTC 11168

[g] *Campylobacter jejuni* subsp. *jejuni* strain 81116 (NCTC: 11828)

[h] *Staphylococcus aureus* strain: MDC 5233(Microbial Depository Center (MDC) (WDM803)

[i] *Salmonella typhimurium*: TA98 and TA100 (Moltox (Molecular Toxicology, Inc., USA).

## 4.5 Conclusions

In conclusion, *A. eupatoria* is of therapeutic interest due to a diverse array of bioactive properties. In terms of correlation between its ethnomedicinal use and the described pharmacological properties, the antimicrobial and anti-inflammatory properties of *A. eupatoria* may explain the main ethnomedicinal use in gastrointestinal disorders as well as being a topical anti-inflammatory agent. Also, the antidiabetic activity relates to its ethnomedicinal use in the context of diabetes, as the antioxidant and hepatoprotective properties may be relevant for its use in liver diseases. The use for pain may relate to the antinociceptive properties as well as to the anti-inflammatory activity, as other pharmacological properties have also been described, namely anti-tumor and neuroprotective.

It is of note that the hepatoprotective properties evidenced in preclinical studies have been confirmed by clinical studies, which have also shown the safety of the clinical use of *A. eupatoria*. Clinical evidence has also emerged suggesting a protective role in cardiovascular disease, metabolic disorders and diabetes.

A major limitation of the available evidence refers to the remarkable absence of knowledge in regard to specific bioactive compounds as well as the mechanisms that underly the diverse bioactivity displayed by this plant species. Moreover, an important lack of consistency was found regarding the methods that have been used to prepare extracts from several parts of *A. eupatoria* and the methods used for phytochemical characterization, as well as the concentrations and doses that have been used in bioactivity and pharmacological assays.

Further research will provide a better perspective on the relationship between the biological and pharmacological properties and its ethnomedicinal use, as well as on the underlying molecular mechanisms.

# Chapter 5: Further research on *Agrimonia eupatoria* L.

Content adapted from:

Malheiros J, et al. Vascular Effects of Polyphenols from *Agrimonia eupatoria* L. and Role of Isoquercitrin in Its Vasorelaxant Potential in Human Arteries. Pharmaceuticals (Basel) 2022, 15(5):638. doi: 10.3390/ph15050638.

Following the results presented in Chapter 3, we aimed to further assess the direct vascular effects of *A. eupatoria* in ITAs. To this purpose, we tested the vascular activity of an infusion, an ethyl acetate (EtOAc) fraction, with the purpose of testing a fraction with a high content of polyphenols. Then, we assayed the three major constituents of these extracts, i.e., isoquercitrin, tiliroside and *p*-coumaric acid. Furthermore, we evaluated the role of the two major endothelial pathways, i.e., nitric oxide (NO) and cyclooxygenase (COX), in its vascular bioactivity. Here, we showed that the extracts of *A. eupatoria* display potent vasorelaxant activity in human arteries, which is mediated by both NO and COX pathways for the infusion and specifically by COX for the EtOAc fraction. Of the tested compounds, isoquercitrin was found to be the responsible for the vasorelaxant properties of the EtOAc fraction of *A. eupatoria*. Further research is warranted to fully evaluate its vasoprotective properties with therapeutic potential in several conditions, e.g., atherosclerosis.

## 5.1 Mechanism(s) underlying the vascular activity of *A. eupatoria* infusion

The next step, was to assess the vasorelaxant activity of the infusion and the role of the two major endothelial pathways of vasorelaxation, i.e. COX and NO. The influence of NO and COX pathways was assessed with incubations with $N^G$-monomethyl-L-arginine (L-NMMA) and indomethacin (1, 10 and 100 μM), respectively.

As can be seen, the infusion induced a marked concentration-dependent vasorelaxation ($R_{max}$ of 88.99±18.89% and 76.88±6.98%, respectively in Fig. V.1A and IC). Incubation with indomethacin (COX inhibitor) produced a significant decrease in the maximal vasorelaxation to the infusion ($p$<0.01 10μM vs control and $p$<0.05 100μM vs control, Fig V.1D). Incubation with L-NMMA (NO synthase inhibitor) elicited significant decreases in maximal vasorelaxation in all tested concentrations (Fig V.IA).

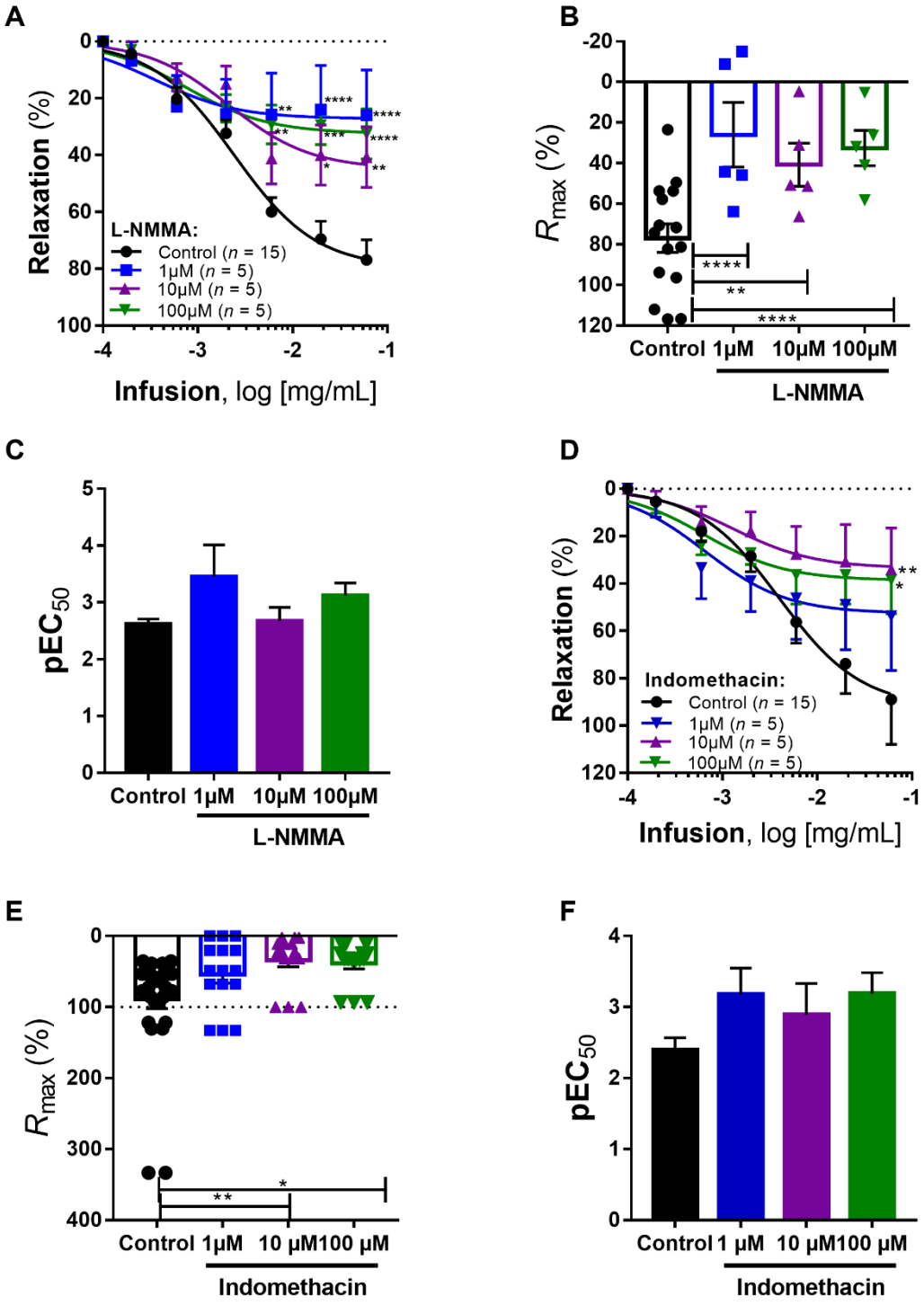


**Figure V.1:** Influence of endothelial nitric oxide synthase blocking with L-NMMA on the vasorelaxation to the infusion (**A**). **B-C,** Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max,}$ **B**) and potency ($pEC_{50,}$). *$p$<0.05 vs. control, **$p$<0.01 vs control, ***$p$<0.001 vs. control, ****$p$<0.0001 vs. control. Significance refers to unpaired two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (**A** and **D**). Influence of COX blocking with indomethacin on the vasorelaxation to the infusion (**D**). **E-F,** Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max,}$ **E**) and potency ($pEC_{50,}$) *$p$<0.5 vs. control, **$p$<0.01 vs. control (D-F).

**Table V.1.** Pharmacological parameters from vasorelaxation activity studies with *A. eupatoria* infusion.

| Studies | Concentration | Maximal effect[1] | Potency[2] | *n* |
|---|---|---|---|---|
| *Role of COX (indomethacin)* | Control | 89.99±18.89 | 2.40±0.17 | 15 |
| | 1 μM | 53.77±22.91** | 3.18±0.36 | 5 |
| | 10 μM | 34.03±17.36* | 2.89±0.44 | 5 |
| | 100 μM | 38.44±14.18 | 3.19±0.29 | 5 |
| *Role of NO (L-NMMA)* | Control | 76.88±6.98 | 2.62±0.09 | 15 |
| | 1 μM | 26.00±15.91**** | 3.45±0.56 | 5 |
| | 10 μM | 40.73±10.62** | 2.67±0.24 | 5 |
| | 100 μM | 32.51±8.7**** | 3.12±0.22 | 5 |

Results presented as mean ± SEM and *n* corresponds to the number of experiments. [1] Maximal relaxation for studies on the role of COX and NO in vasorelaxation (%$R_{max}$). [2] Potency expressed as $pEC_{50}$ for incubation studies or as $pIC_{50}$ for vasorelaxation studies. *$p$<0.5 vs control, **$p$<0.01 vs control, ****$p$< 0.0001 vs control.

## 5.2 Vascular activity of *the ethyl acetate fraction*

### 5.2.1 Material and methods

Plant material

The EtOAc fraction was obtained from half of the crude extract, which was fractionated by repeated extraction with ethyl acetate (3 × 225 mL), also according to previous published data (Santos et al. 2017). After the extraction, the sample was concentrated with water in a rotavapor at 30 °C and then freeze-dried. The method of extraction is described in section 3.1, subsection 3.1.2.

HPLC-PDA

For the phytochemical characterization of the ethyl acetate fraction, the same conditions described in section 3.1.2 were utilized.

Quantification was carried out using peak area in the chromatograms obtained by HPLC-PDA against external standards at appropriate wavelengths: 315 nm for *p*-coumaric acid and tiliroside and 256 nm for quercetin derivatives. Three independent injections were performed for each sample, injecting 100 μL of extract and standards dissolved in water, except quercetin, which was dissolved in 1:1 methanol in water. The identification of compounds was performed by comparing retention time and their UV spectra with previous analysis (Santos et al. 2017) and commercial reference compounds.

Detection (LOD) and quantification limits (LOQ) were determined from the parameters of the calibration curves represented in Table V.2.

**Table V.2.** Linearity, limit of detection (LOD), limit of quantification (LOQ) of the three standards compounds used as reference.

| Standard compound | Range concentrations (µg/mL) | *n* [1] | Slope | Intercept | $R^2$ | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|---|---|---|---|---|
| *p*-coumaric acid | 2.5-15 | 6 | $8.95 \times 10^6$ | $3.90 \times 10^6$ | 0.9960 | 0.49±0.32 | 2.66±0.21 |
| Quercetin | 2.5-125 | 5 | $3.10 \times 10^6$ | $2.62 \times 10^6$ | 0.9932 | 8.18±3.66 | 29.26±3.26 |
| Tiliroside | 5-25 | 6 | $3.65 \times 10^6$ | $1.11 \times 10^6$ | 0.9979 | 1.30±0.36 | 3.65±0.31 |

The identification of infusion and EtOAc fraction compounds was performed by comparing retention times and their UV spectra with *p*-coumaric acid, isovitexin, isoquercitrin (quercetin-3-*O*-glucoside), ellagic acid and tiliroside and data previously published by other authors.

Vascular activity studies

The effect on basal vascular tone was assessed by CCRCs to the EtOAc fraction (0.002–0.2 mg/mL) or vehicle, and the results were expressed as absolute contraction (in millinewton, mN). The vasorelaxant effect was tested with CCRCs (0.02–0.2 mg/mL) to EtOAc fraction or vehicle, after sustained pre-contraction with noradrenaline (20 µM), as results were expressed as percentage of the maximum contraction to NA (%).The modulatory effect on the noradrenaline-induced contraction was assessed with CCRCs to noradrenaline (0.1–48 µM) before and after 30-min pre-incubations with the EtOAc fraction (0.02, 0.2 and 2 mg/mL) or vehicle, as the results were expressed as the percentage of the maximal contraction in the control curve, i.e., absence of infusion (% $E_{max}$).

The influence of NO and COX pathways was assessed with $N^G$-monomethyl-L-arginine (L-NMMA) and indomethacin (10 µM), respectively. The role of the endothelium on the vasorelaxant effect was also assessed by mechanical endothelium removal (which was confirmed with the absence of a relaxant response to acetylcholine) In these experimental setups, the vehicle (distilled water) did not elicit any changes (data not shown).

Thereafter, the effects of compounds were assessed in the same conditions described above for noradrenaline-induced contraction and the influence of NO and COX with L-NMMA and indomethacin (10 µM, respectively) in the vasorelaxant effect.

The concentrations that were assayed were based on compound quantification as described above, *p*-coumaric acid (1.5 µg/mL), quercetin and isoquercitrin (28 µg/mL), and tiliroside (3 µg/mL). For these experiments, vehicle refers to distilled water (for tiliroside and *p*-coumaric acid) or DMSO in water (for isoquercitrin and quercetin). Tissue viability was tested with potassium chloride (KCl, 60 mM) at the beginning and at the end of all experiments.

The analysis of results was performed as described previously in chapter 3, section 3.1.2.

Reagents

Chemicals used for Krebs–Henseleit buffer preparation and arterial ring viability testing in the pharmacological studies were purchased from Sigma-Aldrich® (St. Louis, MO, USA). The following commercial standards were used in the ITA assays: quercetin 3-*O*-glucoside (Sigma, 17793-10MG-F), quercetin (G Buchs SG. K148-/49/2), *p*-coumaric acid (Sigma, C9008) and tiliroside (Extrasynthese, 1001 S, 20316-62-5). The selective prostacyclin IP receptor antagonist Ro 1138452 hydrochloride (4268) was purchased from Tocris (Bristol, UK).

Chemicals used for phytochemical characterization were purchased from Merck® (Darmstadt, Germany) and correspond to the highest grade commercially available. The reference compounds used were: ellagic acid (Sigma, E2250-5G), *p*-coumaric acid (Sigma, C-9008, Lot: 22H0312), quercetin (G Buchs SG., Buchs, Switzerland, K148-/49/2), quercetin3-*O*-glucoside (Sigma, 17793-10MG-F), tiliroside (Extrasynthese, Genay, France, 1001 S, Lot: 12080209), vitexin (Extrasynthese, 1232 S) and isovitexin (Extrasynthese, 1235 S).

### 5.2.2 Results

Phenolic profile of the EtOAc fraction

This section follows section 3.1, where the methods and results from *A. eupatoria* infusion were previously described.

In the EtOAc chromatogram (Fig V.2), peak 1 was identified as *p*-coumaric acid. Peaks 2 and 4 with wavelength maxima at 272 and 335 was characteristic of apigenin derivatives (Matos et al. 2018). Peak 4 was identified as isovitexin with a commercial standard. Peaks 3, 5, 6, 7, and 10 exhibited UV profile spectra characteristic of quercetin derivatives (Mabry 1970). Specifically, peak 5 was identified as isoquercitrin by

comparison of the retention time with a commercial standard, also reported in the infusion (peak 4), in section 3.1.3. Peaks 3 and 6, through spectrum UV, suggested the presence of quercetin *O*-galloyl-hexoside and quercetin *O*-malonyl-hexoside, respectively. These compounds were previously reported (Santos et al. 2017) and were identified by HPLC-PDA-ESI/$MS^n$, once more these compounds were also present in the infusion (peaks 3 and 5). Peak 8 was identified as ellagic acid, also identified in the infusion (peak in figure III.2, available in section 3) (peak 7). Peaks 9, 14 and 18 showed UV spectra, retention time, and wavelength maxima near 265, 346 nm with a shoulder at 314 nm. This behavior is characteristic of kaempferol derivatives accordingly to Mabry (1970) and Campos and Markhan (2007) and were also identified in Santos et al. (2017).

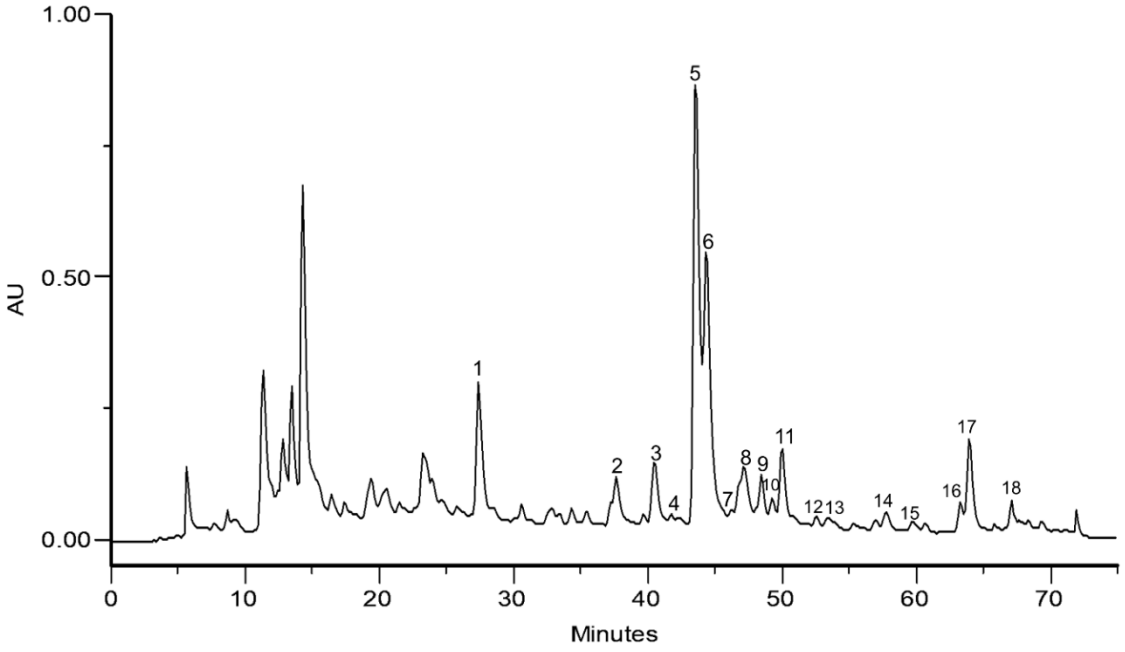


**Figure V.2: HPLC-PDA chromatogram of the ethyl acetate fraction**. Chromatogram was recorded at 280 nm.

Peak 9 was identified as tiliroside, comparing the retention time with a commercial standard. This compound was also identified in the infusion (peak 6), in figure III.2 (available in section 3). Peaks 11, 12, and 13 were also identified as kaempferol derivatives due to the UV profile and wavelength maxima at 264 nm, 346 nm, and a shoulder at 296 nm (Mabry 1970). Finally, peak 12 was identified as kaempferol-*O*-malonyl-hexoside by HPLC-PDA-ESI/$MS^n$ (Santos et al. 2017).

Based on the chromatograms, *p*-coumaric acid, tiliroside and quercetin derivatives were identified as the major constituents in the infusion and the EtOAc

fraction and therefore quantified by HPLC (Table V.3). As can be seen, quercetin derivatives were the major class of compounds in both extracts.

**Table V.3.** Quantification of *p*-coumaric acid, quercetin derivatives and tiliroside in the infusion and the EtOAc fraction of *A. eupatoria* by HPLC-PDA and the respective concentrations in organ bath experiments.

| | **Compound** (tentative identification) | **µg of compound/100 g of extract** | **µg of compound per 2 mg of extract/mL** [1] |
|---|---|---|---|
| **Infusion** | | | |
| Peak 2 | *p*-coumaric acid | 73.00 | 1.470 |
| Peak 3 | Quercetin derivatives [2] | 150.7 | 3.140 |
| Peak 4 | Isoquercitrin | 1500 | 30.16 |
| Peak 5 | Quercetin derivatives [2] | 270.8 | 5.550 |
| Peak 6 | Tiliroside | 147.0 | 2.940 |
| **EtOAc fraction** | | | |
| Peak 1 | *p*-coumaric acid | 70.00 | 1.400 |
| Peak 3 | Quercetin derivatives [2] | 157.9 | 3.150 |
| Peak 5 | Isoquercetrin | 1400 | 28.00 |
| Peak 6 | Quercetin derivatives [2] | 333.5 | 6.670 |
| Peak 9 | Tiliroside | 138.0 | 2.760 |

[1] Major concentration of the extract of *A. eupatoria* used in vascular activity experiments. [2] Results expressed as quercetin equivalent.

