UNIVERSIDADE D
COIMBRA

Daniela Sofia Matias Simões

Study of the vasorelaxant and antioxidant activity of *Cymbopogon citratus*

Dissertação no âmbito do Mestrado em Farmacologia Aplicada, orientada pelo Professor Doutor Diogo André Afonso da Fonseca e pela Professora Doutora Maria Dulce Ferreira Cotrim e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Julho de 2019

1 2 9 0

UNIVERSIDADE D
COIMBRA

Daniela Sofia Matias Simões

Study of the vasorelaxant and antioxidant activity of *Cymbopogon citratus*

Dissertação no âmbito do Mestrado em Farmacologia Aplicada, orientada pelo Professor Doutor Diogo André Afonso da Fonseca e pela Professora Doutora Maria Dulce Ferreira Cotrim e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Julho 2019

"Nas grandes batalhas da vida, o primeiro passo para a vitória
é o desejo de vencer"

Mahatma Gandhi

AGRADECIMENTOS

Ao meu orientador, Professor Doutor Diogo André Afonso da Fonseca, pela constante disponibilidade e aconselhamento, por todo o apoio, exigência, suporte e ensinamentos ao longo da realização prática e na concretização desta dissertação de mestrado.

À minha co-orientadora, Professora Doutora Maria Dulce Cotrim, pela disponibilidade, transmissão de conhecimentos, incentivo, apoio, exigência e presença permanente no desenvolvimento e concretização desta dissertação de mestrado.

À aluna de doutoramento, Jéssica Malheiros, pelo companheirismo e amizade, pela disponibilidade, suporte e troca de conhecimentos essenciais para o desenvolvimento e concretização de toda a prática experimental deste projeto.

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

Ao laboratório de Farmacognosia, Faculdade de Farmácia da Universidade de Coimbra, em especial ao Professor Doutor Artur Figueirinha pela disponibilização dos extratos usados no estudo, pela disponibilidade, apoio e ensinamentos fundamentais para a realização e concretização deste trabalho. Incluo um agradecimento em especial também à aluna de doutoramento Patrícia, pela simpatia, suporte, aconselhamento, e troca de conhecimentos essenciais na concretização de parte do trabalho realizado.

A toda a 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 mamária, crucial para o desenvolvimento deste projeto de mestrado.

A todos os professores da Faculdade de Farmácia da Universidade de Coimbra, por todos os conhecimentos transmitidos, que contribuíram para a minha formação pessoal e profissional, facultando-me as bases científicas cruciais nesta etapa do meu percurso académico.

Ao Miguel, pelo companheirismo e apoio incondicional, por ser porto de abrigo cheio de força e incentivo determinante para a concretização deste projeto de mestrado.
Aos meus familiares e amigos, pelo suporte, amor e carinho.

Aos meus pais, por todos os ensinamentos e saberes, por todo o amor, carinho, suporte e incentivo que permitiram a realização deste trabalho, e por sempre acreditarem em mim.

A todos,
O meu mais sincero e sentido agradecimento

Table of Contents

Abbreviations

A

ABS	Absorbance
ANOVA	Analysis of variance

C

°C	Celsius degrees
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CAD	Coronary artery disease
CC CE	Crude extract of *Cymbopogon citratus*
CC F	Flavonoids fraction of *Cymbopogon citratus* CC
PA	Phenolic acids fraction of *Cymbopogon citratus* CC T
	Tannins fraction of *Cymbopogon citratus*
CO_2	Carbon dioxide
CVD	Cardiovascular diseases

D

DPPH	2,2-diphenyl-1-picrylhydrazyl

E

E_{max}	Maximal effect produced by the agonist
EDHF	Endothelium-derived hyperpolarizing factor

G

g	Gram
µg	Microgram
µg/mL	Microgram/milliliter

H

HIMA	Human internal mammary artery
HPLC	High performance liquid chromatography
HPLC-PDA	High performance liquid chromatography with photodiode-array detection

K

K^+	Potassium
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate

L

µL	Microliter
LDL	Low density lipoprotein

M

µM	Micromolar
mL	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
Mg	Milligrams
$MgCl_2$	Magnesium chloride
mg/mL	Milligrams/milliliter
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
mN	MilliNewtons

N

n	Number of experiences
NaCl	Sodium Chloride
$NaHCO_2$	Sodium bicarbonate
NaH_2PO_4	Monosodium phosphate
nm	Nanometers
NO	Nitric oxide

S

SEM	Standard error of mean

O

O_2	Oxygen

P

pEC_{50}	Negative logarithm of the concentration required to achieve 50% of the maximal effect
PGI2	Prostaglandin I2

R

R_{max}	Maximum percentage of reduction of the pre-contraction to NA

T

TLC	Thin -layer chromatography

U

UV-Vis	Ultraviolet-visible region

List of Figures

I. INTRODUCTION

II. MATERIALS AND METHODS

III. RESULTS AND DISCUSSION

List of Tables

I. INTRODUCTION

III. RESULTS AND DISCUSSION

RESUMO

Cymbopogon citratus, Stapf, também conhecido como capim-limão, é uma planta que pertence à família Poaceac-Gramineae. É originário da Índia, sendo atualmente cultivado em muitos países tropicais e subtropicais. É amplamente utilizado na medicina tradicional, devido aos potenciais efeitos antioxidantes, anti-inflamatórios, antimicrobianos, hipoglicemiantes e anti-hipertensores associados aos compostos bioativos da planta.

Neste projeto de estudo pretendeu-se avaliar e comprovar a atividade vascular e antioxidante do extrato total de folhas secas de *Cymbopogon citratus*, bem como testar o efeito vascular das frações de flavonóides, taninos e ácidos fenólicos, utilizando a artéria mamária interna humana (HIMA) como modelo humano de reatividade vascular.

O extrato total mostrou ter potencial como agente antioxidante, com um EC_{50} = 33,98±1,51 µg/ml. O extrato total (nas concentrações de 0,002 e 2 mg/mL) e as frações de ácidos fenólicos e taninos (na concentração de 1mg/mL) induziram um aumento significativo da contração máxima à noradrenalina, enquanto a concentração de 0,0002 mg/mL de extrato total e 0,2 mg/mL de fração de flavonóides inibiram essa contração. A curva de concentração-resposta da fração de taninos (0,002 a 0,2 mg/mL) apresentou atividade intrínseca de relaxamento na HIMA.

A potencial atividade vasodilatadora e antioxidante demonstrado no presente trabalho suscita a necessidade de aprofundar conhecimentos e realizar novos trabalhos com extratos de diferentes partes da planta ou frações/compostos isolados. A validação e caracterização dos potenciais efeitos farmacológicos de *Cymbopogon citratus*, sustentam o uso desta planta para fins medicinais.

Palavras-chave: *Cymbopogon citratus*; atividade vascular; atividade antioxidante; Artéria Mamária Interna Humana, Doenças Cardiovasculares.

ABSTRACT

Cymbopogon citratus, Stapf, also known as lemongrass, is a plant that belongs to the Poaceac-Gramineae family. It is derived from India, being currently cultivated in many tropical and subtropical countries. *Cymbopogon citratus* is commonly used in folk medicine due to potential antioxidant, anti-inflammatory, antimicrobial, hypoglycemic and anti- hypertensive effects from the bioactive compounds of the plant.

The aim of the present study was to evaluate and verify the vascular activity of the crude extract of dry leaves of *Cymbopogon citratus*, as well as to test the vascular effects of the flavonoid, tannin and phenolic acid fractions using the human internal mammary artery (HIMA) as a human model of vascular reactivity.

The crude extract showed potential as an antioxidant, with an EC_{50} = 33.98±1.51 µg/ml. The crude extract (at the concentrations of 0.002 and 2 mg/mL) and the phenolic acid and tannin fractions (at the concentration of 1 mg/mL) elicited a significant increase in the maximum contraction to noradrenaline. While that the concentration of 0.0002 mg/mL of the crude extract and 0.2 mg/mL of flavonoid fraction inhibited this contraction. The concentration-response curve of the tannin fraction (0.002 to 0.2 mg/mL) presented intrinsic relaxation activity in HIMA.

The potential vasorelaxant and antioxidant activity demonstrated in the present study makes it necessary to deepen knowledge and to carry out new work with extracts from different parts of the plant or isolated fractions/compounds. The validation and characterization of the potential pharmacological effects of *Cymbopogon citratus*, will sustain the use of this plant for medicinal purposes.

Keywords: *Cymbopogon citratus*; vascular activity; antioxidant activity; Human Internal Mammary Artery, Cardiovascular Diseases.

I. INTRODUCTION

Phytotherapy

Phytotherapy was the first Medicine of Man and Animals. The medicinal use of plants goes back thousands of years. For centuries, humans have used plants for food, for clothing, for healing and as drugs of abuse. Over the course of the centuries, they has learnt, to his cost, to distinguish between their beneficial and toxic properties.[1]

Phytotherapy is a field of medicine that uses plants either to treat disease or as health-promoting agents. Almost half of all active principles released between 1981 and 2010 were of natural origin or inspired by natural compounds[2] and 80% of 122 plant derived drugs were related to their original ethnopharmacological purpose[3].

According to the World Health Organization and United Nations, 87.5% of people rely on plant-based traditional medicines for primary health care (Figure I.1).

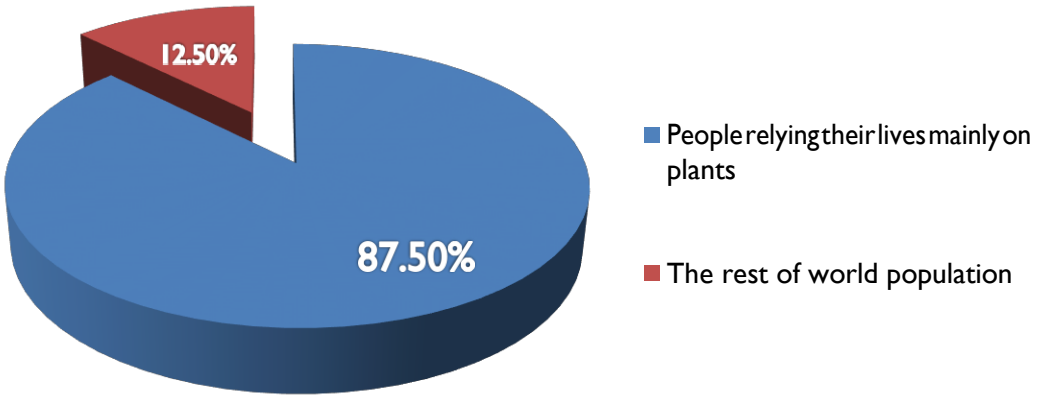

Figure I.1: Use of plant-based traditional medicines in world, in 2014.