Vascular effects

The EtOAc fraction of *A. eupatoria* did not induce any change in basal vascular tone ($E_{max}$ = 0.12±0.32 mN; $pEC_{50}$ = 2.67±3.69, $p<0.05$ vs. infusion, $E_{max}$). Furthermore, the lower concentration (0.02 mg/mL) changed the noradrenaline-induced contraction only in intermediate concentrations of noradrenaline (statistically significant decreases of 27.13% and 27.72%, $p<0.05$ in Fig V.3A), even though no significant differences were detected in efficacy or potency vs control (Table V.4). Furthermore, the higher concentration of the EtOAc fraction (2 mg/mL) elicited a significant decrease of 80.65% in the $E_{max}$ in the noradrenaline CCRCs (Fig. V.3A), thus suggesting a decrease in efficacy.

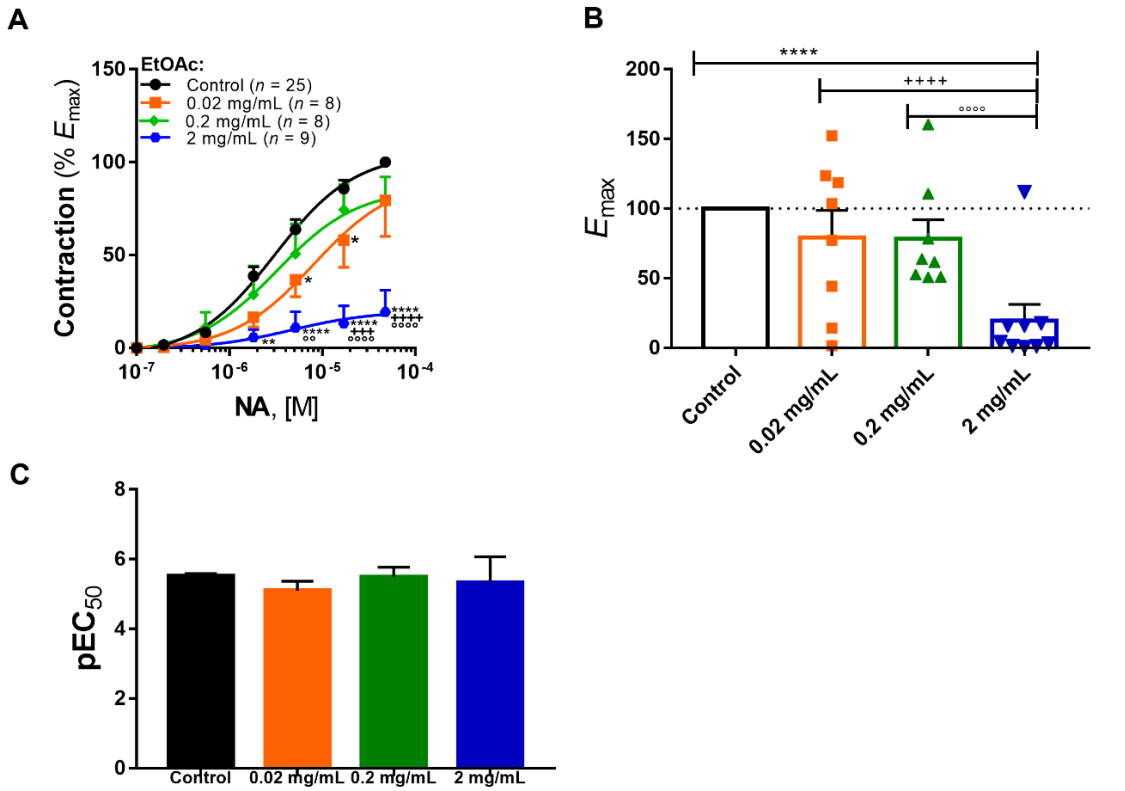


**Figure V.3**: **Vascular activity of the EtOAc fraction**. (**A**) Effect on the noradrenaline-induced contraction. **B-C,** Pharmacological parametersfrom concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50,}$). * $p$<0.05 vs. control, ** $p$<0.01 vs. control, **** $p$<0.0001 vs. control, $^{+++}p$< 0.001 vs. 2 mg/mL, $^{++++}p$<0.0001 vs. 2 mg/mL, $^{\circ\circ}p$<0.01 vs. 2 mg/mL, $^{\circ\circ\circ\circ}p$<0.0001 vs. 2 mg/mL.

**Table V.4**. Pharmacological parameters from vascular activity studies with the EtOAc fraction of *A. eupatoria*.

| Studies (incubation with fraction or compound) | Concentration | Maximal effect[1] | Potency[2] | *n* |
|---|---|---|---|---|
| *Influence on adrenergic contraction (EtOAc fraction)* | Control | 100.00±0.00 | 5.51±0.06 | 25 |
| | 0.02 mg/mL | 79.43±19.32$^{++++}$ | 5.09±0.26 | 8 |
| | 0.2 mg/mL | 78.40±13.63$^{\circ\circ\circ\circ}$ | 5.49±0.27 | 8 |
| | 2 mg/mL | 19.35±11.82**** | 5.30±0.73 | 9 |
| *Role of mediators in vasorelaxation* | Control | 47.95±6.55 | 2.88±0.22 | 11 |
| Indomethacin | 10 μM | 12.45±3.78*** | 3.75±0.76 | 8 |
| L-NMMA | 10 μM | 25.14±17.80 | 3.19±1.25 | 5 |
| Ro 1138452 | 10 μM | 24.33±12.75 | 4.00±1.51 | 4 |

Results presented as mean ± SEM, and *n* corresponds to the number of experiments. [1]Maximal effect expressed as maximal contraction (%$E_{max}$ to noradrenaline) for adrenergic contraction studies or maximal relaxation for studies on the role of COX and NO in vasorelaxation (%$R_{max}$). [2] Potency expressed as $pEC_{50}$ for incubation studies or as $pIC_{50}$ for vasorelaxation studies. *** $p$< 0.001 vs. control, **** $p$< 0.0001 vs. control, $^{++++}p$< 0.0001 vs. 2 mg/mL, $^{\circ\circ\circ\circ}p$< 0.0001 vs. 2 mg/mL.

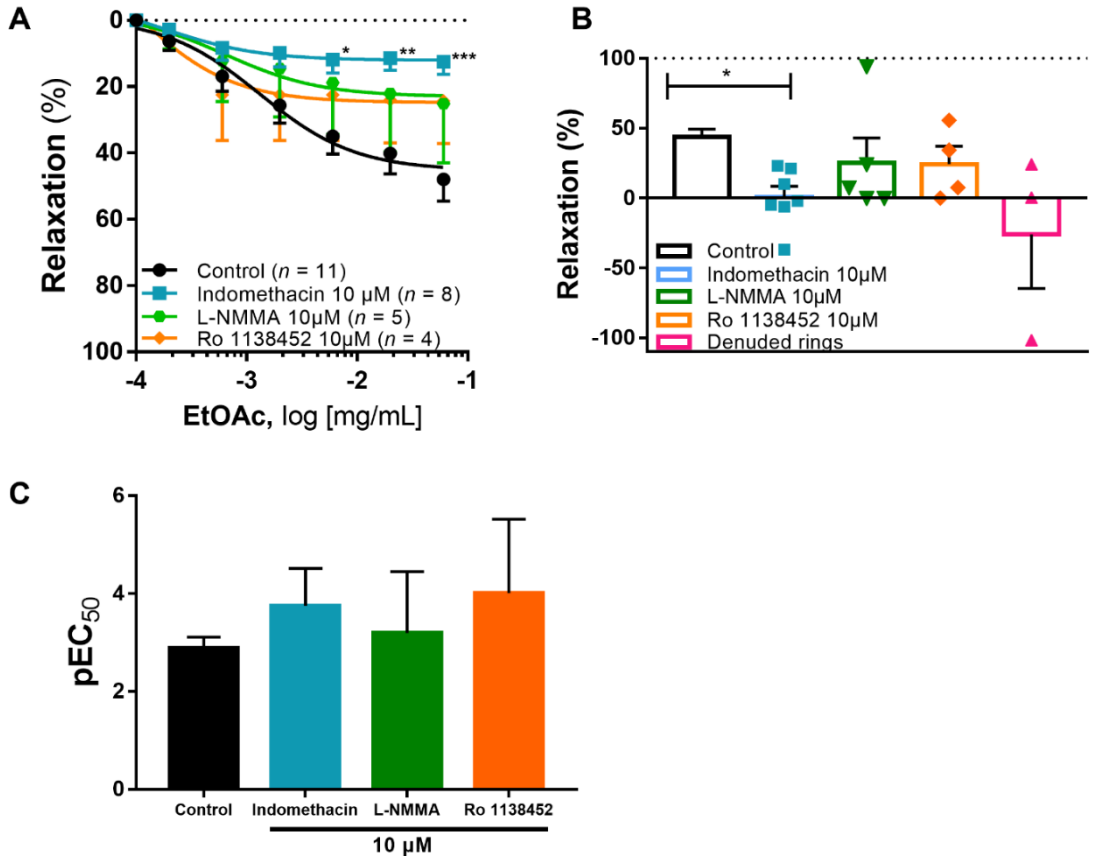


**Figure V.4**: Influence of COX blocking with indomethacin, endothelial nitric oxide synthase blocking with L-NMMA and blocking $PGI_2$ receptors with a selective antagonist Ro 1138452 on the vasorelaxation to EtOAc fraction (**A**). **B-C,** Pharmacological parametersfrom concentration-response curves in A, specifically maximal contraction ($E_{max,}$ **B**) and potency ($pEC_{50,}$). * $p$< 0.05 vs. control, ** $p$< 0.01 vs. control, *** $p$<0.001 vs. control.

The EtOAc fraction elicited a vasorelaxant effect ($R_{max}$ = 47.97±6.55%, Fig V.4A), which was completely abolished by endothelial denudation ($R_{max}$= -25.37±39.05%; data not shown in graph), thus suggesting this effect is endothelium-dependent. Similarly, the role of NO and COX pathways was also evaluated. Incubation with 10 µM L-NMMA produced a non-significant decrease in the vasorelaxation to the EtOAc fraction ($R_{max}$ = 25.14±17.80%, Fig. V.4A). In contrast, incubation with 10 µM indomethacin decreased significantly the vasorelaxation ($R_{max}$ = 12.45±3.87%, $p$<0.001 vs control, Fig. V.4A).

Given this significant decrease of the relaxation with the incubation with indomethacin, we hypothesized that the COX-derived prostanoid, prostacyclin, could be responsible for this vasorelaxant effect. However, the incubation with the IP receptor selective antagonist (Ro 1138452) produced a nonsignificant decrease of the maximal relaxation to EtOAc fraction ($R_{max}$ = 24.33±12.75%, Fig. V.4A).

## 5.3 Vascular activity of isoquercitrin, tiliroside and *p*-coumaric acid

The marked vasorelaxant effect previously observed lead to the investigation of the compounds that could be responsible for this action. Hence, the quantification of the major compounds identified in the infusion and the EtOAc fraction was carried out (Table V.3). Based on this quantification, we extrapolated the amount of compound present in the higher concentration of the EtOAc fraction used in the vascular studies described above (i.e., 2 mg/mL). Thereafter, we used the following concentrations: isoquercitrin (28 µg/mL), tiliroside (3 µg/mL) and *p*-coumaric acid (1.5 µg/mL). Given the predominant composition in quercetin derivatives (Table V.3), we also included quercetin as a reference compound for comparison of vascular effects.

Isoquercitrin elicited a statistically significant decrease of 42.86% ($p< 0.001$ vs. vehicle) in the maximal contraction to noradrenaline ($E_{max}$ = 56.94 ± 8.28%, Fig V.5A) and a potent vasorelaxant activity with $R_{max}$ of 53.29 ± 7.42 ($p< 0.01$ vs. vehicle, Fig V.5D). As seen, incubation with indomethacin decreased significantly the vasorelaxation elicited by isoquercitrin ($R_{max}$ = 15.94 ± 5.10%, $p< 0.05$ vs. isoquercitrin, Fig V.5D), whereas incubation with L-NMMA led to a nonsignificant reduction ($R_{max}$ = 25.16 ± 6.45%, Fig V.5D).In the same concentration, quercetin was able to elicit a more pronounced inhibition of noradrenaline-induced contraction ($E_{max}$ = 35.94 ± 6.25%, $p< 0.0001$ vs. vehicle, Fig V.6A).Moreover, quercetin elicited a marked vasorelaxant effect ($R_{max}$ = 40.21 ± 6.49, $p< 0.05$ vs. vehicle, Fig V.5E), which was not significantly different from isoquercitrin ($p> 0.05$). Differently from isoquercitrin, the vasorelaxant effect of quercetin was not affected by preincubation with indomethacin ($R_{max}$ = 27.74 ± 4.18%) or L-NMMA ($R_{max}$ = 34.12 ± 8.64%).

Both *p*-coumaric acid and tiliroside elicited a potentiation of the noradrenaline contraction ($E_{max}$ of 152.80±28.23% and 139.40±33.10%, respectively, Fig V.6A), even though only *p*-coumaric acid produced a statistically significant changes in intermediate concentrations ($P<0.05$ vs vehicle). In the tested concentrations, these compounds did not elicit any vasorelaxation in the ITA ($R_{max}$ of -11.43±10.69% and -13.69%±3.93% respectively, Fig V.6D).

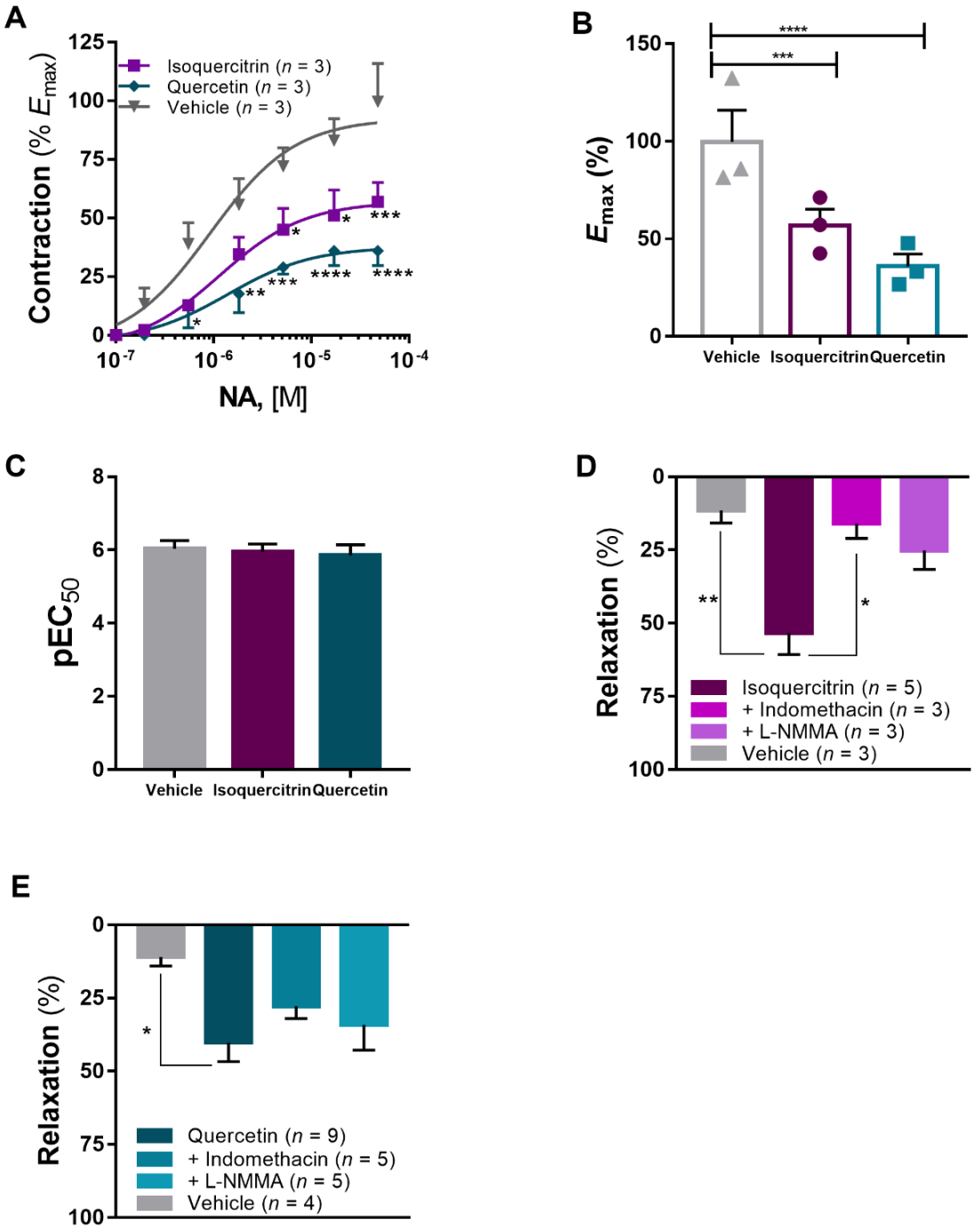


**Figure V.5: Vascular activity of isoquercitrin and quercetin**. (**A**) Effect of isoquercitrin and quercetin (28 µg/mL) on the contraction induced by noradrenaline. **B-C,** Pharmacological parametersfrom concentration-response curves in A, specifically maximal contraction ($E_{max,}$ **B**) and potency ($pEC_{50,}$) Vasorelaxant effect on isoquercitrin (**D**) and quercetin (**E**) and influence of COX and endothelial nitric oxide synthase blocking with indomethacin (10 µM) and L-NMMA (10 µM), respectively. * $p$<0.05, ** $p$<0.01, *** $p$<0.001, **** $p$<0.0001 vs. vehicle.

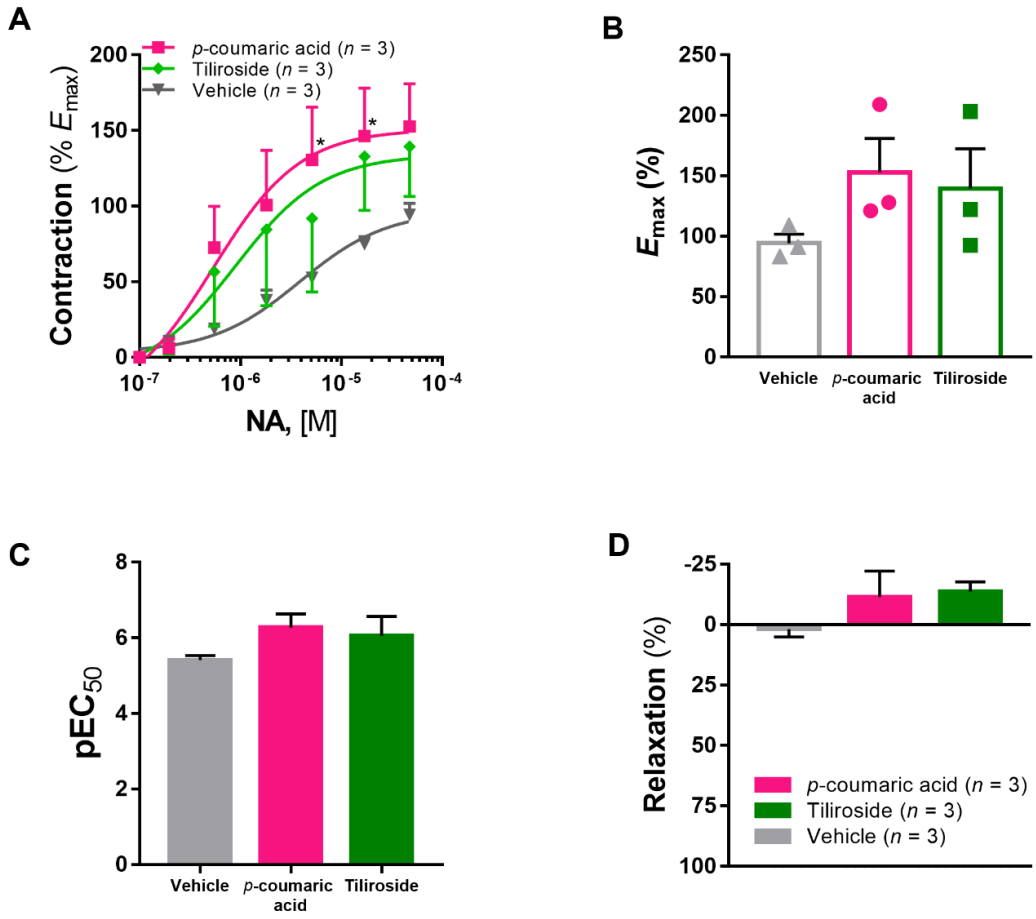


**Figure V.6: Vascular activity of *p*-coumaric (1.5 µg/mL) acid and tiliroside (3 µg/mL).** (**A**) Effect of p-coumaric acid and tiliroside on the noradrenaline-induced contraction. **B-C,** Pharmacological parametersfrom concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$). (**D**) Vasorelaxant effect of *p*-coumaric acid and tiliroside. * $p<0.05$ vs. vehicle.

## 5.4 Discussion

To our knowledge, only one report (Kuczmannová et al. 2016) has focused on the vascular activity of *A. eupatoria* showing that the treatment with the aqueous extract improved acetylcholine-induced vasorelaxation in aortic rings isolated from diabetic animals. Importantly, this improvement was observed only in comparison with untreated diabetic animals but not with healthy controls. Whether such effect was a result from the anti-diabetic properties of this extract or from a direct vascular activity remained to be clarified.

Here, we tested the direct vascular activity of *A. eupatoria* and showed that its infusion exhibits potent vasorelaxant activity in human arteries (Fig III.3D, section 3.1). As a low concentration (0.02 mg/mL) elicited a potentiation of the maximal response to noradrenaline, the infusion displayed a mixed vascular effect thus suggesting some compounds may elicit an increase in vascular tone and others a decrease. Both endothelial pathways (i.e. NO and COX) seem to be involved (Fig V.1). Interestingly, the magnitude of the vasorelaxation (above 76%) could also suggest a direct vascular smooth muscle relaxation (i.e. endothelium-independent), which remains to be confirmed.

A potent vasorelaxant effect was also observed with the EtOAc fraction (Fig V.5), although it was lower compared to the infusion (above 47% and 76%, respectively). In contrast to the observed with the infusion, the EtOAc fraction elicited a decrease in noradrenaline-induced contraction at the higher concentration (2 mg/mL). Furthermore, the COX endothelial pathway was found to be the underlying mechanism for the vasorelaxation of the EtOAc fraction. This difference in vasorelaxant activity, particularly NO-mediated, must be highlighted, as it may result from the extraction process, with a loss of compounds such as procyanidin B2. In fact, endothelium-dependent vasorelaxation to procyanidin B2 has been described in the ITA (Novakovic et al. 2017), which involves NO synthesis and secretion by endothelial cells and partially of prostacyclin and also activation of $BK_{Ca}$ and $K_{ATP}$ channels, as well as $K_V$ and $IK_{Ca}$. Moreover, procyanidins have also been reported to stimulate the production of prostacyclin in the ITA (Aldini et al. 2003). Although we did not identify procyanidin B2 in the extracts that were assayed in our work, the EtOAc fraction was found to be rich in procyanidin dimers in a previous report (Santos et al. 2017). In fact, this compound has been identified in the EtOAc fraction of the same batch of this plant species (Correia et al. 2006; Santos et al. 2017).

Based on the findings, we hypothesized that prostacyclin, which is a fundamental endothelium-derived relaxing factor that increases cAMP leading to vasorelaxation, could be the mediator of this response. However, experiments with the IP receptor antagonist Ro 1138452 did not confirm our hypothesis (Fig V.4A). Beyond prostacyclin, other vasodilator metabolites have been described, such as epoxyeicosatrienoic acids or EETs (namely 14,15-EET and 11,12-EET), cytochrome P450 metabolites and prostaglandin D2 and E2 also via COX (Mihaljević et al. 2020). Previous evidence suggests that 11,12-EET elicits relaxation in ITA through the activation of $BK_{Ca}$ channels and the vanilloid transient receptor potential channel 4 and canonical transient receptor potential channel 1 in smooth muscle cells (Archer et al. 2003; Ma et al. 2015). As to prostaglandins D2 and E2, previous reports have shown no vasorelaxation in the ITA,

rather an EP receptor-mediated vasoconstriction in response to prostaglandin E2 (Foudi et al. 2011; Foudi et al. 2017). Overall, the precise mechanism through which *A. eupatoria* elicits vasorelaxation remains to be fully characterized.

In terms of composition, both extracts displayed a high phenolic content and quercetin derivatives were found to be the predominant compounds, namely isoquercitrin, together with tiliroside and *p*-coumaric acid (Table V.3). Therefore, we hypothesized that these compounds could be responsible for the observed vasorelaxant activity. Based on our findings (Fig V.6), isoquercitrin showed vasoactive properties consistent with the EtOAc fraction (i.e., ability to reduce noradrenaline-induced contraction and potent COX-mediated vasorelaxant activity), thus suggesting this is the compound responsible for such activity. This is a compound known for multiple activities, such as antiviral against human herpes viruses (Kim et al. 2020), antioxidant, anti-inflammatory and anticancer effects, among others (Valentová et al. 2014).

In regard to its cardiovascular activity, previous studies have highlighted the antihypertensive potential of isoquercitrin through an inhibitory effect on angiotensin converting enzyme (Gasparotto et al. 2011). Also, it has been shown to elicit $K^+$ channel- and endothelial NO-mediated vasodilation in perfused rat mesenteric arteries (Gasparotto Junior et al. 2016). Moreover, isoquercitrin displays antiapoptotic activity in vascular endothelial cells, which may be relevant in atherogenesis (Duan et al. 2021; Liu et al. 2021). Atheroprotective properties have also been attributed to enzymatically modified isoquercitrin or EMIQ (i.e., isoquercitrin with malto-oligosaccharides) in atherogenic ApoE-deficient mice (Motoyama et al. 2009). In clinical studies, Bondonno et al. (2016) showed no acute changes in blood pressure or brachial NO-mediated endothelium-dependent relaxation in healthy individuals. More recently, EMIQ was shown to acutely improve brachial flow-mediated dilatation in patients at risk for cardiovascular disease (Bondonno et al. 2020).

Since our extract and the EtOAc fraction contained a high content of quercetin derivatives, namely, isoquercitrin, we aimed to assess the vascular activity of quercetin as a reference compound in human arteries. In our study, quercetin decreased significantly the maximal contractile response to noradrenaline (Figure V.5A) and elicited a potent vasorelaxation (Figure V.5E). To our knowledge, this is the first report on the vasoactivity of quercetin in human arteries, as the observed effects are generally consistent with previous reports on rat aorta (Roghani et al. 2004; Khoo et al. 2010), rat basilar artery (Yuan et al. 2018) and rat tail main artery (Iozzi et al. 2013). Furthermore, indomethacin and L-NMMA did not change significantly the vasorelaxation to quercetin, suggesting that the endothelium is not involved in this vasorelaxant effect

in the ITA. In rat aorta, a previous study showed that the vasorelaxant effect of quercetin is partially dependent on the endothelium nitric oxide synthase and endothelium-derived hyperpolarizing factor (Khoo et al. 2010). More recently, this vasorelaxation partially mediated by NO was confirmed, even though endothelium nitric oxide synthase blocking with L-NAME decreased only the potency and not the maximal relaxation (Yuan et al. 2018). A similar finding was described for COX blocking with indomethacin. Other studies have suggested endothelium-independent relaxation with the involvement of calcium and potassium channels (Iozzi et al. 2013; Hou et al. 2014). Overall, our findings are consistent with this evidence suggesting the involvement of endothelium-independent mechanisms.

In regard to the other tested compounds, *p*-coumaric acid and tiliroside, these elicited no vasorelaxation and no statistically significant effects on noradrenaline-induced maximal contraction at the tested concentrations. In the literature, *p*-coumaric acid has been shown to protect against doxorubicin-induced oxidative stress in the rat heart through free radical scavenging properties (Abdel-Wahab et al. 2003), as well as to display antiplatelet activity (Luceri et al. 2007), but no report has emerged on its vascular activity.

As to tiliroside, its antihypertensive and vasorelaxant effects have been reported (Silva et al. 2013). In that study, the authors reported a dose-dependent decrease in blood pressure in hypertensive rats, a concentration-dependent vasodilation of rat mesenteric arteries, as well as a blockage in the membrane depolarization-induced increase of intracellular $Ca^{2+}$ concentration and decreased voltage-activated peak amplitude of the L-type $Ca^{2+}$ channel current in VSMCs. This antihypertensive potential has been recently confirmed (Lagunas-Herrera et al. 2019), as tiliroside antagonized the transient hypertension evoked by the administration of angiotensin II in a dose-dependent way.

The absence of vasorelaxant effect in our assays, particularly for tiliroside, could be explained by the low concentrations that were used. However, our focus was to assess the compounds responsible for the vasorelaxation to *A. eupatoria*; thus, we extrapolated the concentrations of compounds that were present in the higher concentration of the EtOAc fraction that was used (2 mg/mL), rather than use a range of concentrations or higher concentrations as described in those previous reports.

Some limitations should be considered. First, the variability in the obtained data may derive from the use of human arterial tissue harvested from patients with underlying cardiovascular conditions and therefore a diverse biological background. However, this may also constitute an advantage, as it provides a better translation of the findings to

humans and especially when considering patients with vascular disease. Second, we did not test if direct smooth muscle relaxation is involved in the observed vasorelaxant effects. However, it is unlikely that it is a major mechanism, considering the results obtained after endothelium removal or in the presence of inhibitors of endothelial pathways.

To our knowledge, this is the first report on the direct vascular effects of extracts and compounds from *A. eupatoria*. Collectively, the data demonstrates the vasorelaxant potential of its infusion and EtOAc fraction. Both the COX and NO pathways appear to be involved in the activity of the infusion of *A. eupatoria*, as the COX pathway was found to be the major pathway involved in the effects elicited by the EtOAc fraction and specifically isoquercitrin, even though the specific mediators remain unclear.

## 5.5 Additional assays with *A. eupatoria*

Once the infusion and the EtOAc fraction demostraded a marked vasoactivity and the mechanisms underlying the vasorelaxation were described (chapter 3 and 5), the next step of our work was tentatively identifying the compound responsible for the referred activity.

For this purpose, the first step was to obtain enriched fractions of quercetin and tiliroside, the major components present in the infusion and EtOAc.

The fractions were utilized mainly in for phytochemical characterization and the modulatory effect on adrenergic contraction. The method of fractionation and the resuls obtaneid are described below.

**Preparation of quercetin- and tiliroside-enriched fractions**

Flash chromatography was used to fractionate the ethyl acetate fraction. Aliquots of 25 mg/mL of the subfraction were chromatographed in a reverse phase C18 column Buchi® (40x150 mm, with particle diameter between 40 and 63 µm) (Flawil, Switzerland). The mobile phase was propelled by two pumps Buchi® Pump Module C-605 (Flawil, Switzerland), taking up the water and methanol used in the gradient: 0% B (0-17 min), 80% B (17-25 min) and 100% B (25-31 min) at a constant flow rate of 10 mL/min (Fig V.7). After control with HPLC, six fractions were obtained (F1, F1B, F1C, F2, F3 and F4).

After control with HPLC, fractions F1C (quercetin enriched fraction) and F4 (tiliroside enriched fraction) were utilized.

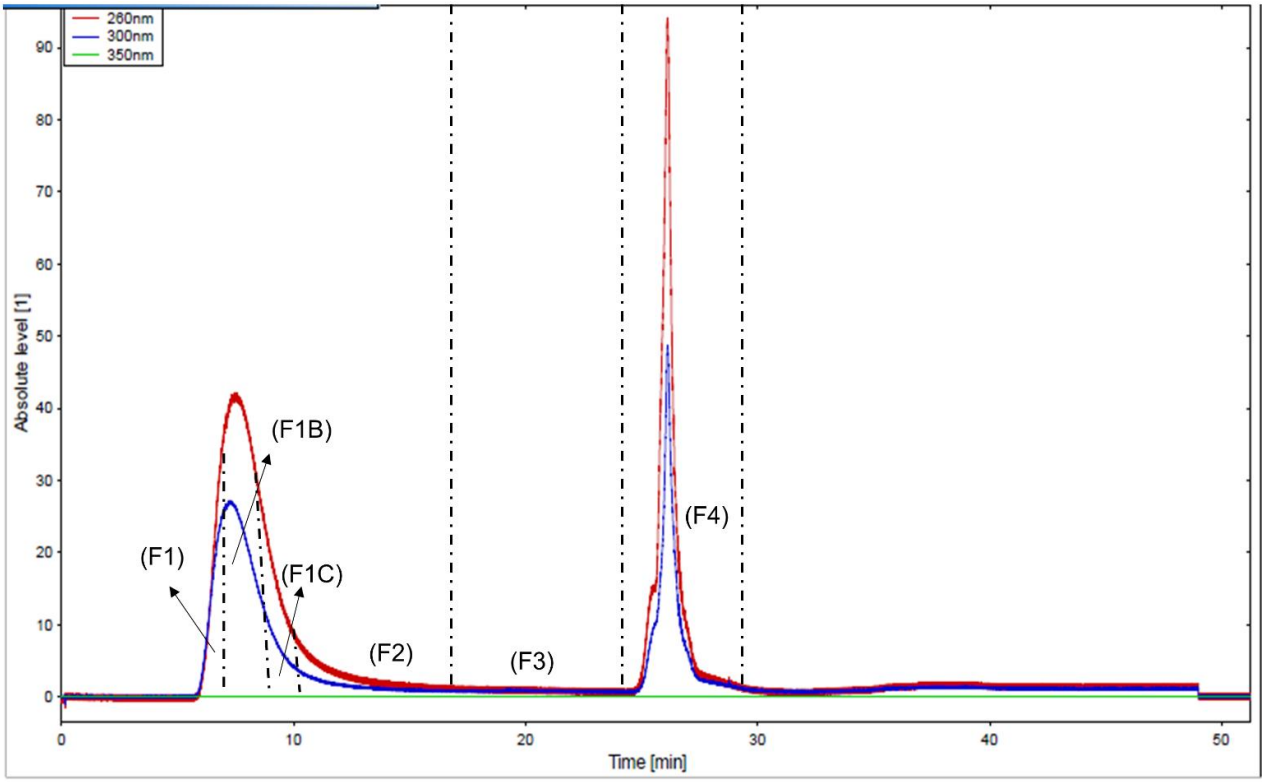


**Figure V.7**: **Chromatogram obtained with Flash Chromatography for the fractionation of ethyl acetate fraction of *A. eupatoria***. **(F1)**: 5,8-7 min; **(F1B)**:7-8,5 min; **(F1C)**: 8,5-10 min; **(F2)**: 10-17 min; **(F3)**: 17-25 min; **(F4)**: 25-31 min.

HPLC-PDA

HPLC Gilson (Gilson, Middleton, WI, USA) were used for the characterization of the fractions. It was equipped with two pumps (models 305 and 306), mixer (model 811), manometer (model 805) and an automatic injector (Gilson 234) coupled to a photodiode detector (model 107) and control and processing station Unipoint System data (Unipoint® 2.10)

The samples (100 µL) were injected in Waters® a Spherisorb S5 ODS-2 column (250 × 4.6 mm i.d., 5 µm) protected with a guard cartridge C18 (30 × 4 mm i.d., 5 µm) (Nucleosil, Macherey-Nagel, Duren, Germany) and eluted at a flow rate of 1 mL/min and 24 °C. Mobile phase consisted of 5% formic acid (v/v) (eluent A) and methanol (eluent B). The gradient used was: 0-60 min (5-50%B), 60-70 min (50-100%B), 70-75 min (100-100%B). Chromatographic profiles was recorded at 280 nm.

Vascular activity studies

Vascular tissue preparation was carried out as previously described. Similarly, the modulatory effect on the noradrenaline-induced contraction was assessed with CCRCs to noradrenaline (0.1 - 48 μM) before and after 30-min pre-incubations with the quercetin or tiliroside enriched fractions (0.002, 0.02 and 0.2 mg/mL), as the results were expressed as the percentage of the maximal contraction in the control curve, i.e. absence of infusion (%$E_{max}$).

Data were generally expressed as mean ± SEM unless specified otherwise, as *n* indicates the number of patients. potency was expressed as the negative logarithm of the effective concentration (in mol/L or M) of noradrenaline able to induce half of the maximum contraction ($pEC_{50}$, -log [M]). Efficacy was expressed as %$E_{max}$ (maximal contraction, %).

Statistical analysis was performed using GraphPad Prism 7® (GraphPad Inc., La Jolla, CA, USA). First, the normality of data was accessed with Shapiro-Wilk test. The analysis of CCRCs was performed generally by two-way ANOVA with Tukey's multiple comparisons test to identify differences in specific concentrations, including maximal effects.

## Phenolic profile and vascular activity of the quercetin-enriched fraction

Regarding the quercetin-enriched fraction (Fig V.8), peak 1 suggests an apigenin derivative to a wavelength maximum at 272 and 335 nm and its spectral profile (Matos et al. 2018). Peaks 2,3,4 were 3-*O*-glycosylated quercetin derivatives, as suggested by their UV spectra profile, with band I near 350 nm with absorptivity lower than band II with wavelength maxima near 250 and shoulder approximately at 265 and 295 nm (Mabry 1970). Peak 5 suggested a kaempferol derivate, due to spectra prolife wavelength maxima near 265 and 346, with a shoulder near 314 nm (Mabry 1970), and finally, peak 6, with wavelength maximum near 248 and 371 nm with a shoulder 322 nm, thus suggesting a presence of an ellagic acid derivative (Couto et al. 2020).

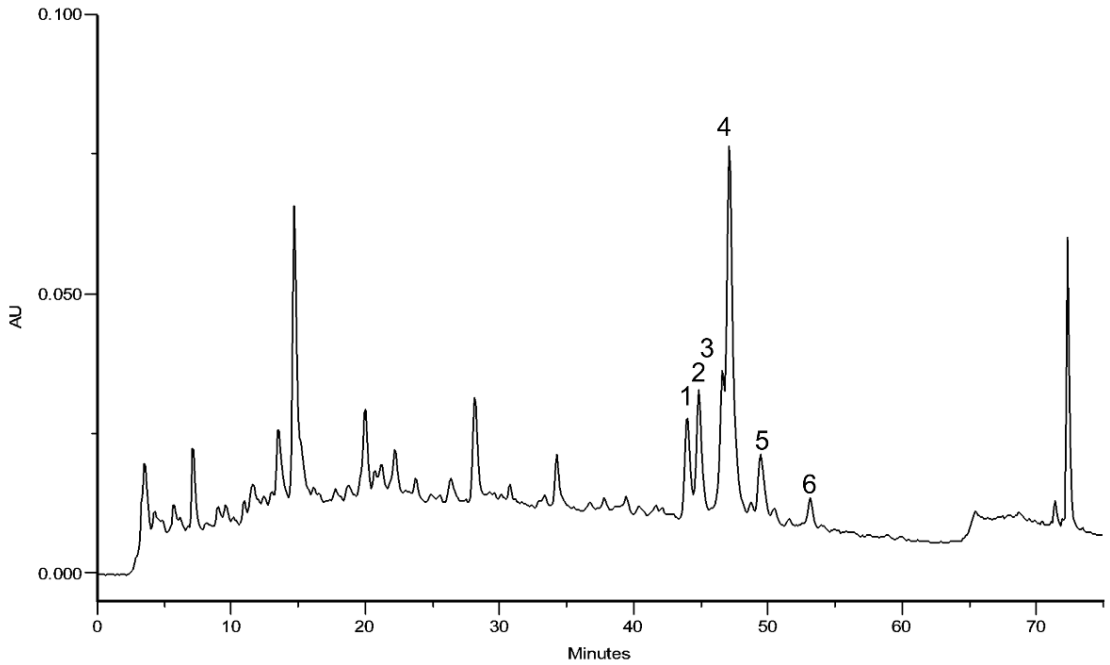


**Figure V.8: HPLC-PDA chromatogram of quercetin-enriched fraction**. Chromatogram were recorded at 280 nm.

In terms of vascular activity, even though in the lowest concentration (0.002 mg/mL) only presents two replicates, the quercetin-enriched fraction did not induce a statistically significant effect in the noradrenaline-induced maximal vascular contraction. No significant changes were observed in terms of potency (Fig V.9, Table V.5).