Phytotherapy uses the whole plant or parts (root, flower, leaves, fruits or seeds, barks, juices, sap or resins, wood, etc.). All plants are a renewable source of specialized metabolites, i.e., they are a source of primary and secondary metabolites. Primary metabolites are compounds involved in the pathways of biosynthesis and breakdown of proteins, fatty acids, nucleic acids and carbohydrates. Secondary metabolites are generally not essential for the growth, development or reproduction of an organism and are produced either as a result of the organism adapting to its surrounding environment or are produced to act as a possible defense mechanism against predators to assist in the survival of the organism, as alkaloids, polyphenols and terpenoids. The biosynthesis of secondary metabolites is derived from the fundamental processes of photosynthesis, glycolysis and the

Krebs cycle to afford biosynthetic intermediates which results in the formation of natural products[3, 4].

Natural products have enormous structural and functional chemical variability which makes them interesting for the research of new bioactive compounds. However its complexity make that they are isolated in low quantities and obtaining is hampered by rapid synthetic methods.

Plants may form the basis of food supplements, cosmetics, perfumes, medicines, medical devices and biocides. However, medicinal plants are increasingly important for the development of new drugs as:

i. Herbal medicines;
ii. Direct use of the constituents as therapeutic agents;
iii. Basic material for the hemisynthesis of medicinal products;
iv. Natural prototype for obtaining synthetic derivatives.

According to INFARMED (Dir. 2001/83 / EC)[5], herbal medicinal products are derived from plants which have curative or preventive properties relating to diseases, with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic. So, as with any other drug, herbal medicinal products are only approved by regulatory agencies and subsequently marketed after going through several steps in order to meet the fundamental requirements of product quality, efficacy and safety. The specifications are fundamentally the same regardless of whether the drug candidate is a synthetic, semisynthetic substance or a product isolated from a natural source.

Thus, the drug approval process comprises four major phases[6]:

i. Identification of the molecular compounds (i.e., lead compounds);
ii. Optimization of prototypes through chemical tools, with a view to development of biopharmaceutical properties;
iii. Preclinical pharmacology and toxicology;
iv. Clinical pharmacology and toxicology.

Although these products are quite safe and have fewer adverse effects it is important to respect the prescribed dosage and to know the possible drug and dietary interactions for the treatment to be effective.

Cymbopogon citratus, Stapf

The genus *Cymbopogon* is composed of 144 species and belongs to the Gramineae family. It is native to India but is currently widely distributed in the tropical and subtropical regions of Asia, Africa and South and Central America[7]. The genus *Cymbopogon* is known worldwide for its high content of essential oil, widely used in food industry, perfumery, cosmetology and pharmaceuticals[8, 9].

Cymbopogon citratus, Stapf, also known as lemongrass is ranked as one of the most widely distributed of the genus and it is used in every part of the world due to its physicochemical characteristics, including flavor, lemony smell, color, strength and intensity, but also for physiological reasons[10].

The chemical composition of the plant varies according to its geographical localization, so it is associated with many different traditional uses. In addition, the use of plant parts such as leaves, stem or root (Figure I.2) or the plant as a whole also contributes to the therapeutic variability associated with *Cymbopogon citratus*.

Figure I.2: *Cymbopogon citratus* Stapf, Poaceac-Gramineae family. Available from [11].

Cymbopogon citratus has associated numerous traditional applications, described in Table I.1.

Table I.1: Traditional use of *Cymbopogon citratus* in the world.

Country	Extract	Traditional application	Reference
Argentina	Infusion of leaves	Colds; cardiotonic; cough.	[12, 13]
	Decoction of leaves	Stomachic; hypotensive; sore throat, empacho, emetic.	[7, 13]
Bolivia	Decoction of leaves	Stomach pain; swellings; tranquilizer.	[14]
Brazil	Infusion of leaves	Antispasmodic; analgesic; anti-inflammatory; antipyretic; diuretic; sedative; headache; muscle aches; rheumatism; diarrhea; pre-partum pain.	[7, 15, 16]
	Infusion of whole plant	Hypertension; stomach ache; gastritis; ulcer; diarrhea; ingestion, intestinal colic.	[17]
	Bath with leaves	Flu with scratching throat, witchcraft, envy, laziness.	[15]
China	Bath with leaves	Relieve pains.	[18]
Congo	Decoction of leaves	Cough; gastric disorders; diarrhea; fever; malaria; edemas; digestive stimulant.	[19]
Cuba	Decoction of fresh leaves	Sedative; hypotensive effect; fever.	[20]
	Decoction of dried leaves	Hypotensive; catarrh; rheumatism	[7]
Ecuador	Infusion of leaves	Gastritis; relaxant; stomach pain; diarrhea.	[21]
Egypt	Infusion of dried leaves and stem	Renal antispasmodic; diuretic.	[7]
Ghana	Poultice of leaves	Boils; swelling.	[22]
Honduras	Decoction of leaves	Lactation.	[23]
India	Essential oil	Gastric troubles; cholera; carminative; analgesic; antibacterial; antifungal; antipyretic.	[7, 24, 25]
	Bath with dried leaves	Severe headache; antipyretic.	[7]
	Infusion of whole plant	Sedative; digestive problems; muscle relaxant; spasms; flatulence; catarrh.	[7, 24]

Indonesia	Infusion of the whole plant Essential oil	Emmenagogue. Sedative; antiseptic; antiphlogistic.	[7] [26]
Malaysia	Infusion of the whole plant	Emmenagogue.	[7]
Mexico	Infusion of the whole plant Infusion of leaves	Grippe. Stomach ache; cough.	[27] [28, 29]
Nepal	Infusion of the whole plant	Respiratory tract infections	[30]
Nicaragua	Infusion of leaves	Backache; abdominal pain; postpartum abdominal pain; lactation; fever; digestive disorders.	[31]
Nigeria	Decoction of leaves Infusion of leaves	Malaria, diarrhea. Yellow fever.	[32, 33]
Portugal	Infusion of leaves	Analgesic gastric; intestinal anti-inflammatory; renal antispasmodic	[34]
Thailand	Infusion of the dried whole plant Infusion of the dried root	Gastric disorder. Diabetes.	[7]
Tonga	Infusion of leaves	Morning sickness.	[35]
USA	Infusion of the whole plant	Heal wounds; bone fractures.	[7]

Briefly, we can say that *Cymbopogon citratus* is associated with the following potential bioactivities: anti-tumor; anti-carcinogenic; anti-inflammatory; antidiarrheal; antiprotozoan; antibacterial; antimycobacterial; antifungal; antimalarial; and antimutagenic. Furthermore, it is believed to have potential antinociceptive; anti-amebic; antioxidant; anti-hypertensive; hematologic; hypocholesterolemic; hypoglycemic/hypolipidemic; neurobehavioral/ neuropharmacodinamic[7, 10] effects.

Cymbopogon citratus, as most plants, is rich in flavonoids, phenolic acids and tannins.

Flavonoids are the most common polyphenols found in plants. This polyphenols having a benzo-γ-pyrone structure, represented in Figure I.3, i.e. they are composed of an aromatic ring (A) fused to a heterocyclic ring (C) which in turn are linked, via a single carbon-carbon bond, to another aromatic ring (B).

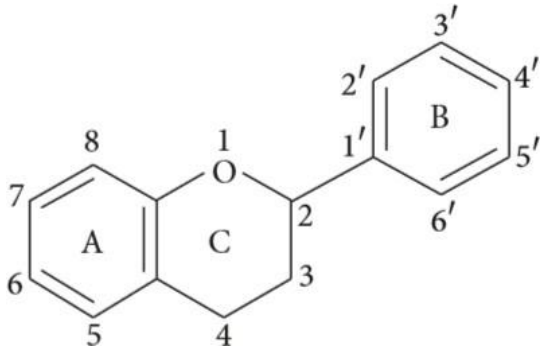

Figure I.3: Representation of chemical structure of flavonoids. Reproduced from Kumar and Pandey[36].

Because of chemical complexity and structural diversity, they are further classified into distinct subclasses such as flavonols, anthocyanins, proanthocyanidins, flavones, flavanones, aurones, isoflavones, and neoflavonoids.

Although they all share the same basic structure, the different groups of flavonoids result from the structural differences related with the level of oxidation and the pattern of substitution of the heterocyclic ring, and the derivatives of these large groups will in turn differentiating among themselves by the substitution pattern of the aromatic rings[4, 36].

Currently, flavonoids are considered health promoters, since they are associated with antibacterial, antiviral, anti-inflammatory, antiplatelet, antioxidant, antithrombotic, free radical scavenger and vasorelaxant effects[37-39]. Of course, this diversity of effects associated with flavonoids as a whole depends on their structural class, degree of hydroxylation, different substitutions and conjugations, and degree of polymerization of the molecule[36].

Tannins are polyphenols of varying molecular size and complexity, present in plant extracts. The classical tannin division was based on their resistance or not, to hydrolysis in the presence of hot water or in the enzymes tannases[40].

Tannins can be classified as hydrolysable (Figure I.4) or non-hydrolysable/condensed (Figure I.5). Hydrolysable tannins contain a central glucose molecule linked to gallic acid molecules (gallotannins) or hexahydrofenhydenic acid (ellagitannins) and are readily decomposed by acids[41].

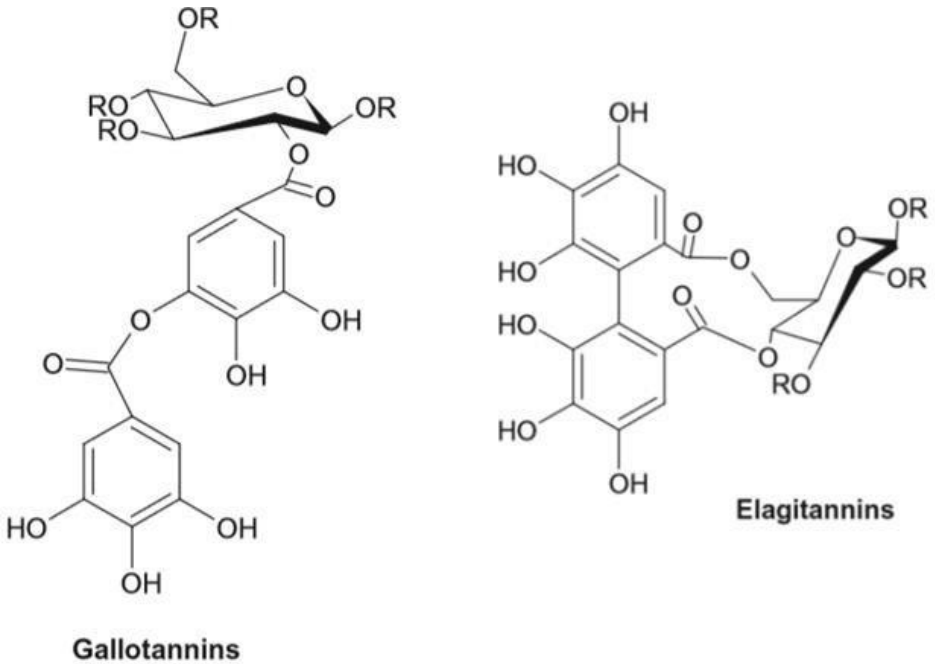

Figure I.4: Chemical structure of hydrolysable tannins. Reproduced from Sieniawska and Baj[40]

Non-hydrolysable/condensed tannins, also called proanthocyanidins, are formed by the successive condensation of catechins with a degree of polymerization between two and greater than fifty catechins being reached. Structural complexity of proanthocyanidins are due to the structural rearrangement that came of frequently derivatizations as *O*-methylation, *C*- and *O*-glycosylation and *O*-galloylation which hinders hydrolysis[40, 42].