**Table V.5**: Influence of the quercetin-enriched fraction on the parameters of efficacy (%$E_{max}$) and potency ($pEC_{50}$) of the noradrenaline-induced contractile response.

| Concentration | %$E_{max}$ | $pEC_{50}$ | *n* |
|---|---|---|---|
| Control | 100.00±0.00 | 6.07±0.20 | 8 |
| 0.002 mg/mL | 113.68±38.21 | 6.99±1.49 | 2 |
| 0.02 mg/mL | 75.42±42.89 | 7.16±2.40 | 3 |
| 0.2 mg/mL | 128.81±8.15 | 6.76±0.44 | 3 |

Values presented in mean ± SEM and *n* correspond to the number of experiments (n) performed in each experimental setting.

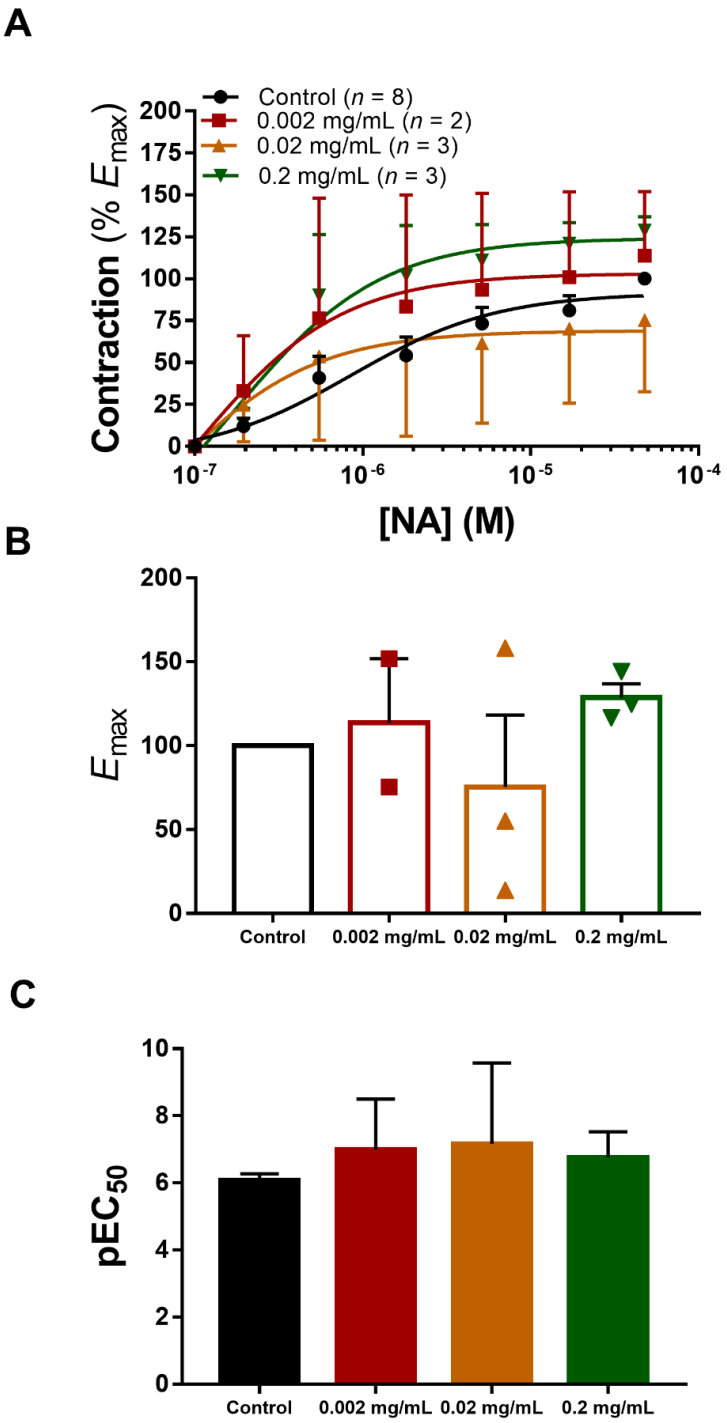


**Figure V.9: Vascular activity of quercetin-enriched fraction**. **A,** Cumulative concentration-response curves to noradrenaline (NA) in the absence or presence of several concentrations of quercetin-enriched fraction. **B-C**, Pharmacological parameters from concentration-response curves in A, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$).

**Phenolic profile and vascular activity of the tiliroside-enriched fraction**

In the phenolic profile of the tiliroside-enriched fraction (Fig V.10), only peaks 1 and 2 were identified. Both presented wavelength near 265 and 346 nm with a shoulder near 314 nm. This behavior suggests the presence of kaempferol*O*-*p*-coumaroyl-glucoside (tiliroside) (Mabry 1970).

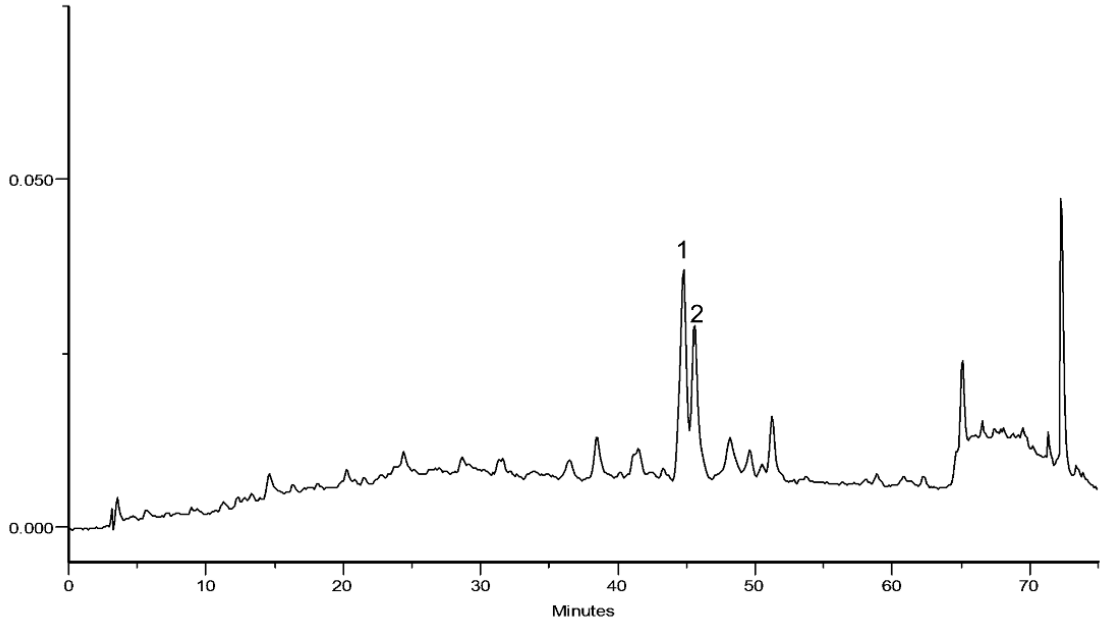


**Figure V.10**: **HPLC-PDA chromatogram of tiliroside-enriched fraction**. Chromatogram was recorded at 280 nm.

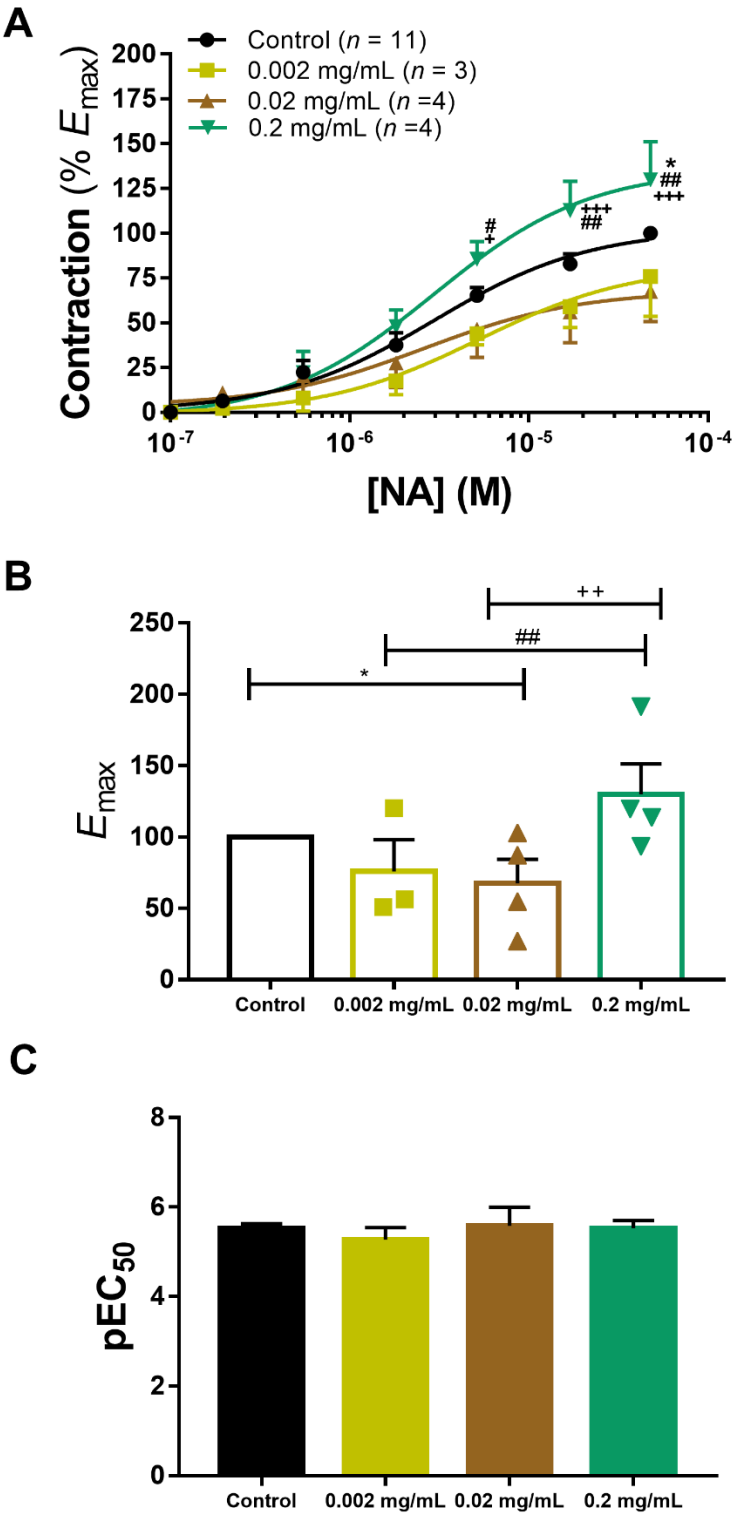


**Figure V.11: Vascular activity of tiliroside-enriched fraction. A,** Cumulative concentration-response curves to noradrenaline (NA) in the absence or presence of several concentrations of fraction. **B-C**, Pharmacological parameters from concentration-response curves in **A**, specifically maximal contraction ($E_{max}$, **B**) and potency ($pEC_{50}$). *$p$<0.05 vs. control; $^{\#}p$<0.05 0.002 mg/mL vs. 0.2 mg/mL; $^{\#\#}p$<0.01 0.002 mg/mL vs. 0.2 mg/mL; $^{+}p$< 0.05 0.02 mg/mL vs. 0.2 mg/mL; $^{+++}p$< 0.001 0.02 mg/mL vs. 0.2 mg/mL.

Concerning the tiliroside-enriched fraction, a significant potentiation of the maximal effect of the noradrenaline-induced contraction (about 29.93%, $p<0.05$ vs control) was observed for the higher concentration of the fraction (0.2 mg/mL). No significant changes were observed in terms of potency (Fig V.11 and Table V.6).

**Table V.6**: Influence of tiliroside-enriched fraction and tiliroside enriched fraction on the parameters of efficacy (%$E_{max}$) and potency ($pEC_{50}$) of the noradrenaline-induced contractile response.

| Concentration | %$E_{max}$ | $pEC_{50}$ | *n* |
|---|---|---|---|
| Control | 100.00±0.00 | 5.53±0.10 | 11 |
| 0.002 mg/mL | 75.87±22.32$^{\#\#}$ | 5.28±0.26 | 3 |
| 0.02 mg/mL | 67.66±16.91$^{+++}$ | 5.58±0.41 | 4 |
| 0.2 mg/mL | 129.93±21.38$^{*}$ | 5.53±0.17 | 4 |

Values presented in mean ± SEM and *n* correspond to the number of experiments (*n*) performed in each experimental setting. $^{*}p<0.05$ vs. control, $^{\#\#}p<0.01$ 0.002 mg/mL vs. 0.2 mg/mL. $^{+++}p< 0.001$ 0.02 mg/mL vs. 0.2 mg/mL

As flavonoids are largely encountered in plants, this has led to an extensive body of scientific literature on their potential therapeutic use. In the vascular area, flavonoids have been shown to induce endothelium-dependent relaxation in many *in vitro* experiments (Woodman et al. 2005). However, our group could not confirm these results in ITAs. The results previous demonstrated were realized with the content of 55 fractionations.

To proceed with the vasorelaxant effects of this fraction, a large amount of plant and solvents would be required, thus making unfeasible to continue the assays with the enriched fractions. Therefore, our group settled to continue the vascular assays with the commercial standards previously identified in the infusion and the ethyl acetate fraction (chapter 5, section 5.3).

### Role of the receptor IP on the vasorelaxation of Isoquercitrin

The influence of the prostacyclin receptor (IP receptor) was evaluated with the selective antagonist Ro1138452 (10μM).

As can be seen in Figure V.12, isoquercitrin elicited vasorelaxant activity with $R_{max}$= 31.39 ± 5.67%. Incubation with the Ro 1138452 did not decrease the vasorelaxation elicited by isoquercitrin ($R_{max}$ = 42.10 ± 11.57%). This result suggests that

the IP receptor is not involved in the vasorelaxation produced by isoquercitrin, confirming the results obtained with the EtOAc fraction.

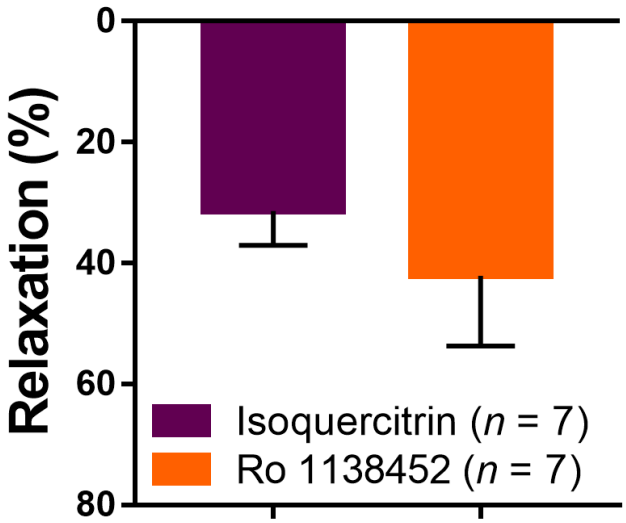


**Figure V.12**: Influence of a selective antagonist Ro 1138452 on the vasorelaxation to isoquercitrin.

# Chapter 6: Discussion

A diet rich in polyphenols can protect against chronic pathologies by the modulation of numerous physiological processes, namely cellular redox potential, enzymatic activity, cell proliferation and signaling transduction pathways (Luca et al. 2020). For example, literature evidence suggests that grape juice, wine and extracts from a variety of vegetables and fruits rich in polyphenols displays vasodilator effects, inducing endothelium-dependent vasorelaxation (Fitzpatrick et al. 1993; Andriambeloson et al. 1997; Perez-Vizcaino et al. 2006).

The vascular endothelium plays a major role in vascular tone, blood flow and control of vascular inflammatory reactions. The loss of the phenotype, that results in an endothelial dysfunction can lead to vasoconstriction and inflammation of the blood vessel (Frombaum et al. 2012b). These features are a common pathophysiological pathway to development and early evolution of cardiovascular diseases(Perez-Vizcaino et al. 2006).

In our research, our aim was to evaluate the vasoactivity of three plants: *A. eupatoria*, *F. vesca* L. and *U. dioica.* Regarding specifically to the vasorelaxant effects, *F. vesca and U. dioica* have been described in the literature. However, our findings showed that, both extracts of *F. vesca* and *U. dioica extract* did not elicit vasorelaxant effects of human arteries.

In contrast, *A. eupatoria* exhibited a marked vasoactivity, which lead to multiple assays with the objective of elucidating the mechanisms and then the compounds responsible for its vasoactivity.

In this context, both the infusion and the ethyl acetate fraction, were samples enriched in polyphenols. Considering the mechanism underlying the vasoactivity observed in ITAs, based on our results, the infusion bioactivity was mediated through NO and COX products. In contrast, the mechanism of the ethyl acetate fraction seems only to involve COX-derived products. This shift of the mechanism can be explained due to many reasons, mainly as loss of compounds as explained in chapter 5, section 5.4.

Our hypothesis was that the vasorelaxation elicited by the infusion was produced by procyanidin B2 through NO pathway and isoquercitrin through COX pathway (Fig VI.1). Although procyanidin B2 was not fully identified in our extract, Correia et al. (2006) identified this compound in the same batch of *A. eupatoria* utilized in this work. In addition, Novakovic et al. (2017) demonstrated that procyanidin B2 elicited an endothelium-dependent vasorelaxation in ITAs.

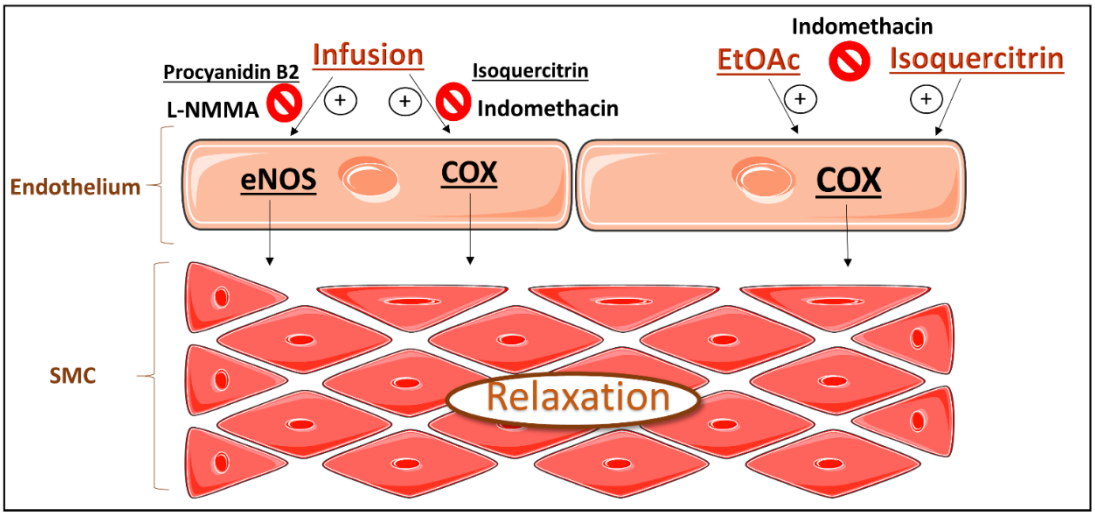


**Figure VI.1**: **Proposed mechanism underlying the vasoactivity of *A. eupatoria*.** Briefly, after the procyanidin B2 stimuli, the endothelium NO through eNOS activation, which can be blocked with L-NMMA. Then, NO diffuses from the endothelium to the smooth muscle leading to activation of guanylyl cyclase in VSMCs, with production of cGMP and thus eliciting vasorelaxation. Furthermore, phospholipase A2 converts membrane phospholipids into arachidonic acid, which is converted into cyclic endoperoxides that subsequently are transformed into prostanoids with vasorelaxant effect.

Flavonoids have gained considerable attention, specifically due to their broad spectrum of health beneficial effects for the treatment of CVDs (Salvamani et al. 2014). Numerous studies support the hypothesis that flavonoids, a major group of compounds among the polyphenols, display protective effects *in vitro,* on the endothelium-derived NO, on the vessel wall, particularly on the endothelial function, with multiple mechanisms underlying their activity (Perez-Vizcaino et al. 2006).

The protection of blood vessels displayed by the polyphenols can be explained due to their ability to act specifically in endothelial cells to increase the synthesis of vasoprotective factors such as NO and EDHF, reduce the formation of COX-derived vasoconstrictors in addition to prevent apoptosis induced by oxidative damage and inhibit the release or action of endothelial derived constrictors, i.e. ET-1. Moreover, in the vascular smooth muscle cells, the reduction of the oxidative stress, partly by decreasing the expression of NADPH oxidase and also the receptors of angiotensin-1(Andriantsitohaina et al. 2012). In VSMCs, flavonoids inhibit directly the contractile and proliferative response induced by several pathological stimuli (Andriantsitohaina et al. 2012).

An improvement of endothelial function, reduction of blood pressure and the associated cardiac, vascular and renal damage and a reduction in the expression and the activity of prooxidative enzymes such as membrane NADPH oxidase was observed in

several animal models of endothelial function with the administration of flavonoids. In addition, although limited, clinical studies have also shown that foods rich in flavonoids improve endothelial function in patients with hypertension, atherosclerosis and coronary disease (Andriantsitohaina et al. 2012; Salvamani et al. 2014)

The three major actions suggested to be related with the decrease of coronary heart disease by dietary flavonoids are the following: preventing low-density lipoproteins (LDLs) from oxidizing; decrease of the ability of platelets in the blood to clot and improvement of coronary vasodilation (Mojžišová and Kuchta 2001). In fact, Perez-Vizcaino et al. (2006) reported an endothelium-dependent relaxation of flavonoids, especially anthocyanin delphinidin and the flavone chrysin.

Flavonols, such as quercetin, are generally more active as vascular relaxant and as antioxidants and offer possible therapeutic agents to improve vascular function after ischemia and reperfusion since their antioxidant and $Ca^{2+}$ inhibitory actions may be beneficial in this setting (Woodman et al. 2005).

Specifically, quercetin derivatives are described as one of the most abundant polyphenolic flavonoid groups, present in fruits and vegetables and are responsible for many biological and health promoting effects in a wide range of diseases(Ebrahimpour et al. 2020). For example, quercetin acts as a direct antioxidant, scavenging ROS such as superoxide anion and peroxynitrite. This capacity has been attributed due two characteristics presented in the molecule: the catechol and -OH groups (Andriantsitohaina et al. 2012; Ebrahimpour et al. 2020)

Isoquercitrin (quercetin-3-*O*-glucoside) is one of the major glycosidic forms of the natural flavonol quercetin (Valentová et al. 2014) and is commonly found in medicinal herbs, fruits, vegetables and plant-derived foods and beverages (Valentová et al. 2014).

In our phytochemistry analysis, we identified isoquercitrin as the major compound in the infusion and EtOAc fraction of *A. eupatoria*. Moreover, this compound exhibited a vasoactivity mainly mediated by cyclooxygenase products, presenting the same behavior of the ethyl acetate fraction.

Interestingly, isoquercitrin showed an endothelium-dependent vasorelaxation however, this mechanism was not confirmed with quercetin. This fact suggests that the pattern of substitution of the compound might play an important role in the mechanism underlying their activity.

The role of the IP receptor was assessed through incubation with a selective antagonist (Ro1138452). However, the relaxation to the ethyl acetate fraction or to isoquercitrin was not statistically significantly blunted or abolished, thus suggesting that other cyclooxygenase products may be involved in the vasorelaxation, rather than

prostacyclin (Fig VI.2). Therefore, the receptors that mediate the relaxation remain unclear.

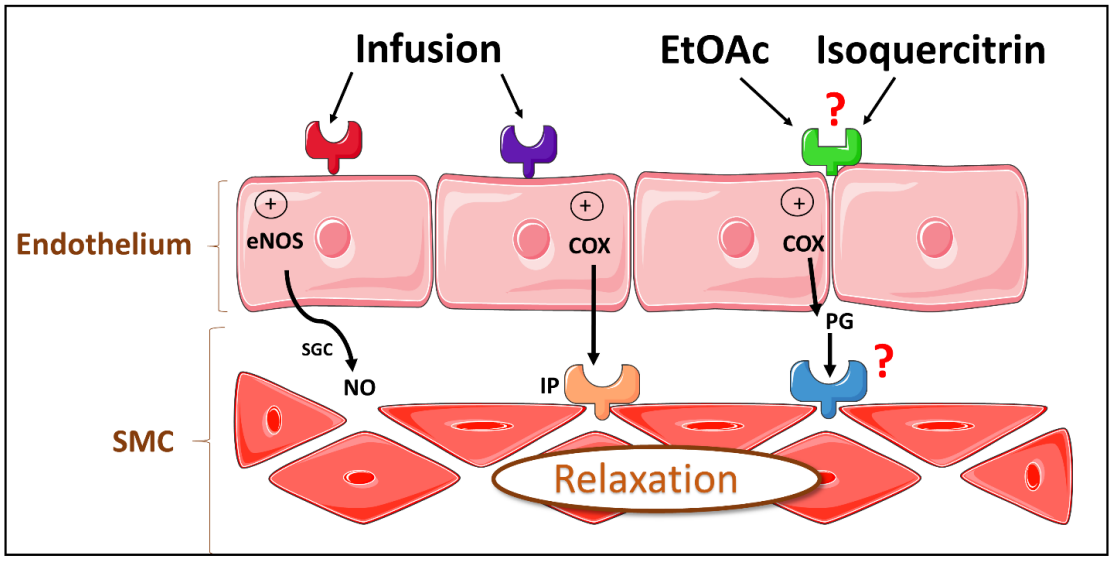


**Figure VI.2**: Our first hypothesis was that the vasorelaxation elicited by isoquercitrin was mediated by the IP receptors. However, in vascular assays with the selective inhibitor Ro 1138452, the relaxation was not statistically significant abolished, thus the receptors that mediate the vasorelaxation to isoquercitrin remain unclear.

Of note, the vasorelaxation elicited by the ethyl acetate fraction and isoquercitrin points to another perspective regarding the pharmacological pathways of vasorelaxation of the ITA. Since the literature points towards NO as the major factor involved in its vasorelaxation, our results suggested that cyclooxygenase products may play an important role.

Since the vasorelaxation to ethyl acetate fraction and isoquercitrin seems to be through other pathway than prostacyclin, our group hypothesized other mechanism that could be involved in the vasoactivity observed. For example, the ITA synthesizes EDHF, a $K^+$ channel activator that persists after inhibition of NO and prostaglandin synthesis. EDHF hyperpolarizes and relaxes SMCs. However, the identity of EDHF in humans is unknown. Archer et al. (2003) suggested that that EDHF is11,12-epoxyeicosatrienoic acid (11,12-EET). Second, that is generated by cytochrome P450-2C, CYP450-2C and finally, that causes relaxation by promoting an opening od SMC large-conductance $Ca^{2+}$ activated $K^+$ channels ($BK_{ca}$).

The employment of ITA as an arterial model presents some limitations. The arterial segments are from patients undergoing coronary revascularization, who generally display a variable clinical profile with multiple cardiovascular risk factors that could interfere with several pathways of regulation of vascular function, e.g. endothelial

function, hypoxia-induced oxidative stress and potential damage or interference because of the harvesting or isolation techniques thus eventually contributing to an increased variability of results (Fonseca et al. 2014). Nevertheless, the use of this arterial model can be advantageous from a translational point of view, as it mimics the human vascular system more closely in comparison to animal-derived tissue.

In the last two decades, the absorption and metabolism of dietary flavonoids have been extensively studied (Xiao 2017).That is an important aspect that should be considered in clinical scenario of administration of *A. eupatoria* extracts to patients with vascular disorders, who typically exhibit reduced endothelial NO production.

Although polyphenols have a low oral bioavailability due to an extensive biotransformation mainly mediated by phase I and phase II reaction in enterocytes and liver and by gut microbiota, most polyphenols have displayed significant biological activity (Luca et al. 2020).

Quercetin and related flavonoids are absorbed in the small intestine. Levels of unconjugated flavonoids in plasma are low, and it is suggested that the most of *in vivo* effects of flavonoids may be due to their conjugated metabolites (Perez-Vizcaino et al. 2006).

In an overview of isoquercitrin, Valentová et al. (2014) specifically described the occurrence, preparation, bioavailability, pharmacokinetics, toxicology and biological activity of isoquercitrin and "enzymatically modified (α-glucosylated) isoquercitrin" (EMIQ). In this report, some bioactivities are attributed to isoquercitrin, such as chemoprotective, antioxidant, protection to cardiovascular disorders, diabetes and allergic reactions. Hence, the commercial interest in this compound has increased, since it is easy to obtain by an enzymatic hydrolysis of rutin (Valentová et al. 2014).

According to Xiao (2017), quercetin-3-*O*-glucoside is rapidly absorbed in humans. After the absorption, the flavonoids are bound to albumin and transported to the liver via portal vein. Occasionally, if a flavonoid glycoside is not absorbed in the small intestine, it can be metabolized by the colonic microflora into its aglycone in the large intestine. Following the absorption, flavonoid glycosides are metabolized by phase II enzymes in small intestine and then in the liver.

Gasparotto et al. (2011) demonstrated that the intravenous administration of isoquercitrin caused a significant reduction in the hypertensive response to angiotensin I, but not angiotensin II in normotensive rats. Results showed that the hypotensive effects caused by the hydroethanolic extract as well as by its semi-purified fraction of *Tropaeolum majus* L., may be associated with the high levels of the flavonoid isoquercitrin

found in this plant. In addition, isoquercitrin-induced hypotension in rats is an event dependent on the inhibition of angiotensin II generation by ACE.

Of note, a randomized, double-blind, placebo-controlled, crossover trial was conducted on 37 pre-hypertensive adults, to evaluate the effect of supplementation with capsules with quercetin-3-*O*-glucoside. The intermediate markers of cardiovascular risk factors were analyzed for 4 weeks. Results showed that the supplementation reduced sE-selectin and z-score for endothelial dysfunction, biomarkers that indicate the dysfunction of the endothelium and inflammation (Dower, Geleijnse, Gijsbers, Schalkwijk, et al. 2015; Dower, Geleijnse, Gijsbers, Zock, et al. 2015).

In terms of metabolism, isoquercitrin is extensively metabolized in the intestine and in the liver and biotransformation of this compounds includes deglycosylation and then, formation of conjugated and methylated derivatives of quercetin or degradation to phenolic acids and carbon dioxide (Valentová et al. 2014)

The process of deglycosilation by small intestine by epithelial cell β-glucosidases it is a critical step in the absorption and metabolism of flavonoid glycosides (Walle et al. 2005). These glycosides are generally absorbed as their aglycones after hydrolyzation along the digestive tract. Absorption of flavonoid glycosides mainly depends on their permeability. Nonetheless, the flavonoid glycosides have a high solubility in water to diffuse across the cellular membrane. Therefore, the absorption requires hydrolyzing their sugar group (Kottra and Daniel 2007; Chen et al. 2011).

Previous published literature indicates that isoquercitrin has a higher solubility in water and bioavailability than the aglycone quercetin, and can be found intact in plasma and tissues after oral application, however is mostly deglycosylated before absorption, thus being a source in quercetin in humans. Meanwhile, there is lack of information concerning the biodistribution and fate of the degradation products *in vivo* (Valentová et al. 2014).

The flavonoid glycosides are commonly hydrolyzed to their aglycones to produce effects *in vivo* (Walle et al. 2005) since the aglycones are characterized of being more hydrophobic and may be easily absorbed by the epithelial cells through passive diffusion (Walle et al. 2005).

Quercetin was also evaluated in our vascular assays, and according to the literature, it is suggested that quercetin might exert benefit in cardiovascular diseases and has been extensively studied over the years (Perez-Vizcaino et al. 2006; Terao 2017).

In addition, quercetin has been reported to improve endothelium-dependent vasorelaxation in aorta and decrease of systolic blood pressure, and reduction of cardiac hypertrophy and proteinuria in hypertensive rats (Salvamani et al. 2014).

Quercetin exhibits significant benefits, such as inhibition of LDL oxidation, endothelium-independent vasodilator effects, reduction of adhesion molecules and other inflammatory markers, also protection of nitric oxide and endothelial function under conditions of oxidative stress and platelet antiaggregant effects (Patel et al. 2018).The possible pathways of the anti-hypertensive action of quercetin include vasodilator effects, VSMC apoptosis/proliferation ROS scavenger, renal effects and inhibition and downregulation of NADPH oxidase (Patel et al. 2018).

Moreover, extensive studies using cell cultures and experimental animals, strongly suggested the anti-atherosclerotic effect potential of quercetin (Terao et al. 2008; Terao 2017).

Oxidative stress can trigger increased activity of MMP-2 in aortas of different models of hypertension.Pereira et al. (2018) hypothesized that quercetin could reduce increased MMP-2 activity by decreasing oxidative stress in aortas of hypertensive rats and then ameliorate hypertension-induced vascular remodeling. In their results, quercetin reduced the hypertension-induced vascular remodeling, oxidative stress and MMP-2 activity in aortas (Pereira et al. 2018).

Quercetin is absorbed at the small intestine level by passive diffusion or by complex formation with membrane transporters (Luca et al. 2020). Following the absorption, quercetin suffers biotransformation in the small intestine, colon, liver and kidney (Luca et al. 2020). The first stage of metabolism is considered deglycosylation. Flavonoids and their derivatives may undergo hydroxylation, methylation and reduction in the liver (Xiao 2017). After, the aglycones are sulfated or glucuronidated to from flavonoids metabolites (Xiao 2017).

The oral supplementation of quercetin was investigated in three different doses. The daily supplementation of healthy humans with graded concentrations of quercetin increased plasma quercetin concentrations, however, did not affect antioxidant status, oxidized LDL, inflammation, or metabolism (Egert et al. 2008).

Quercetin metabolites were also investigated regarding their role in NO bioavailability and endothelial function (Luca et al. 2020).

Multiple metabolites (Q3'S, Q3GA, I3GA) partially prevented the impairment of endothelial-derived NO response under conditions of high oxidative stress induced by a SOD inhibitor (diethyldithiocarbamic acid, DETCA) in endothelium-denuded rat aortic rings. In addition, quercetin, Q3S and Q3GA inhibited NADPH oxidase-derived superoxide release, whereas only quercetin and Q3GA prevented the endothelial dysfunction induced by endothelin-1(Luca et al. 2020).

According with Andriantsitohaina et al. (2012), in a compilation of multiple evidence of several authors, quercetin elicits vasodilation. First, Perez-Vizcaino et al. (2006) and Perez-Vizcaino et al. (2009) indicated that quercetin caused an endothelium-dependent vasodilation through production of NO, and Khoo et al. (2010)suggests that is possible by increasing eNOS phosphorylation. Quercetin was also proposed to increase NO release, leading to hyperpolarization-dependentand $Ca^{2+}$entry in isolated cultured endothelial cells. The result of this effect in endothelium-dependent vasodilatation that is inhibited by eNOS inhibitors and charybdotoxin, thus demonstrating that the quercetin effect is dependent on both the NO/cyclic guanylyl monophosphate pathway and EDHF. In addition, quercetin also increased eNOS and VEGF messenger RNA (mRNA) expression in culture of HUVEC (Nicholson et al. 2010).

Interestingly, Dagher et al. (2021) explored the efficacy of quercetin to reduce inflammation, myocardial injury and senescence in patients undergoing CABG following an acute coronary syndrome, since after this outcome, patients exhibited an elevated inflammatory profile. A phase II, prospective, randomized, double-blind and placebo-controlled clinical trial was designed where participants were randomly assigned to quercetin supplementation or placebo, starting 2 days before surgery until the seventh postoperative day. The primary endpoint consisted in examination of the effects of quercetin on blood inflammatory cytokines and markers of myocardial injury and senescence. The second endpoint consisted in the assessment of the endothelial (dys)function by looking at *ex vivo* vascular reactivity and mRNA expressionof endothelial cells from the wall of discarded segments of ITA.

According to Dagher et al. (2021), the preventive intake of quercetin might help limit the inflammatory response triggered by CABG and consequently, help in postoperative complications in patients suffering from an acute coronary syndrome.

In fact, the results of this trial provided valuable information regarding a novel approach to improve biological, and potentially clinical outcomes post-CABG (Dagher et al. 2021).

# Chapter 7: Conclusions and Future perspectives

In this work, we aimed to screen the vascular activity of leaves extracts from plants which have been previously characterized in terms of phytochemical composition and main biological effects, specifically *A. eupatoria*, *F. vesca* and *U. dioica*.

A vasorelaxant effect has been described in the literature for *F. vesca*. However, in our study, *F. vesca* extracts did not elicit vasorelaxant effects of human arteries harvested from patients with underlying coronary artery disease. In fact, the leaves extract elicited a significant potentiation on the noradrenaline contractile response. Differences between our findings and previous studies could be due to differences in extract preparation and composition and in vascular bed. Further studies should be carried out to fully understand the clinical applicability of *F. vesca* extracts in cardiovascular disorders.

*U. dioica* has been recognized as a natural product for hypertension. Previous studies have suggested its anti-hypertensive potential may result from direct vascular or cardiac effects. In human arteries, we showed that the hydroalcoholic extract of aerial parts of *U. dioica* did not elicit vasorelaxant effects, but modulated the adrenergic vascular contraction. Together with the findings from previous studies, this suggests that the vasorelaxant properties may be attributable to compounds found in the ground parts rather than in the aerial parts. Furthermore, this also confirms the existence of compounds with affinity to human vascular adrenergic receptors which could be responsible for the anti-hypertensive properties of this plant species. The specific compounds and underlying mechanisms remain to be fully characterized.

In contrast, *A. eupatoria* exhibited a marked vasoactivity, which lead to multiple assays with the objective to elucidate the mechanisms responsible for its vasoactivity.

To our knowledge, this is the first report on the direct vascular effects of extracts and compounds from *A. eupatoria.* In our study, we demonstrated that an infusion of *A. eupatoria* may exhibit a mixed vascular activity, in which some compounds may promote mild vasoconstriction while others elicit pronounced vasorelaxation. Interestingly, the EtOAc fraction elicited a decrease in the contraction to noradrenaline, while maintaining a marked vasorelaxant effect. In our study, we showed also that this remarkable vasorelaxation involves both the COX and the NO endothelial pathways, while the COX pathway exhibits a central role in the vasorelaxant effect of the EtOAc fraction. Isoquercitrin, a major compound found in the infusion and EtOAc fraction, showed a marked COX-mediated vasorelaxation, and this result corroborates the activity observed for the EtOAc fraction. Overall, these findings suggest *A. eupatoria* exhibits vasoprotective properties and warrant further research to clarify the mechanistic basis

for this vascular activity (i.e., identify the specific targets and mediators) and to validate the therapeutic potential in several conditions, e.g., atherosclerosis.

From a clinical perspective, the administration of extracts of *A. eupatoria* in patients with vascular disorders could be interesting since it is a major source of polyphenols, specifically, quercetin derivatives thus acting as vasoprotective. In fact, this class of compounds are described in the literature to exert multiple beneficial effects effect in CVDs.

As future perspectives, first, it would be interesting to fully characterize the procyanidins present in the infusion and EtOAc fraction of *A. eupatoria* and then proceed with their quantification. In order to confirm our hypothesis that the vasorelaxation of about 80% of the infusion was elicited both by procyanidin B2 and isoquercitrin, it would be interesting to test solutions with the respective extract amount of procyanidin B2 and isoquercitrin. And finally, quantify the metabolites of NO and prostacyclin, so the pathways of vasorelaxation can be fully confirmed.

# References

Abdel-Wahab MH, El-Mahdy MA, Abd-Ellah MF, Helal GK, Khalifa F, Hamada FMA. 2003. Influence of *p*-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. Pharmacol Res. 48(5):461–465. https://doi.org/10.1016/S1043-6618(03)00214-7.

Abdulazeez MA, Muhammad SA, Saidu Y, Sallau AB, Arzai AA, Tabari MA, Hafiz A, Gwarzo MY, Manosroi J, Idi A, et al. 2021. A systematic review with meta-analysis on the antihypertensive efficacy of Nigerian medicinal plants. J Ethnopharmacol. 279. https://doi.org/10.1016/j.jep.2021.114342.

Ad'hiah AH, Al-Bederi ONH, Al-Sammarrae KW. 2013. Cytotoxic effects of *Agrimonia eupatoria* L. against cancer cell lines in vitro. Journal of the Association of Arab Universities for Basic and Applied Sciences. 14(1):87–92. https://doi.org/10.1016/j.jaubas.2013.01.003

Aldini G, Carini M, Piccoli A, Rossoni G, Facino RM. 2003. Procyanidins from grape seeds protect endothelial cells from peroxynitrite damage and enhance endothelium-dependent relaxation in human artery: New evidences for cardio-protection. Life Sci. 73(22):2883–2898. https://doi.org/10.1016/S0024-3205(03)00697-0.

Al-Snafi AE. 2015. THE PHARMACOLOGICAL AND THERAPEUTIC IMPORTANCE OF *AGRIMONIA EUPATORIA*-A REVIEW. Asian Journal of Pharmaceutical Science & Technology. 5(2):112–117.

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. 2017. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants. 6(4). https://doi.org/10.3390/plants6040042.