The extensive structural variability of the tannins causes they may act as non-absorbable, these are usually complex structures with binding properties which may produce local effects; or as absorbable, these are usually low molecular weight structures which are readily absorbed and produce systemic effects[40].

It is believed that the tannins may exhibit cardioprotective, antidiabetic and antiobesity effects. Moreover, tannins are also known for their anti-inflammatory, anti- oxidant, antimicrobial, antiviral and antimutagenic and anticarcinogenic effects. Nevertheless, the ingestion of large amounts of tannins may be harmful, since tannins may have antinutritional, cytotoxic and carcinogenic potential.[40, 43]

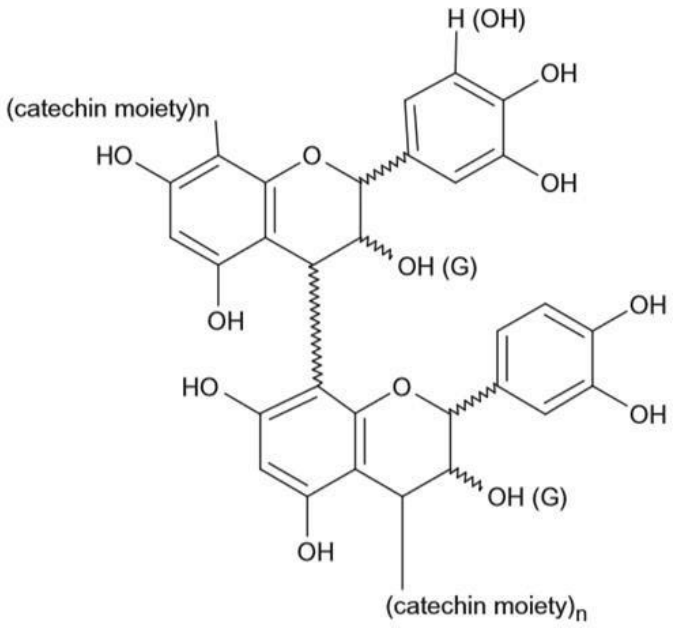

Figure I.5: Chemical structure of non-hydrolysable tannins. Reproduced from Sieniawska and Baj[40]

Several studies related the possible relation between tannins and the development of spontaneous tumors. For example, a study reported that tannins applied to burns or injected subcutaneously might cause tumors in experimental animals. Another study that investigated the effect of different tannin solutions on the carcinogenic action of benzo[α]pyrene, concluded that the appearance of tumors was accelerated in mice treated with tannins solution after a single topical application of benzo[α]pyrene. Betel nuts, that are rich in tannins, are also described as potent carcinogenic compounds. Other authors pointed out that natural tannins were regarded as possible etiological agents for nasal cancer in shoe- making workers[43]. Nevertheless, the correlation between tannins and these types of cancer might not reflect a true cause-effect relationship, because of the environmental influence, occupation, heavy smoking, high consumption of alcoholic beverages, poor nutrition, and use of emetics are strong influencers of cancer.[43] On the order hand, the anticarcinogenic potential of tannins may be related to their antioxidant effects, which are important in protecting against cellular oxidative damage.

An undesirable effect associated to tannins is the antinutricional effect. A study that evaluated the gastrointestinal digestion and absorption of nutrients in rat intestine, showed that the presence of polyphenols can inhibit sugar absorption in rat jejunum perfused *in situ*. Also, it was found that tannins may reduce metallic ions such as Cr^{6+}, Fe^{3+} and Cu^{2+} to Cr^{3+}, Fe^{2+} and Cu^{+}, respectively, thus reducing their intestinal absorption.[44]

Another study investigated the effect of proteins in the food matrix on the inhibitory activity of hydrolysable tannins, using wheat flour and wheat starch. The wheat starch does not contain proteins. The presence of flour proteins reduced inhibitory activity of hydrolysable tannins by possibly interfering with the binding of hydrolysable tannins to α-amylase when they were incorporated into a real food system such as wheat flour. At high protein concentrations, galloyl groups of hydrolysable tannins tend to interact with the hydrophobic sidechains of amino acids form a hydrophobic mono-layer. In addition, the hydrogen bonds between phenolic groups and polar groups of proteins force the protein aggregation. This non-specific protein precipitating property of tannins allowed it to bind to flour proteins, difficulty the binding with α-amylase, significantly reducing the effect of hydrolysable tannins in inhibiting starch digestion.[45]

Cytotoxic effects associated to tannins were also described. A study used human tumor cell lines cultured *in vitro* to evaluate the cytotoxic effects of polyphenols. In this experiment human duodenum cancer cells, human non-small cell lung cancer cells and human colon cancer cells were used. This study demonstrated that some of tannins structures produced a significant cytotoxicity in most of the cancer cell lines tested, due to the inhibition of cell proliferation by these compounds and the incorporation of galloyl groups enhanced the cytotoxic effects of these compounds.[46]

Therefore, the dosage and kind of tannins are critical to different and antagonist effects.

Phenolic acids are polyphenols that derivate from non-phenolic molecules of benzoic and cinnamic acid, into divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids.

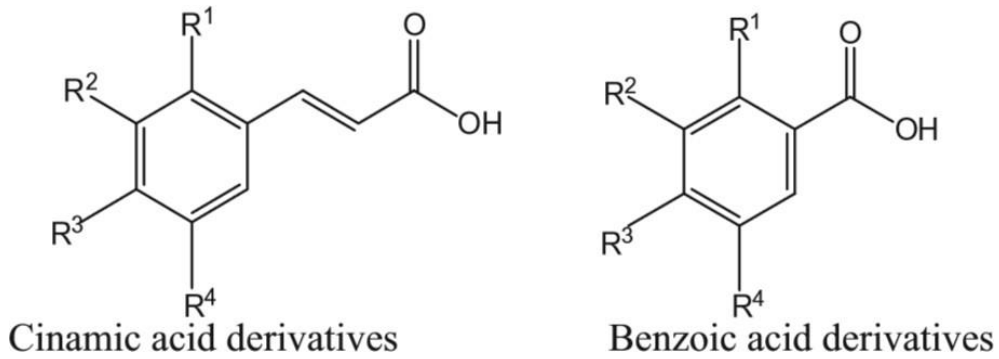

Figure I.6: Representation of chemical structure of phenolic acids. Reproduced from Heleno *et al.*[47]

Chemically, these compounds have at least on aromatic ring in which at least one hydrogen is substituted by a hydroxyl group and a carboxylic acid function at the benzene ring (Figure I.6). The derivatives differ in the degree of hydroxylation and methoxylation of the aromatic ring [47-49].

Different bioactive properties have been attributed to phenolic acids, namely antitumor, antimicrobial, and antioxidant[47].

Cardiovascular diseases

Cardiovascular diseases (CVD) are the leading cause of death worldwide and are projected to remain among the most important contributors to mortality by 2030[50]. According to the World Health Organization, approximately 17.9 million people died of CVD in 2016, around the world, representing almost a third of the total number of deaths that year. Of these deaths, 85% are due to heart attacks and strokes[51]. According to statistics on CVD in 2015, it is estimated that 1.91 million deaths were caused by diseases of the circulatory system, in the EU, namely by CVD and stroke[52]. In Portugal, CVD is also the main cause of death, with stroke and congenital heart disease accounting for 6.1% and 6.0%, respectively, of the total years of disability-adjusted life in 2015[50].

Cardiovascular diseases are disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, rheumatic heart disease, peripheral arterial disease, congenital heart disease, deep vein thrombosis, pulmonary embolism, among other conditions[51].

In terms of etiology, CVD are multifactorial and are associated with numerous risk factors that may or may not be modifiable. The main modifiable CVD risk factors are smoking, alcohol consumption, unhealthy diet, obesity, arterial hypertension, dyslipidemia, diabetes, psychological stress and a sedentary lifestyle[50, 53]. Age, sex and other hereditary factors are also an increased risk for the development of cardiovascular diseases, however they are non- modifiable factors.

It is known that, in some way, polyphenols have positive effects in the prevention and treatment of cardiovascular diseases. However, the diversity and variability of polyphenols may have different significant effects on cardiovascular disease. Therefore, polyphenols have different effects on free radical scavenging, prevention of LDL oxidation, anti-inflammatory

and anti-allergic properties, endothelial function, modulation on the renin-angiotensin- aldosterone axis, and others[54, 55].

Currently, there are some studies that report the potential cardiovascular effect of some constituents of *Cymbopogon citratus*. Animal model trials have demonstrated that lemongrass oil has anti-hyperlipidemic activity, as it reduces serum cholesterol, triglycerides levels and atherogenic index[56]; and antihypertensive activity, since it has an effect on blood pressure reduction, and it induce relaxation in vascular smooth[57, 58].

Human Internal Mammary artery (HIMA)

Coronary artery disease (CAD) is one of the health problems, within cardiovascular diseases, which has associated a major morbidity and mortality rate.

Coronary arteries present characteristics of an elastic artery composed of three main layers: tunica intima, tunica media and tunica adventitia[59]. The development of atherosclerotic lesions, i.e. asymmetric focal thickenings of the innermost layer of the artery, the intima, is at the basis of CAD. The atheroma plaque is composed of inflammatory and immune cells, 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[60].

Coronary revascularization as a therapeutic strategy has been widely accepted for many years, though the procedures have been constantly developed and expanded.

Due to its unique properties, such as a lower incidence of atherosclerosis, better graft patency and lower incidence of vasospasm[61], the human internal mammary artery (HIMA), which is also known as internal thoracic artery, has long been considered the best graft to use in this type of surgery. The HIMA is derived from the subclavian artery and is located on the internal face of the anterior chest wall. The artery is accompanied by a pair of internal thoracic veins. The HIMA supplies blood to the pericardium, phrenic nerve, sternum, anterior chest wall, pectoralis major muscle, mammary gland, anterior abdominal wall, and the diaphragm[62].

The HIMA is the only peripheral artery in the human body that is elastic, being composed of an intima layer that is limited by a well-formed internal elastic lamina and a media layer that is formed by elastic lamellae. This elastic layer separates the arterial intima from the media and may act as a barrier, protecting the media from the effect of any noxious

luminal stimuli and protecting the intima by preventing the inward migration of smooth muscle cells[62].