Andrade DML de, Borges LL, Torres IMS, da Conceição EC, Rocha ML. 2016. Jabuticaba-induced endothelium-independent vasodilating effect on isolated arteries. Arq Bras Cardiol. 107(3):223–229. https://doi.org/10.5935/abc.20160118.

Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC, Andriantsitohaina R. 1997. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. Br J Pharmacol. 120(6):1053–1058. https://doi.org/10.1038/sj.bjp.0701011.

Andriambeloson E, line Magnier C, le Haan-Archipoff G, Lobstein A, Anton R, Beretz A, Claude Stoclet J, Andriantsitohaina R. 1998. Biochemical and Molecular Roles of Nutrients Natural Dietary Polyphenolic Compounds Cause Endothelium-Dependent Vasorelaxation in Rat Thoracic Aorta 1,2. J Nutr. 128:2324–2333.

Andriantsitohaina R, Auger C, Chataigneau T, Étienne-Selloum N, Li H, Martínez MC, Schini-Kerth VB, Laher I. 2012. Molecular mechanisms of the cardiovascular protective effects of polyphenols. British Journal of Nutrition. 108(9):1532–1549. https://doi.org/10.1017/S0007114512003406.

Anwar MA, al Disi SS, Eid AH. 2016. Anti-hypertensive herbs and their mechanisms of action: Part II. Front Pharmacol. 6. https://doi.org/10.3389/fphar.2016.00050.

de Araújo FF, de Paulo Farias D, Neri-Numa IA, Pastore GM. 2021. Polyphenols and their applications: An approach in food chemistry and innovation potential. Food Chem. 338. https://doi.org/10.1016/j.foodchem.2020.127535.

Archer SL, Gragasin FS, Wu X, Wang S, McMurtry S, Kim DH, Platonov M, Koshal A, Hashimoto K, Campbell WB, et al. 2003. Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle BKCa channels. Circulation. 107(5):769–776. https://doi.org/10.1161/01.CIR.0000047278.28407.C2.

Arts IC, Hollman PC, Feskens EJ, Mesquita HBB de, Kromhout D. 2001. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. The American Journal of Clinical Nutrition. 74:227–232.

Bae H, Kim H-J, Shin M, Lee H, Yin CS, Ra J, Kim J. 2010. Inhibitory effect of Agrimoniae Herba on lipopolysaccharide-induced nitric oxide and proinflammatory cytokine production in BV2 microglial cells. Neurol Res. 32(sup1):53–57. https://doi.org/10.1179/016164109x12537002794002.

Bagdonaite E, Jakstas V, Raudonis R, Janulis V. 2013. Chlorogenic acid, rutin and hyperoside content in *Fragaria vesca*, *F. viridis* and *F. moschata* in Lithuania. Nat Prod Res. 27(2):181–184. https://doi.org/10.1080/14786419.2012.660634.

Baker DD, Chu M, Oza U, Rajgarhia V. 2007. The value of natural products to future pharmaceutical discovery. Nat Prod Rep. 24(6):1225–1244. https://doi.org/10.1039/b602241n.

Barnes EC, Kumar R, Davis RA. 2016. The use of isolated natural products as scaffolds for the generation of chemically diverse screening libraries for drug discovery. Nat Prod Rep. 33(3):372–381. https://doi.org/10.1039/c5np00121h.

Benito S, Lopez D, Saâ MP, Buxaderas S, Saâ J, Puig-Parellada P, Mitjavila MT. 2002. A flavonoid-rich diet increases nitric oxide production in rat aorta. Br J Pharmacol. 135:910–916.

Bensch K, Tiralongo J, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E. 2011. Investigations into the antiadhesive activity of herbal extracts against *Campylobacter jejuni*. Phytotherapy Research. 25(8):1125–1132. https://doi.org/10.1002/ptr.3384.

Bernatova I. 2018. Biological activities of (−)-epicatechin and (−)-epicatechin-containing foods: Focus on cardiovascular and neuropsychological health. Biotechnol Adv. 36(3):666–681. https://doi.org/10.1016/j.biotechadv.2018.01.009.

Bilia AR, Palme E, Marsili A, Pistelli L, Morelli I. 1993. A FLAVONOL GLYCOSIDE FROM *AGRIMONIA EUPATORIA*. Phytochemistry. 32(4):1078–1079.

Bishop-Bailey D, Pepper JR, Larkin SW, Mitchell JA. 1998. Differential Induction of Cyclooxygenase-2 in Human Arterial and Venous Smooth Muscle Role of Endogenous Prostanoids.

Bondonno NP, Bondonno CP, Rich L, Mas E, Shinde S, Ward NC, Hodgson JM, Croft KD. 2016. Acute effects of quercetin-3-O-glucoside on endothelial function and blood pressure: a randomized dose-response study. Am J Clin Nutr. 104(1):97–103. https://doi.org/10.3945/AJCN.116.131268.

Bondonno NP, Bondonno CP, Ward NC, Woodman RJ, Hodgson JM, Croft KD. 2020. Enzymatically modified isoquercitrin improves endothelial function in volunteers at risk of cardiovascular disease. Br J Nutr. 123:182–189. https://doi.org/10.1017/S0007114519002137.

Borović ML, Borović S, Perić M, Vuković P, Marinković J, Todorović V, Radak Đ, Lačković V. 2010. The internal thoracic artery as a transitional type of artery: a morphological and morphometric study. Histol Histopathol. 25(5):561–576.

del Bubba M, Checchini L, Chiuminatto U, Doumett S, Fibbi D, Giordani E. 2012. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of polyphenolic composition of four cultivars of *Fragaria vesca* L. berries and their comparative evaluation. Journal of Mass Spectrometry. 47(9):1207–1220. https://doi.org/10.1002/jms.3030.

Buyel JF. 2018. Plants as sources of natural and recombinant anti-cancer agents. Biotechnol Adv. 36(2):506–520. https://doi.org/10.1016/j.biotechadv.2018.02.002.

Byun EB, Sung NY, Yang MS, Song DS, Byun EH, Kim JK, Park JH, Song BS, Lee JW, Park SH, et al. 2014. Procyanidin C1 causes vasorelaxation through activation of the endothelial no/cGMP pathway in thoracic aortic rings. J Med Food. 17(7):742–748. https://doi.org/10.1089/jmf.2013.2978.

Calderón-Montaño JM, Burgos-Morón E, Pérez-Guerrero C, López-Lázaro M. 2011. A Review on the Dietary Flavonoid Kaempferol. Reviews in Medicinal Chemistry. 11:298–344.

Camejo-Rodrigues J, Ascensão L, Bonet MÀ, Vallès J. 2003. An ethnobotanical study of medicinal and aromatic plants in the Natural Park of "Serra de São Mamede" (Portugal). J Ethnopharmacol. 89(2–3):199–209. https://doi.org/10.1016/S0378-8741(03)00270-8.

Campos M da G, Markhan KR. 2007. Structure information from HPLC and on-line measured absorption spectra: Flavones, Flavonols and Phenolic Acids. 1st ed. Coimbra: Imprensa da Universidade de Coimbra.

Cardoso O, Donato MM, Luxo C, Almeida N, Liberal J, Figueirinha A, Batista MT. 2018. Anti-Helicobacter pylori potential of *Agrimonia eupatoria* L. and *Fragaria vesca*. J Funct Foods. 44:299–303. https://doi.org/10.1016/j.jff.2018.03.027.

Carvalho AR, Costa G, Figueirinha A, Liberal J, Prior JAV, Lopes MC, Cruz MT, Batista MT. 2017. *Urtica spp*.: Phenolic composition, safety, antioxidant and anti-inflammatory

activities. Food Research International. 99:485–494. https://doi.org/10.1016/j.foodres.2017.06.008.

Chaves AA, Joshi MS, Coyle CM, Brady JE, Dech SJ, Schanbacher BL, Baliga R, Basuray A, Bauer JA. 2009. Vasoprotective endothelial effects of a standardized grape product in humans. Vascul Pharmacol. 50(1–2):20–26. https://doi.org/10.1016/j.vph.2008.08.004.

Cheng YC, Sheen JM, Hu WL, Hung YC. 2017. Polyphenols and Oxidative Stress in Atherosclerosis-Related Ischemic Heart Disease and Stroke. Oxid Med Cell Longev. 2017. https://doi.org/10.1155/2017/8526438.

Chen Y, Wang J, Jia X, Tan X, Hu M. 2011. Role of intestinal hydrolase in the absorption of prenylated flavonoids present in yinyanghuo. Molecules. 16(2):1336–1348. https://doi.org/10.3390/molecules16021336.

Cho YM, Kwon JE, Lee M, Lea Y, Jeon D-Y, Kim HJ, Kang SC. 2018. *Agrimonia eupatoria* L. (Agrimony) Extract Alters Liver Health in Subjects with Elevated Alanine Transaminase Levels: A Controlled, Randomized, and Double-Blind Trial. J Med Food. 21(3):282–288. https://doi.org/10.1089/jmf.2017.4054.

Copland A, Nahar L, Tomlinson CTM, Hamilton V, Middleton M, Kumarasamy Y, Sarker SD. 2003. Antibacterial and free radical scavenging activity of the seeds of *Agrimonia eupatoria*. Fitoterapia. 74(1–2):133–135. https://doi.org/10.1016/S0367-326X(02)00317-9.

Correia H, González-Paramás A, Amaral MT, Santos-Buelga C, Batista MT. 2006. Polyphenolic profile characterization of *Agrimonia eupatoria* L. by HPLC with different detection devices. Biomed Chromatogr. 20(1):88–94. https://doi.org/10.1002/bmc.533.

Correia HS, Batista MT, Dinis TCP. 2007. The activity of an extract and fraction of *Agrimonia eupatoria* L. against reactive species. BioFactors. 29(2–3):91–104. https://doi.org/10.1002/biof.552029209.

Couto J, Figueirinha A, Batista MT, Paranhos A, Nunes C, Gonçalves LM, Marto J, Fitas M, Pinto P, Ribeiro HM, Pina ME. 2020. *Fragaria vesca* L. extract: A promising cosmetic

ingredient with antioxidant properties. Antioxidants. 9(2). https://doi.org/10.3390/antiox9020154.

Cwikla C, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E. 2010. Investigations into the antibacterial activities of phytotherapeutics against *Helicobacter pylori* and *Campylobacter jejuni*. Phytotherapy Research. 24(5):649–656. https://doi.org/10.1002/ptr.2933.

Dagher O, Mury P, Noly P-E, Fortier A, Lettre G, Thorin E, Carrier M. 2021. Design of a Randomized Placebo-Controlled Trial to Evaluate the Anti-inflammatory and Senolytic Effects of Quercetin in Patients Undergoing Coronary Artery Bypass Graft Surgery. Front Cardiovasc Med. 8. https://doi.org/10.3389/fcvm.2021.741542.

Devkota HP, Paudel KR, Khanal S, Baral A, Panth N, Adhikari-Devkota A, Jha NK, Das N, Singh SK, Chellappan DK, et al. 2022. Stinging Nettle (*Urtica dioica* L.): Nutritional Composition, Bioactive Compounds, and Food Functional Properties. Molecules. 27(16). https://doi.org/10.3390/molecules27165219.

Dhouibi R, Moalla D, Ksouda K, ben Salem M, Hammami S, Sahnoun Z, Zeghal KM, Affes H. 2018. Screening of analgesic activity of Tunisian *Urtica dioica* and analysis of its major bioactive compounds by GCMS. Arch Physiol Biochem. 124(4):335–343. https://doi.org/10.1080/13813455.2017.1402352.

Dias MI, Barros L, Morales P, Cámara M, Alves MJ, Oliveira MBPP, Santos-Buelga C, Ferreira ICFR. 2016. Wild: *Fragaria vesca* L. fruits: A rich source of bioactive phytochemicals. Food Funct. 7(11):4523–4532. https://doi.org/10.1039/c6fo01042c.

Dias MI, Barros L, Oliveira MBPP, Santos-Buelga C, Ferreira ICFR. 2015. Phenolic profile and antioxidant properties of commercial and wild *Fragaria vesca* L. roots: A comparison between hydromethanolic and aqueous extracts. Ind Crops Prod. 63:125–132. https://doi.org/10.1016/j.indcrop.2014.10.021.

Dias MI, Barros L, Sousa MJ, Oliveira MBPP, Santos-Buelga C, Ferreira ICFR. 2017. Enhancement of nutritional and bioactive compounds by in vitro culture of wild *Fragaria vesca* L. vegetative parts. Food Chem. 235:212–219. https://doi.org/10.1016/j.foodchem.2017.05.060.

DiNatale JC, Crowe-White KM. 2022. Effects of resveratrol supplementation on nitric oxide-mediated vascular outcomes in hypertension: A systematic review. Nitric Oxide. 129:74–81. https://doi.org/10.1016/j.niox.2022.10.005.

Al Disi SS, Anwar MA, Eid AH. 2016. Anti-hypertensive herbs and their mechanisms of action: Part I. Front Pharmacol. 6(JAN). https://doi.org/10.3389/fphar.2015.00323.

Dower JI, Geleijnse JM, Gijsbers L, Schalkwijk C, Kromhout D, Hollman PC. 2015. Supplementation of the pure flavonoids epicatechin and quercetin affects some biomarkers of endothelial dysfunction and inflammation in (pre)hypertensive adults: A randomized double-blind, placebo-controlled, crossover trial. Journal of Nutrition. 145(7):1459–1463. https://doi.org/10.3945/jn.115.211888.

Dower JI, Geleijnse JM, Gijsbers L, Zock PL, Kromhout D, Hollman PCH. 2015. Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: A randomized, double-blind, placebo-controlled, crossover trial. American Journal of Clinical Nutrition. 101(5):914–921. https://doi.org/10.3945/ajcn.114.098590.

Duan H, Zhang Q, Liu J, Li R, Wang D, Peng W, Wu C. 2021. Suppression of apoptosis in vascular endothelial cell, the promising way for natural medicines to treat atherosclerosis. Pharmacol Res. 168. https://doi.org/10.1016/J.PHRS.2021.105599.

Duarte J, Peâ Rez-Palencia R, Vargas F, Ocete MA, Peâ Rez-Vizcaino F, Zarzuelo A, Tamargo J. 2001. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. Br J Pharmacol. 133:117–124.

Dulger B, Gonuz A. 2004. Antimicrobial Activity of SomeTurkish Medicinal Plants. Pakistan Journal of Biological Sciences. 7(9):1559–1562.

Ebrahimpour S, Zakeri M, Esmaeili A. 2020. Crosstalk between obesity, diabetes, and alzheimer's disease: Introducing quercetin as an effective triple herbal medicine. Ageing Res Rev. 62. https://doi.org/10.1016/j.arr.2020.101095.

Egert S, Wolffram S, Bosy-Westphal A, Boesch-Saadatmandi C, Wagner AE, Frank J, Rimbach G, Mueller MJ. 2008. Daily Quercetin Supplementation Dose-Dependently

Increases Plasma Quercetin Concentrations in Healthy Humans 1,2. J Nutr. 138:1615–1621.

Esposito S, Bianco A, Russo R, Di Maro A, Isernia C, Pedone PV. 2019. Therapeutic Perspectives of Molecules from *Urtica dioica* Extracts for Cancer Treatment. Molecules. 24(15). https://doi.org/10.3390/molecules24152753.

European Medicines Agency. 2015. Committee on Herbal Medicinal Products (HMPC) European Union herbal monograph on *Agrimonia eupatoria* L., herba.

European Medicines Agency. 2018. Assessment report on *Fragaria vesca* L., *Fragaria moschata* Weston, *Fragaria viridis* Weston and *Fragaria* x *ananassa* (Weston) Duchesne ex Rozier, folium. www.ema.europa.eu/contact.

Fitzpatrick DF, Hirschfield SL, Coffey RG. 1993. Endothelium-dependent vasorelaxing activity of wine and other grape products.

Fonseca DA, Antunes PE, Antunes MJ, Cotrim MD. 2018. Vasomotion as an oscillatory sign of functional impairment in the human internal thoracic artery: A study based on risk factors and vessel reactivity. Exp Physiol. 103(7):1030–1038. https://doi.org/10.1113/EP087002.

Fonseca DA, Antunes PE, Cotrim MD. 2014. Endothelium-dependent vasoactivity of the human internal mammary artery. Coron Artery Dis. 25(3):266–274. https://doi.org/10.1097/MCA.0000000000000097.

Fonseca DA, Ferreira M, Campos MG, Antunes PE, Antunes MJ, Cotrim MD. 2020. Vascular effects of a polyphenolic fraction from *Oxalis pes-caprae L.*: role of α-adrenergic receptors Sub-types. Nat Prod Res. 34(23):3369–3372. https://doi.org/10.1080/14786419.2018.1564291.

Fonseca DA, Antunes PE, Cotrim MD. 2017. Ultrastructural and histomorphologic properties of the internal thoracic artery: Implications for coronary revascularization. Coron Artery Dis. 28(6):518–527. https://doi.org/10.1097/MCA.0000000000000527.

Foudi N, Kotelevets L, Gomez I, Louedec L, Longrois D, Chastre E, Norel X. 2011. Differential reactivity of human mammary artery and saphenous vein to prostaglandin E 2: Implication for cardiovascular grafts Keywords prostaglandin E2; human mammary artery; human saphenous vein; EP3 receptor; EP4 receptor; vascular tone. Br J Pharmacol. 163:826–834. https://doi.org/10.1111/j.1476-5381.2011.01264.x.

Foudi N, Ozen G, Amgoud Y, Louedec L, Choqueux C, Badi A, Kotelevets L, Chastre E, Longrois D, Norel X. 2017. Decreased vasorelaxation induced by iloprost during acute inflammation in human internal mammary artery. Eur J Pharmacol. 804:31–37. https://doi.org/10.1016/j.ejphar.2017.03.060.

Fraga CG, Croft KD, Kennedy DO, Tomás-Barberán FA. 2019. The effects of polyphenols and other bioactives on human health. Food Funct. 10(2):514–528. https://doi.org/10.1039/c8fo01997e.

Frank T, Netzel G, Kammerer DR, Carle R, Kler A, Kriesl E, Bitsch I, Bitsch R, Netzel M. 2012. Consumption of *Hibiscus sabdariffa* L. aqueous extract and its impact on systemic antioxidant potential in healthy subjects. J Sci Food Agric. 92(10):2207–2218. https://doi.org/10.1002/jsfa.5615.

Frombaum M, Le Clanche S, Bonnefont-Rousselot D, Borderie D. 2012a. Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: Potential benefits to cardiovascular diseases. Biochimie. 94(2):269–276. https://doi.org/10.1016/j.biochi.2011.11.001.

Frombaum M, Le Clanche S, Bonnefont-Rousselot D, Borderie D. 2012b. Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: Potential benefits to cardiovascular diseases. Biochimie. 94(2):269–276. https://doi.org/10.1016/j.biochi.2011.11.001.

Galisteo M, García-Saura MF, Jiménez R, Villar IC, Wangensteen R, Zarzuelo A, Vargas F, Duarte J. 2004. Effects of quercetin treatment on vascular function in deoxycorticosterone acetate-salt hypertensive rats. Comparative study with verapamil. Planta Med. 70(4):334–341. https://doi.org/10.1055/s-2004-818945.

Gallagher AM, Flatt PR, Duffy G, Abdel-Wahab YHA. 2003. The effects of traditional antidiabetic plants on in vitro glucose diffusion. Nutrition Research. 23(3):413–424. https://doi.org/10.1016/S0271-5317(02)00533-X.

Galleano M, Bernatova I, Puzserova A, Balis P, Sestakova N, Pechanova O, Fraga CG. 2013. Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism. IUBMB Life. 65(8):710–715. https://doi.org/10.1002/iub.1185.

Galleano M, Pechanova O, Fraga CG. 2010. Hypertension, Nitric Oxide, Oxidants, and Dietary Plant Polyphenols. Curr Pharm Biotechnol. 11(8):837–848.

Garcia-Oliveira P, Fraga-Corral M, Pereira AG, Lourenço-Lopes C, Jimenez-Lopez C, Prieto MA, Simal-Gandara J. 2020. Scientific basis for the industrialization of traditionally used plants of the Rosaceae family. Food Chem. 330. https://doi.org/10.1016/j.foodchem.2020.127197.

García-Saura MF, Galisteo M, Concepción Villar I, Bermejo A, Zarzuelo A, Vargas F, Duarte J. 2005. Effects of chronic quercetin treatment in experimental renovascular hypertension. Mol Cell Biochem. 270:147–155.

Gaspar MC, Fonseca DA, Antunes MJ, Frigerio C, Gomes NGM, Vieira M, Santos AE, Cruz MT, Cotrim MD, Campos MG. 2018. Polyphenolic characterisation and bioactivity of an *Oxalis pes-caprae* L. leaf extract. Nat Prod Res. 32(6):732–738. https://doi.org/10.1080/14786419.2017.1335728.