Pediculation and skeletonization are techniques have been developed to harvest the HIMA. The pedicled harvesting technique involves collecting the graft from the vessel together with the adjacent intact tissues, which include veins, lymphatic vessels and nerves. The skeletonization procedure involves the surgical dissection of the HIMA from the perivascular tissue. The advantages of the skeletonization procedure are longer vessel length for grafting and minimized chest-wall trauma with reduced risk of sternal wound infection because vein, muscle, and accompanying endothoracic tissues are left in place and collateral sternal blood supply is preserved. However, the skeletonization procedure is technically more demanding and time consuming, and the risk of arterial injury is increased as the vessel is handled directly and the margin of error is small in the absence of a myofascial tissue buffer. Furthermore, pedicled harvesting has been preferred by many surgeons[63].

Patients who require coronary revascularization usually present multiple risk factors, which can interfere with regulation of the endothelial function. Endothelium is a cellular layer crucial involved in cardiovascular homeostasis and the major regulator of vessel function. The endothelium of the HIMA is itself unique with a significantly higher basal production of vasodilators such as nitric oxide and prostacyclin[62, 63]. Other molecules are also produced by the endothelium, such as the Endothelium-Derived Hyperpolarizing Factor (EDHF), and vasoconstrictors, e.g. endothelin[63].

Several diseases and cardiovascular risk factors have been shown to interfere with the endothelial function promoting the production of vasoconstrictors, inhibiting the production of vasodilators or both, and thus eventually leading to endothelial dysfunction. Despite the HIMA being considered an atherosclerosis-resistant vessel cardiovascular risk factors previously described have been associated with structural changes, in the HIMA.

Objectives

In the present work aimed to study the vascular and antioxidant activity of the crude extract and fractions of phenolic acids, flavonoids and tannins of *Cymbopogon citratus*. Vascular activity of the crude extract and fractions was assessed in organ bath experiments with the HIMA as a human model of vascular reactivity and antioxidant activity of the crude extract with the DPPH assay.

II. MATERIALS AND METHODS

2.1 Plant material and extraction

Dry leaves of *Cymbopogon citratus* were purchased from ERVITAL® (Mezio, Castro D'Aire, Portugal) and a voucher specimen was deposited in the herbarium of the University of Coimbra, Faculty of Pharmacy.

For extraction, Figueirinha, *et al.*[8], used boiling water for infusion preparation. The extract was prepared by adding 150 mL of water to 5 g of the powdered plant material. After extraction, the extract was filtered under vacuum and its volume made up to 150 mL with the water. Then, an essential oil-free infusion was subsequently prepared from the infusion extract. So, an infusion obtained, as described above, was repeatedly washed with n- hexane to remove the less polar compounds. The aqueous phase was concentrated on a rotatory evaporator to a small volume and then freeze-dried.

To the powdered plant material resulting from lyophilization, water was added and centrifuged. After centrifugation, the fractionation process was started. The fractionation process, described in Figure II.1, was monitored by TLC and HPLC.

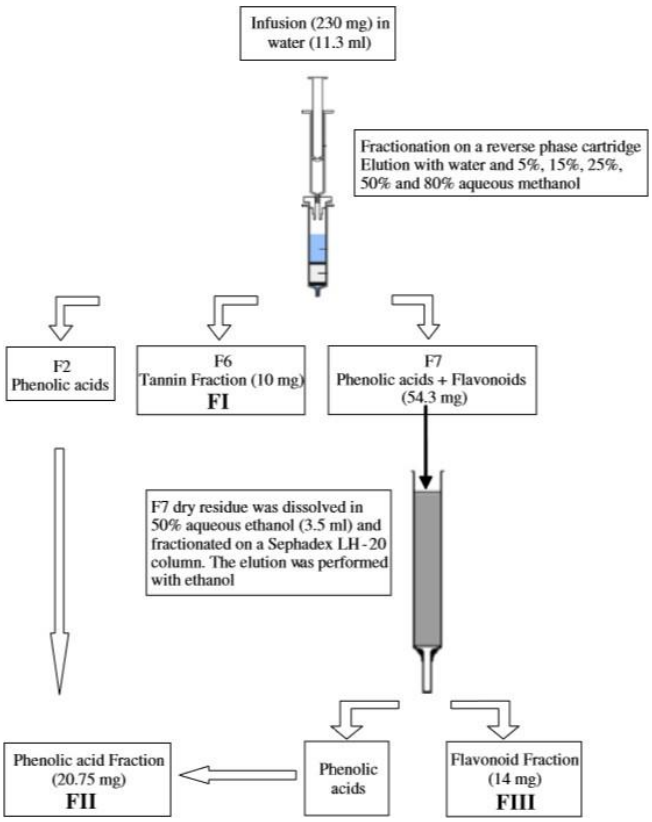

Figure II.1: Scheme of the process used for the fractionation of the *Cymbopogon citratus* leaves infusion. Reproduced by Figueirinha, *et al.*[8]

The aqueous solution was fractionated on a reverse phase cartridge Chromabond® C18, eluting with water, giving fraction F_1 and aqueous methanol solutions, then 5% methanol, 15% methanol, 25% methanol, 50% methanol and 80% methanol, giving fractions F_2, F_3, F_4, F_5, F_6 and F_7, respectively. Dry residue of F_7 was recovered in 50% aqueous ethanol and fractionated by gel chromatography on a Sephadex® LH-20 column, using ethanol as the mobile phase. Two different sub-fractions were obtained from F_7: sub- fraction F_{7a} that containing phenolic acids and sub-fraction F_{7b} that containing flavonoids.