Gasparotto A, Gasparotto FM, Lourenço ELB, Crestani S, Stefanello MEA, Salvador MJ, da Silva-Santos JE, Marques MCA, Kassuya CAL. 2011. Antihypertensive effects of isoquercitrin and extracts from *Tropaeolum majus* L.: Evidence for the inhibition of angiotensin converting enzyme. J Ethnopharmacol. 134(2):363–372. https://doi.org/10.1016/j.jep.2010.12.026.

Gasparotto Junior A, dos Reis Piornedo R, Assreuy J, da Silva-Santos JE. 2016. Nitric oxide and Kir6.1 potassium channel mediate isoquercitrin-induced endothelium-dependent and independent vasodilation in the mesenteric arterial bed of rats. Eur J Pharmacol. 788:328–334. https://doi.org/10.1016/J.EJPHAR.2016.08.006.

Gasperotti M, Masuero D, Mattivi F, Vrhovsek U. 2015. Overall dietary polyphenol intake in a bowl of strawberries: The influence of *Fragaria spp*. in nutritional studies. J Funct Foods. 18:1057–1069. https://doi.org/10.1016/j.jff.2014.08.013.

Ghosh D, Scheepens A. 2009. Vascular action of polyphenols. Mol Nutr Food Res. 53(3):322–331. https://doi.org/10.1002/mnfr.200800182.

Gião MS, Gomes S, Madureira AR, Faria A, Pestana D, Calhau C, Pintado ME, Azevedo I, Malcata FX. 2012. Effect of in vitro digestion upon the antioxidant capacity of aqueous extracts of *Agrimonia eupatoria*, *Rubus idaeus*, *Salvia sp.* and *Satureja montana*. Food Chem. 131(3):761–767. https://doi.org/10.1016/j.foodchem.2011.09.030.

Gião MS, Pereira CI, Fonseca SC, Pintado ME, Malcata FX. 2009. Effect of particle size upon the extent of extraction of antioxidant power from the plants *Agrimonia eupatoria*, Salvia sp. and Satureja montana. Food Chem. 117(3):412–416. https://doi.org/10.1016/j.foodchem.2009.04.020.

Giessler C, Wangemann T, Silber RE, Dhein S, Brodde OE. 2002. Noradrenaline-induced contraction of human saphenous vein and human internal mammary artery: Involvement of different α-adrenoceptor subtypes. Naunyn Schmiedebergs Arch Pharmacol. 366(2):104–109. https://doi.org/10.1007/s00210-002-0582-6.

Ginovyan M, Ayvazyan A, Nikoyan A, Tumanyan L, Trchounian A. 2020. Phytochemical Screening and Detection of Antibacterial Components from Crude Extracts of Some Armenian Herbs Using TLC-Bioautographic Technique. Curr Microbiol. 77(7):1223–1232. https://doi.org/10.1007/s00284-020-01929-0.

Gómez-Guzmán M, Jiménez R, Sánchez M, Zarzuelo MJ, Galindo P, Quintela AM, López-Sepúlveda R, Romero M, Tamargo J, Vargas F, et al. 2012. Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension. Free Radic Biol Med. 52(1):70–79. https://doi.org/10.1016/j.freeradbiomed.2011.09.015.

Granica S, Kluge H, Horn G, Matkowski A, Kiss AK. 2015. The phytochemical investigation of *Agrimonia eupatoria* L. and Agrimonia procera Wallr. as valid sources of

*Agrimoniae* herba-The pharmacopoeial plant material. J Pharm Biomed Anal. 114:272–279. https://doi.org/10.1016/j.jpba.2015.05.027.

Granica S, Krupa K, Klebowska A, Kiss AK. 2013. Development and validation of HPLC-DAD-CAD-MS3 method for qualitative and quantitative standardization of polyphenols in *Agrimoniae eupatoriae* herba (Ph. Eur). J Pharm Biomed Anal. 86:112–122. https://doi.org/10.1016/j.jpba.2013.08.006.

Gray AM, Flatt PR. 1998. Actions of the traditional anti-diabetic plant, *Agrimony eupatoria* (agrimony): Effects on hyperglycaemia, cellular glucose metabolism and insulin secretion. Br J Nutr. 80(1):109–114. https://doi.org/10.1017/s0007114598001834.

Grosso G, Godos J, Currenti W, Micek A, Falzone L, Libra M, Giampieri F, Forbes-Hernández TY, Quiles JL, Battino M, et al. 2022. The Effect of Dietary Polyphenols on Vascular Health and Hypertension: Current Evidence and Mechanisms of Action. Nutrients. 14(3). https://doi.org/10.3390/nu14030545.

Hansson GK. 2005. Inflammation, Atherosclerosis, and Coronary Artery Disease. The New England journal of medicine . 352:1685–95.

Hedrington MS, Davis SN. 2019. Considerations when using alpha-glucosidase inhibitors in the treatment of type 2 diabetes. Expert Opin Pharmacother. 20(18):2229–2235. https://doi.org/10.1080/14656566.2019.1672660.

He G-W. 2015. Cautions on use of distal internal thoracic artery and its bifurcations. J Thorac Cardiovasc Surg. 149(4):1050–1051. https://doi.org/10.1016/j.jtcvs.2014.12.046

He M, Min JW, Kong WL, He XH, Li JX, Peng BW. 2016. A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia. 115:74–85. https://doi.org/10.1016/j.fitote.2016.09.011.

Hou X, Liu Y, Niu L, Cui L, Zhang M. 2014. Enhancement of voltage-gated K+ channels and depression of voltage-gated Ca2+ channels are involved in quercetin-induced vasorelaxation in rat coronary artery. Planta Med. 80(6):465–472. https://doi.org/10.1055/s-0034-1368320.

Huang Y, Wai N, Chan K, Lau CW, Yao XQ, Chan FL, Chen ZY. 1999. Involvement of endothelium/nitric oxide in vasorelaxation induced by purified green tea (-)epicatechin. Biochimica et Biophysica Acta . 1427:322–328.

Ibrahim DS, Abd El-Maksoud MAE. 2015. Effect of strawberry (*Fragaria* × *ananassa*) leaf extract on diabetic nephropathy in rats. Int J Exp Pathol. 96(2):87–93. https://doi.org/10.1111/iep.12116.

Iozzi D, Schubert R, Kalenchuk VU, Neri A, Sgaragli G, Fusi F, Saponara S. 2013. Quercetin relaxes rat tail main artery partly via a PKG-mediated stimulation of KCa1.1 channels. Acta Physiol. 208(4):329–339. https://doi.org/10.1111/apha.12083.

Ivanova D, Gerova D, Chervenkov T, Yankova T. 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J Ethnopharmacol. 96(1–2):145–150. https://doi.org/10.1016/j.jep.2004.08.033.

Ivanova D, Vankova D, Kiselova-Kaneva Y. 2011. ANTIOXIDATIVE POTENTIAL OF *AGRIMONIA EUPATORIA* L. Science & Technologies. I(1):20–25.

Ivanova D, Vankova D, Nashar M. 2013. Agrimonia eupatoria tea consumption in relation to markers of inflammation, oxidative status and lipid metabolism in healthy subjects. Arch Physiol Biochem. 119(1):32–37. https://doi.org/10.3109/13813455.2012.729844.

Je HG, Hong SM, Je HD, Sohn UD, Choi YS, Seo SY, Min YS, Chung SJ, Shin YK, Lee TJ, et al. 2014. The inhibitory effect of vitexin on the agonist-induced regulation of vascular contractility. Pharmazie. 69(3):224–228. https://doi.org/10.1691/ph.2014.3803.

Kamyab R, Namdar H, Torbati M, Ghojazadeh M, Araj-Khodaei M, Fazljou SMB. 2021. Medicinal plants in the treatment of hypertension: A review. Adv Pharm Bull. 11(4):601–617. https://doi.org/10.34172/APB.2021.090.

Kanodia L, Borgohain M, Das S. 2011. Effect of fruit extract of *Fragaria vesca* L. on experimentally induced inflammatory bowel disease in albino rats. Indian J Pharmacol. 43(1):18–21. https://doi.org/10.4103/0253-7613.75660.

Kanodia L, Das S. 2009. A comparative study of analgesic property of whole plant and fruit extracts of *Fragaria vesca* in experimental animal models. Bangladesh J Pharmacol. 4(1):35–38. https://doi.org/10.3329/bjp.v4i1.1049.

Kasote DM, Katyare SS, Hegde M v., Bae H. 2015. Significance of antioxidant potential of plants and its relevance to therapeutic applications. Int J Biol Sci. 11(8):982–991. https://doi.org/10.7150/ijbs.12096.

Khoo NKH, White CR, Pozzo-Miller L, Zhou F, Constance C, Inoue T, Patel RP, Parks DA. 2010. Dietary flavonoid quercetin stimulates vasorelaxation in aortic vessels. Free Radic Biol Med. 49(3):339–347. https://doi.org/10.1016/j.freeradbiomed.2010.04.022.

Kim CH, Kim JE, Song YJ. 2020. Antiviral activities of quercetin and isoquercitrin against human herpesviruses. Molecules. 25(10). https://doi.org/10.3390/molecules25102379.

Kirsch J, Jung A, Hille K, König B, Hannig C, Kölling-Speer I, Speer K, Hannig M. 2020. Effect of *fragaria vesca*, hamamelis and tormentil on the initial bacterial colonization in situ. Arch Oral Biol. 118. https://doi.org/10.1016/j.archoralbio.2020.104853.

Kiselova Y, Ivanova D, Chervenkov T, Gerova D, Galunska B, Yankova T. 2006. Correlation between the In Vitro Antioxidant Activity and Polyphenol Content of Aqueous Extracts from Bulgarian Herbs. Phytother Res. 20:961–965. https://doi.org/10.1002/ptr.

Kiselova Y; NM; ID. 2011. Effects of Dietary Administration of *Agrimonia Eupatoria* L. in Experimental Model of Metabolic Syndrome. Obes Rev. 12:155.

Komiazyk M, Palczewska M, Sitkiewicz I, Pikula S, Groves P. 2019. Neutralization of cholera toxin by Rosaceae family plant extracts. BMC Complement Altern Med. 19(1). https://doi.org/10.1186/s12906-019-2540-6.

Kottra G, Daniel H. 2007. Flavonoid glycosides are not transported by the human Na +/glucose transporter when expressed in *Xenopus laevis* oocytes, but effectively inhibit electrogenic glucose uptake. Journal of Pharmacology and Experimental Therapeutics. 322(2):829–835. https://doi.org/10.1124/jpet.107.124040.

Kraler S, Libby P, Evans PC, Akhmedov A, Schmiady MO, Reinehr M, Camici GG, Lüscher TF. 2021. Resilience of the Internal Mammary Artery to Atherogenesis: Shifting From Risk to Resistance to Address Unmet Needs. Arterioscler Thromb Vasc Biol. 41(8):2237–2251. https://doi.org/10.1161/ATVBAHA.121.316256.

Kubínová R, Jankovská D, Bauerová V. 2012. ANTIOXIDANT AND α-GLUCOSIDASE INHIBITION ACTIVITIES AND POLYPHENOL CONTENT OF FIVE SPECIES OF *AGRIMONIA* GENUS. Acta fytotechnica et zootechnica. 2:38–41.

Kubínová R, Švajdlenka E, Jankovská D. 2016. Anticholinesterase, antioxidant activity and phytochemical investigation into aqueous extracts from five species of *Agrimonia* genus. Nat Prod Res. 30(10):1174–1177. https://doi.org/10.1080/14786419.2015.1043552.

Kuczmannová A, Balažová A, Račanská E, Kameníková M, Fialová S, Majerník J, Nagy M, Gál P, Mučaji P. 2016. *Agrimonia eupatoria* L. and *Cynara cardunculus* L. water infusions: Comparison of anti-diabetic activities. Molecules. 21(5):1–12. https://doi.org/10.3390/molecules21050564.

Kuczmannová A, Gál P, Varinská L, Treml J, Kováč I, Novotný M, Vasilenko T, Dall'Acqua S, Nagy M, Mučaji P. 2015. *Agrimonia eupatoria* L. and *Cynara cardunculus* L. water infusions: Phenolic profile and comparison of antioxidant activities. Molecules. 20(11):20538–20550. https://doi.org/10.3390/molecules201119715.

Kumar S, Pandey AK. 2013. Chemistry and biological activities of flavonoids: An overview. The Scientific World Journal. 2013. https://doi.org/10.1155/2013/162750.

Kwon DH, Kwon HY, Kim HJ, Chang EJ, Kim MB, Yoon SK, Song EY, Yoon DY, Lee YH, Choi IS, Choi YK. 2005. Inhibition of hepatitis B virus by an aqueous extract of *Agrimonia eupatoria* L. Phytotherapy Research. 19(4):355–358. https://doi.org/10.1002/ptr.1689.

Lagunas-Herrera H, Tortoriello J, Herrera-Ruiz M, Belen Martínez-Henández G, Zamilpa A, Santamaría LA, Lorenzana MG, Lombardo-Earl G, Jiménez-Ferrer E. 2019. Acute and Chronic Antihypertensive Effect of Fractions, Tiliroside and Scopoletin from *Malva parviflora*. Biol Pharm Bull. 18(1):18–25.

Landmesser U, Drexler H. 2007. Endothelial function and hypertension. Curr Opin Cardiol. 22:316–320.

Leblais V, Krisa S, Valls J, Courtois A, Abdelouhab S, Vila AM, Mérillon JM, Muller B. 2008. Relaxation induced by red wine polyphenolic compounds in rat pulmonary arteries: Lack of inhibition by NO-synthase inhibitor. Fundam Clin Pharmacol. 22(1):25–35. https://doi.org/10.1111/j.1472-8206.2007.00546.x

Lee KH, Rhee KH. 2016. Anti-nociceptive effect of *agrimonia eupatoria* extract on a cisplatin-induced neuropathic model. African Journal of Traditional, Complementary and Alternative Medicines. 13(5):139–144. https://doi.org/10.21010/ajtcam.v13i5.18.

Lee KY, Hwang L, Jeong EJ, Kim SH, Kim YC, Sung SH. 2010. Effect of Neuroprotective Flavonoids of *Agrimonia eupatoria* on Glutamate-Induced Oxidative Injury to HT22 Hippocampal Cells. Biosci Biotechnol Biochem. 74(8):1704–1706. https://doi.org/10.1271/bbb.100200.

Legssyer A, Ziyyat A, Mekhfi H, Bnouham M, Tahri A, Serhrouchni M, Hoerter J, Fischmeister R. 2002. Cardiovascular effects of *Urtica dioica* L. in isolated rat heart and aorta. Phytotherapy Research. 16(6):503–507. https://doi.org/10.1002/ptr.1087.

Liberal J, Costa G, Carmo A, Vitorino R, Marques C, Domingues MR, Domingues P, Gonçalves AC, Alves R, Sarmento-Ribeiro AB, et al. 2019. Chemical characterization and cytotoxic potential of an ellagitannin-enriched fraction from Fragaria vesca leaves. Arabian Journal of Chemistry. 12(8):3652–3666. https://doi.org/10.1016/j.arabjc.2015.11.014.

Liberal J, Francisco V, Costa G, Figueirinha A, Amaral MT, Marques C, Girão H, Lopes MC, Cruz MT, Batista MT. 2014. Bioactivity of *Fragaria vesca* leaves through inflammation, proteasome and autophagy modulation. J Ethnopharmacol. 158:113–122. https://doi.org/10.1016/j.jep.2014.09.043.

Liu L, Huang S, Xu M, Gong Y, Li D, Wan C, Wu H, Tang Q. 2021. Isoquercitrin protects HUVECs against high glucose-induced apoptosis through regulating p53 proteasomal degradation. Int J Mol Med. 48(1). https://doi.org/10.3892/ijmm.2021.4955.

Luca SV, Macovei I, Bujor A, Miron A, Skalicka-Woźniak K, Aprotosoaie AC, Trifan A. 2020. Bioactivity of dietary polyphenols: The role of metabolites. Crit Rev Food Sci Nutr. 60(4):626–659. https://doi.org/10.1080/10408398.2018.1546669.

Luceri C, Giannini L, Lodovici M, Antonucci E, Abbate R, Masini E, Dolara P. 2007. p-Coumaric acid, a common dietary phenol, inhibits platelet activity in vitro and in vivo. Br J Nutr. 97(3):458–463. https://doi.org/10.1017/S0007114507657882.

Mabry TMKTM. 1970. The systematic identification of flavonoids. Berlin: Springer-Verlag.
Machha A, Rais Mustafa M. 2005. Chronic Treatment with Flavonoids Prevents Endothelial Dysfunction in Spontaneously Hypertensive Rat Aorta. J Cardiovasc Pharmacol. 46:36–40.

Malheiros J, Simões DM, Antunes PE, Figueirinha A, Cotrim MD, Fonseca DA. 2022a. Vascular Effects of Polyphenols from *Agrimonia eupatoria* L. and Role of Isoquercitrin in Its Vasorelaxant Potential in Human Arteries. Pharmaceuticals. 15(5). https://doi.org/10.3390/ph15050638.

Malheiros J, Simões DM, Antunes PE, Figueirinha A, Cotrim MD, Fonseca DA. 2022b. Vascular effects of *Fragaria vesca* L. in human arteries. Nat Prod Res. https://doi.org/10.1080/14786419.2022.2152448.

Malheiros J, Simões DM, Figueirinha A, Cotrim MD, Fonseca DA. 2022. *Agrimonia eupatoria* L.: An integrative perspective on ethnomedicinal use, phenolic composition and pharmacological activity. J Ethnopharmacol. 296. https://doi.org/10.1016/j.jep.2022.115498.

Manolova L. 2003. Natural Pharmacy. 1st ed. Pittsburgh: Dorrance Publishing Co, Inc.

Marunaka Y, Marunaka R, Sun H, Yamamoto T, Kanamura N, Inui T, Taruno A. 2017. Actions of quercetin, a polyphenol, on blood pressure. Molecules. 22(2). https://doi.org/10.3390/molecules22020209.

Matos P, Figueirinha A, Paranhos A, Nunes F, Cruz P, Geraldes CFGC, Cruz MT, Batista MT. 2018. Bioactivity of Acanthus mollis – Contribution of benzoxazinoids and

phenylpropanoids. J Ethnopharmacol. 227(May):198–205. https://doi.org/10.1016/j.jep.2018.09.013.

Ma Y, Zhang P, Li J, Lu J, Ge J, Zhao Z, Ma X, Wan S, Yao X, Shen B. 2015. Epoxyeicosatrienoic acids act through TRPV4-TRPC1-KCa1.1 complex to induce smooth muscle membrane hyperpolarization and relaxation in human internal mammary arteries. Biochim Biophys Acta Mol Basis Dis. 1852(3):552–559. https://doi.org/10.1016/j.bbadis.2014.12.010.

Mihaljević Z, Matić A, Stupin A, Frkanec R, Tavčar B, Kelava V, Bujak IT, Kolobarić N, Kibel A, Drenjančević I. 2020. Arachidonic acid metabolites of cyp450 enzymes and hif-1α modulate endothelium-dependent vasorelaxation in sprague-dawley rats under acute and intermittent hyperbaric oxygenation. Int J Mol Sci. 21(17):1–21. https://doi.org/10.3390/ijms21176353.

Mojžišová G, Kuchta M. 2001. Dietary Flavonoids and Risk of Coronary Heart Disease. Physiol Res. 50:529–535.

Motoyama K, Koyama H, Moriwaki M, Emura K, Okuyama S, Sato E, Inoue M, Shioi A, Nishizawa Y. 2009. Atheroprotective and plaque-stabilizing effects of enzymatically modified isoquercitrin in atherogenic apoE-deficient mice. Nutrition. 25(4):421–427. https://doi.org/10.1016/J.NUT.2008.08.013.

Mudnic I, Modun D, Brizic I, Vukovic J, Generalic I, Katalinic V, Bilusic T, Ljubenkov I, Boban M. 2009. Cardiovascular effects in vitro of aqueous extract of wild strawberry (*Fragaria vesca*, L.) leaves. Phytomedicine. 16(5):462–469. https://doi.org/10.1016/j.phymed.2008.11.004.

Muruzović M, Mladenović KG, Stefanović OD, Vasić SM, Čomić LR. 2016. Extracts of *Agrimonia eupatoria* L. as sources of biologically active compounds and evaluation of their antioxidant, antimicrobial, and antibiofilm activities. J Food Drug Anal. 24(3):539–547. https://doi.org/10.1016/j.jfda.2016.02.007.

Nakatsu K, Pym J, Witte S, Marks GS, Bhp JE. 1991. Adrenoceptors of the human internal thoracic artery. J Physiol Pkarmacol. 69:792–797.

Newman DJ, Cragg GM. 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod. 75(3):311–335. https://doi.org/10.1021/np200906s.

Newman DJ, Cragg GM. 2016. Natural Products as Sources of New Drugs from 1981 to 2014. J Nat Prod. 79(3):629–661. https://doi.org/10.1021/acs.jnatprod.5b01055.

Nicholson SK, Tucker GA, Brameld JM. 2008. Effects of dietary polyphenols on gene expression in human vascular endothelial cells. Proceedings of the Nutrition Society. 67(1):42–47. https://doi.org/10.1017/S0029665108006009.

Nicholson SK, Tucker GA, Brameld JM. 2010. Physiological concentrations of dietary polyphenols regulate vascular endothelial cell expression of genes important in cardiovascular health. British Journal of Nutrition. 103(10):1398–1403. https://doi.org/10.1017/S0007114509993485.

Novakovic A, Marinko M, Jankovic G, Stojanovic I, Milojevic P, Nenezic D, Kanjuh V, Yang Q, He GW. 2017. Endothelium-dependent vasorelaxant effect of procyanidin B2 on human internal mammary artery. Eur J Pharmacol. 807(February):75–81. https://doi.org/10.1016/j.ejphar.2017.04.015.

Novakovic A, Marinko M, Vranic A, Jankovic G, Milojevic P, Stojanovic I, Nenezic D, Ugresic N, Kanjuh V, Yang Q, He GW. 2015. Mechanisms underlying the vasorelaxation of human internal mammary artery induced by (-)-epicatechin. Eur J Pharmacol. 762:306–312. https://doi.org/10.1016/j.ejphar.2015.05.066.

Nyakudya TT, Tshabalala T, Dangarembizi R, Erlwanger KH, Ndhlala AR. 2020. The potential therapeutic value of medicinal plants in the management of metabolic disorders. Molecules. 25(11). https://doi.org/10.3390/molecules25112669.

Oak MH, Auger C, Belcastro E, Park SH, Lee HH, Schini-Kerth VB. 2018. Potential mechanisms underlying cardiovascular protection by polyphenols: Role of the endothelium. Free Radic Biol Med. 122:161–170. https://doi.org/10.1016/j.freeradbiomed.2018.03.018.

Orcic D, Franciškovic M, Bekvalac K, Svircev E, Beara I, Lesjak M, Mimica-Dukic N. 2014. Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-

performance liquid chromatography coupled with tandem mass spectrometric detection. Food Chem. 143:48–53. https://doi.org/10.1016/j.foodchem.2013.07.097.

Paluch Z, BIRICZOVÁ L, PALLAG G, MARQUES EC, VARGOVÁ N, KMONÍČKOVÁ E. 2020. The therapeutic effects of *Agrimonia eupatoria* L. Physiol Res. 69(Suppl 4):S555–S571. https://doi.org/10.33549/PHYSIOLRES.934641.

Pan Y, Liu T, Wang X, Sun J. 2022. Research progress of coumarins and their derivatives in the treatment of diabetes. J Enzyme Inhib Med Chem. 37(1):616–628. https://doi.org/10.1080/14756366.2021.2024526.

Parsamanesh N, Asghari A, Sardari S, Tasbandi A, Jamialahmadi T, Xu S, Sahebkar A. 2021. Resveratrol and endothelial function: A literature review. Pharmacol Res. 170:1–14. https://doi.org/10.1016/j.phrs.2021.105725.

Patel R v., Mistry BM, Shinde SK, Syed R, Singh V, Shin HS. 2018. Therapeutic potential of quercetin as a cardiovascular agent. Eur J Med Chem. 155:889–904. https://doi.org/10.1016/j.ejmech.2018.06.053.

Pei K, Ou J, Huang J, Ou S. 2016. p-Coumaric acid and its conjugates: Dietary sources, pharmacokinetic properties and biological activities. J Sci Food Agric. 96(9):2952–2962. https://doi.org/10.1002/jsfa.7578.

Penumathsa SV, Maulik N. 2009. Resveratrol: A promising agent in promoting cardioprotection against coronary heart disease. Can J Physiol Pharmacol. 87(4):275–286. https://doi.org/10.1139/Y09-013.

Pereira SC, Parente JM, Belo VA, Mendes AS, Gonzaga NA, do Vale GT, Ceron CS, Tanus-Santos JE, Tirapelli CR, Castro MM. 2018. Quercetin decreases the activity of matrix metalloproteinase-2 and ameliorates vascular remodeling in renovascular hypertension. Atherosclerosis. 270:146–153. https://doi.org/10.1016/j.atherosclerosis.2018.01.031.

Perez-Vizcaino F, Duarte J, Andriantsitohaina R. 2006. Endothelial function and cardiovascular disease: Effects of quercetin and wine polyphenols. Free Radic Res. 40(10):1054–1065. https://doi.org/10.1080/10715760600823128.

Perez-Vizcaino F, Duarte J, Jimenez R, Santos-Buelga C, Osuna A. 2009. Antihypertensive effects of the flavonoid quercetin. Pharmacological reports. 61:67–75.

Pietta P, Minoggio M, Bramati L. 2003. PLANT POLYPHENOLS: STRUCTURE, OCCURRENCE AND BIOACTIVITY. Studies in Natural Products Chemistry. 28:257–312.

Popiolek-Kalisz J, Fornal E. 2022. The Effects of Quercetin Supplementation on Blood Pressure – Meta-Analysis. Curr Probl Cardiol. 47(11). https://doi.org/10.1016/j.cpcardiol.2022.101350.

Potenza MA, Marasciulo FL, Tarquinio M, Tiravanti E, Colantuono G, Federici A, Kim J-A, Quon MJ, Montagnani M. 2007. EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. Am J Physiol Endocrinol Metab. 292:1378–1387. https://doi.org/10.1152/ajpendo.00698.2006.

Pukalskienė M, Slapšytė G, Dedonytė V, Lazutka JR, Mierauskienė J, Venskutonis PR. 2018. Genotoxicity and antioxidant activity of five *Agrimonia* and *Filipendula* species plant extracts evaluated by comet and micronucleus assays in human lymphocytes and Ames Salmonella/microsome test. Food and Chemical Toxicology. 113:303–313. https://doi.org/10.1016/j.fct.2017.12.031.

Qayyum R, Qamar HM ud D, Khan S, Salma U, Khan T, Shah AJ. 2016. Mechanisms underlying the antihypertensive properties of *Urtica dioica*. J Transl Med. 14(1):1–13. https://doi.org/10.1186/s12967-016-1017-3.

Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, Nishigaki I. 2013. The vascular endothelium and human diseases. Int J Biol Sci. 9(10):1057–1069. https://doi.org/10.7150/ijbs.7502.

Rao AA, Sridhar GR, Das UN. 2007. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitus and Alzheimer's disease. Med Hypotheses. 69(6):1272–1276. https://doi.org/10.1016/j.mehy.2007.03.032.

Rasines-Perea Z, Teissedre PL. 2017. Grape Polyphenols' effects in human cardiovascular diseases and diabetes. Molecules. 22(1). https://doi.org/10.3390/molecules22010068.

Rivera L, Morón R, Zarzuelo A, Galisteo M. 2009. Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. Biochem Pharmacol. 77(6):1053–1063. https://doi.org/10.1016/j.bcp.2008.11.027.

Roghani M, Baluchnejadmojarad T, Reza Vaez-Mahdavi M, Roghani-Dehkordi F. 2004. Mechanisms underlying quercetin-induced vasorelaxation in aorta of subchronic diabetic rats: An in vitro study. Vascul Pharmacol. 42(1):31–35. https://doi.org/10.1016/j.vph.2004.12.001.

Roschek B, Fink RC, Mcmichael M, Alberte RS. 2009. Nettle Extract (*Urtica dioica*) Affects Key Receptors and Enzymes Associated with Allergic Rhinitis. Phytother Res. 23:920–926. https://doi.org/10.1002/ptr.

Sabri NAM, Lee SK, Murugan DD, Ling WC. 2022. Epigallocatechin gallate (EGCG) alleviates vascular dysfunction in angiotensin II-infused hypertensive mice by modulating oxidative stress and eNOS. Sci Rep. 12(1). https://doi.org/10.1038/s41598-022-21107-5.

Sajja LR, Mannam G. 2015. Internal thoracic artery: Anatomical and biological characteristics revisited. Asian Cardiovasc Thorac Ann. 23(1):88–99. https://doi.org/10.1177/0218492314523629.

Salvamani S, Gunasekaran B, Shaharuddin NA, Ahmad SA, Shukor MY. 2014. Antiartherosclerotic effects of plant flavonoids. Biomed Res Int. 2014. https://doi.org/10.1155/2014/480258.

Samaha AA, Fawaz M, Salami A, Baydoun S, Eid AH. 2019. Antihypertensive Indigenous Lebanese Plants: Ethnopharmacology and a Clinical Trial. Biomolecules 2019, Vol 9, Page 292. 9(7):292. https://doi.org/10.3390/BIOM9070292.

Sánchez M, Galisteo M, Vera R, Villar IC, Zarzuelo A, Tamargo J, Pé Rez-Vizcaíno F, Duarte J. 2006. Quercetin downregulates NADPH oxidase, increases eNOS activity and prevents endothelial dysfunction in spontaneously hypertensive rats. J Hypertens. 24:75–84.

Dos Santos TC, Gomes TM, Pinto BAS, Camara AL, De Andrade Paes AM. 2018. Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy. Front Pharmacol. 9(OCT). https://doi.org/10.3389/fphar.2018.01192.

Santos TN, Costa G, Ferreira JP, Liberal J, Francisco V, Paranhos A, Cruz MT, Castelo-Branco M, Figueiredo IV, Batista MT. 2017. Antioxidant, Anti-Inflammatory, and Analgesic Activities of *Agrimonia eupatoria* L. Infusion. Evid Based Complement Alternat Med. 2017. https://doi.org/10.1155/2017/8309894.

Schini-Kerth VB, Auger C, Étienne-Selloum N, Chataigneau T. 2010. Polyphenol-Induced Endothelium-Dependent Relaxations: Role of NO and EDHF. Cardiovascular Pharmacology: Endothelial Control. 60:133–175. https://doi.org/10.1016/S1054-3589(10)60004-X.

Sen T, Samanta SK. 2014. Medicinal plants, human health and biodiversity: A broad review. Adv Biochem Eng Biotechnol. 147:59–110. https://doi.org/10.1007/10_2014_273.

Shah AA, Dar TA, Dar PA, Ganie SA, Kamal MA. 2017. A Current Perspective on the Inhibition of Cholinesterase by Natural and Synthetic Inhibitors. Curr Drug Metab. 18(2):96–111. https://doi.org/10.2174/1389200218666161123122734.

Sigowska P, Zimoch M, Baska A, Kazik J, Leis K, Grześk G. 2022. Genistein in the Treatment of Hypertension: A Review. Endocr Metab Immune Disord Drug Targets. 22(14):1367–1377. https://doi.org/10.2174/1871530322666220510125115.

Silva GC, Pereira AC, Rezende BA, da Silva JPF, Cruz JS, de Souza MDF v., Gomes RA, Teles YCF, Cortes SF, Lemos VS. 2013. Mechanism of the antihypertensive and vasorelaxant effects of the flavonoid tiliroside in resistance arteries. Planta Med. 79(12):1003–1008. https://doi.org/10.1055/s-0032-1328765.

Simões DM, Malheiros J, Antunes PE, Figueirinha A, Cotrim MD, Fonseca DA. 2020. Vascular activity of infusion and fractions of Cymbopogon citratus (DC) Stapf. in human arteries. J Ethnopharmacol. 258. https://doi.org/10.1016/j.jep.2020.112947.

Soares-Bezerra RJ, Calheiros AS, Ferreira NC da S, Frutuoso V da S, Alves LA. 2013. Natural products as a source for new anti-inflammatory and analgesic compounds through the inhibition of purinergic P2X receptors. Pharmaceuticals. 6(5):650–658. https://doi.org/10.3390/ph6050650.

Soares SS, Bekbolatova E, Cotrim MD, Sakipova Z, Ibragimova L, Kukula-Koch W, Giorno TBS, Fernandes PD, Fonseca DA, Boylan F. 2019. Chemistry and pharmacology of the kazakh *crataegus almaatensis* pojark: An asian herbal medicine. Antioxidants. 8(8). https://doi.org/10.3390/antiox8080300.

Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El Bedoui J, Chataigneau M, Schini-Kerth VB. 2004. Vascular protection by dietary polyphenols. Eur J Pharmacol. 500:299–313. https://doi.org/10.1016/j.ejphar.2004.07.034.

Sun J, Liu X, Yang T, Slovin J, Chen P. 2014. Profiling polyphenols of two diploid strawberry (*Fragaria vesca*) inbred lines using UHPLC-HRMSn. Food Chem. 146:289–298. https://doi.org/10.1016/j.foodchem.2013.08.089.

Suzuki A, Yamamoto N, Jokura H, Yamamoto M, Fujii A, Tokimitsu I, Saito I. 2006. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. J Hypertens. 24:1065–1073.

Swanston-Flatt SK, Day C, Bailey CJ, Flat PR. 1990. Traditional plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice. Diabetologia. 33:462–464.

Tahri A, Yamani S, Legssyer A, Aziz M, Mekhfi H, Bnouham M, Ziyyat A. 2000. Acute diuretic, natriuretic and hypotensive effects of a continuous perfusion of aqueous extract of *Urtica dioica* in the rat. J Ethnopharmacol. 73(1–2):95–100. https://doi.org/10.1016/S0378-8741(00)00270-1.

Teka T, Zhang L, Ge X, Li Y, Han L, Yan X. 2022. Stilbenes: Source plants, chemistry, biosynthesis, pharmacology, application and problems related to their clinical Application-A comprehensive review. Phytochemistry. 197. https://doi.org/10.1016/j.phytochem.2022.113128

Terao J. 2017. Factors modulating bioavailability of quercetin-related flavonoids and the consequences of their vascular function. Biochem Pharmacol. 139:15–23. https://doi.org/10.1016/j.bcp.2017.03.021.

Terao J, Kawai Y, Murota K. 2008. Vegetable flavonoids and cardiovascular disease. Asia Pac J Clin Nutr. 17(S1):291–293.

Testai L, Chericoni S, Calderone V, Nencioni G, Nieri P, Morelli I, Martinotti E. 2002. Cardiovascular effects of *Urtica dioica* L. (Urticaceae) roots extracts: in vitro and in vivo pharmacological studies. Journal of Ethnopharmacology . 81:105–109.

Thomas Fleming. 2000. PDR for Herbal Medicines. 4th ed. Montvale: Medical Economics Company.

Tomlinson CTM, Nahar L, Copland A, Kumarasamy Y, Mir-Babayev NF, Middleton M, Reid RG, Sarker SD. 2003. Flavonol glycosides from the seeds of *Agrimonia eupatoria* (Rosaceae). Biochem Syst Ecol. 31(4):439–441. https://doi.org/10.1016/S0305-1978(02)00170-9.

Torres-Fuentes C, Suárez M, Aragonès G, Mulero M, Ávila-Román J, Arola-Arnal A, Salvadó MJ, Arola L, Bravo FI, Muguerza B. 2022. Cardioprotective Properties of Phenolic Compounds: A Role for Biological Rhythms. Mol Nutr Food Res. 66(21). https://doi.org/10.1002/mnfr.202100990.

Tsao R. 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients. 2(12):1231–1246. https://doi.org/10.3390/nu2121231.

Upton R. 2013. Stinging nettles leaf (*Urtica dioica* L.): Extraordinary vegetable medicine. J Herb Med. 3(1):9–38. https://doi.org/10.1016/j.hermed.2012.11.001.

Vajic UJ, Grujic-Milanovic J, Miloradovic Z, Jovovic D, Ivanov M, Karanovic D, Savikin K, Bugarski B, Mihailovic-Stanojevic N. 2018. *Urtica dioica* L. leaf extract modulates blood pressure and oxidative stress in spontaneously hypertensive rats. Phytomedicine. 46:39–45. https://doi.org/10.1016/j.phymed.2018.04.037

Valentová K, Vrba J, Bancířová M, Ulrichová J, Křen V. 2014. Isoquercitrin: Pharmacology, toxicology, and metabolism. Food Chem Toxicol. 68:267–282. https://doi.org/10.1016/j.fct.2014.03.018.

Veeresham C. 2012. Natural products derived from plants as a source of drugs. J Adv Pharm Technol Res. 3(4):200–201. https://doi.org/10.4103/2231-4040.104709.

Veluswamy SK, Babu AS, Sundar LM. 2016. Complementary Role of Herbal Medicine and Exercise in Cardiovascular Disease Prevention and Management: A Review of Evidence. Curr Pharm Des. 23(8):1253–1264. https://doi.org/10.2174/1381612822666161010122252.

Vennat B, Pourrat A, Pourrat H, Gross D, Bastide P, Bastide J. 1988. Procyanidins from the Roots of *Fragaria vesca*: Characterization and Pharmacological Approach. Chem Pharm Bull. 36(2):828–833.

Venskutonis PR, Škemaite M, Ragažinskiene O. 2007. Radical scavenging capacity of *Agrimonia eupatoria* and *Agrimonia procera*. Fitoterapia. 78(2):166–168. https://doi.org/10.1016/j.fitote.2006.10.002.

Venskutonis PR, Škemaite M, Sivik B. 2008. Assessment of radical scavenging capacity of *Agrimonia* extracts isolated by supercritical carbon dioxide. Journal of Supercritical Fluids. 45(2):231–237. https://doi.org/10.1016/j.supflu.2008.01.012.

Vera R, Sánchez M, Galisteo M, Villar IC, Jimenez R, Zarzuelo A, Pérez-Vizcaíno F, Duarte J. 2007. Chronic administration of genistein improves endothelial dysfunction in spontaneously hypertensive rats: Involvement of eNOS, caveolin and calmodulin expression and NADPH oxidase activity. Clin Sci. 112(3–4):183–191. https://doi.org/10.1042/CS20060185.

Verma T, Sinha M, Bansal N, Yadav SR, Shah K, Chauhan NS. 2021. Plants Used as Antihypertensive. Nat Prod Bioprospect. 11(2):155–184. https://doi.org/10.1007/s13659-020-00281-x.

Wallerath T, Li H, Gödtel-Ambrust U, Schwarz PM, Förstermann U. 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human

endothelial NO synthase. Nitric Oxide. 12(2):97–104. https://doi.org/10.1016/j.niox.2004.12.004.

Walle T, Browning AM, Steed LL, Reed SG, Walle UK. 2005. Human Nutrition and Metabolism Flavonoid Glucosides Are Hydrolyzed and Thus Activated in the Oral Cavity in Humans. Human Nutrition and Metabolism. 135(1): 48-52.

Wichtl M. 2004. Herbal drugs and phytopharmaceuticals: a handbook for practice on a scientific basis. Stuttgart: Medpharm GmbH Scientific Publishers.

Woodman OL, Meeker WF, Boujaoude M. 2005. Vasorelaxant and Antioxidant Activity of Flavonols and Flavones : Structure – Activity Relationships. 46(3):302–309.

World Health Organization. 2022. Hypertension.

Xiao J. 2017. Dietary flavonoid aglycones and their glycosides: Which show better biological significance? Crit Rev Food Sci Nutr. 57(9):1874–1905. https://doi.org/10.1080/10408398.2015.1032400.

Xu JW, Ikeda K, Yamori Y. 2004a. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. Hypertension. 44(2):217–222. https://doi.org/10.1161/01.HYP.0000135868.38343.c6.

Xu JW, Ikeda K, Yamori Y. 2004b. Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase. FEBS Lett. 574(1–3):176–180. https://doi.org/10.1016/j.febslet.2004.08.027.

Yoon SJ, Koh EJ, Kim CS, Zee OP, Kwak JH, Jeong WJ, Kim JH, Lee SM. 2012. Agrimonia eupatoria protects against chronic ethanol-induced liver injury in rats. Food and Chemical Toxicology. 50(7):2335–2341. https://doi.org/10.1016/j.fct.2012.04.005.

Yuan TY, Niu ZR, Chen D, Chen YC, Zhang HF, Fang LH, Du GH. 2018. Vasorelaxant effect of quercetin on cerebral basilar artery in vitro and the underlying mechanisms study. J Asian Nat Prod Res. 20(5):477–487. https://doi.org/10.1080/10286020.2018.1463995.

Ziyyat A, Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M, Benjelloun W. 1997. Phytotherapy of hypertension and diabetes in oriental Morocco. J Ethnopharmacol. 58(1):45–54. https://doi.org/10.1016/S0378-8741(97)00077-9.