This fractionation provided three major fractions: FI corresponds to tannins fraction (F_6); FII, corresponds to phenolic acids fraction, comes from joining fraction F_2 and sub-fraction F_{7a}; and FIII corresponds to flavonoids fraction, comes from sub-fraction F_{7b}. The fractions were taken to dryness reduced pressure (40°C).

2.2 Vascular activity studies

Samples of HIMA were harvested with the approval by the Ethics Committees of Coimbra University Hospitals (reference PC-388/08) and Faculty of Medicine of University of Coimbra (reference CE-117/18), from patients after informed consent and undergoing myocardial revascularization. Distal segments of HIMA were dissected as a pedicle from the anterior internal surface of the chest after the sternal incision from 50 patients (42 males and 8 females), with an age between 45 and 80 years-old. The distal segments of HIMA remaining after surgery were placed in cold (4°C) physiologic saline solution - Krebs- Henseleit bicarbonate buffer - composed with 119 mM NaCl, 15 mM $NaHCO_2$, 4.6 mM KCl,
1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.5 mM $CaCl_2$ and 5.5 mM Glucose aerated with 95% O_2 and 5% CO_2 and pH 7.4 - and transferred in an isothermal container with ice to the Laboratory of Pharmacology and Pharmaceutical Care, Faculty of Pharmacy of University of Coimbra, Portugal. The experiments started with artery isolation in a Petri dish with Krebs- Henseleit solution (Figure II.2), by removing the surrounding perivascular tissue (i.e. adipose, connective and muscular tissue) with scissors. After isolation, the arteries were cut into small rings of 3 mm in length. The arteries were mounted on two platinum wires arranged so as to stand on opposite sides and without crossing and then suspended in organ bath chambers, filled with 10 mL of physiologic solution and maintained at 37°C with a PanLab® thermostat.

Figure II.2: Tissue preparation: Removal of surrounding tissue and cut the arteries in small rings (3mm), mounted on platinum wires and then suspended in organ bath chambers, in Laboratory.

The wires were attached to AD Instruments® force transducers which allowed the recording of isometric tension of vascular rings during the experiments. The tension of each ring was adjusted to an optimal resting tension of ~2 g that corresponded to the equilibrium state. The rings of HIMA were washed each 30 minutes, with Krebs-Henseleit bicarbonate buffer, in order to eliminate a possible interference from drugs administered to the patients, during the stabilization period of 2 hours. After the stabilization period, changes in isometric tension were measured using the PowerLab® data acquisition package (Figure II.3) and all data was collected in gram (g) and then converted to milliNewton (mN).

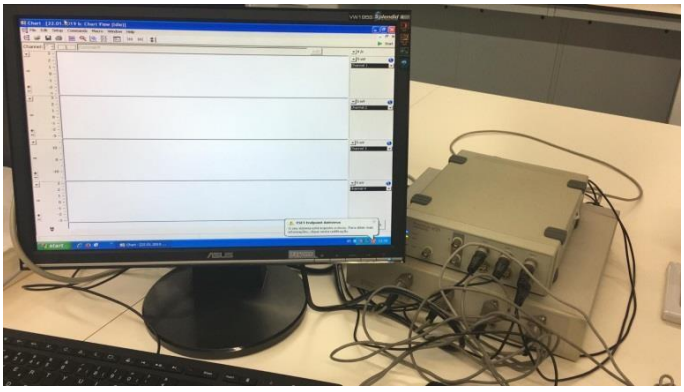

Figure II.3: The PowerLab® system.

➔ Experimentalprotocol

In order to evaluate the viability of the tissue throughout the experimental period, HIMA rings were stimulated with a single-dose of 60mM potassium chloride (KCl) at the

beginning of each experiment. After washing and total relaxation of rings, vasoreactivity of HIMA rings was assessed.

The intrinsic activity of the crude extract of leaves of *Cymbopogon citratus*, i.e., the ability to directly stimulate a receptor or a set of receptors, was evaluated by obtaining concentration-response curves to the extract. These curves were obtained by cumulative addition of increasing doses of extract (0.002-0.2 mg/mL), each dose being added when the ring tension reached a plateau, after the previous dose.

The vasorelaxant activity was studied for the crude extract and the three fractions (phenolic acids, flavonoids and tannins). This experiment started with a precontraction with noradrenaline (20 µM) to assess the vasodilation, through cumulative concentration-response curves, after a plateau was reached on the contractile response. The curves were performed through cumulative addition of increased doses of crude extract and fractions (0.002-0.2 mg/mL), in the same conditions previously described.

The interaction of extract with adrenergic system was evaluated for the crude extract as well as for the three fractions. So, the influence of the crude extract of *Cymbopogon citratus* as well as each fraction was evaluated through comparison of two cumulative concentration-response curves to noradrenaline. The first noradrenaline curve, was performed through cumulative addition of increasing doses of noradrenaline (0.1 to 48 µM), each dose being added when the ring tension reached a plateau, after the previous dose. After this curve, the rings were washed. After relaxation and stabilization of the rings, we were performed incubation with different concentrations of extract: 0.0002, 0.002, 0.02, 0.2 and 2 mg/mL of crude extract; 0.2, 1 and 2 mg/mL of phenolic acids; 0.2 and 1 mg/mL of flavonoids and 0.2 and 1 mg/mL of tannins, for a period of 30 minutes.

After the period of incubation, another cumulative concentration-response curve to noradrenaline was performed, in the same conditions of previous curve. The time difference between the noradrenaline curves was approximately 1 hour, to minimize the risk of tachyphylaxis. Tachyphylaxis is the progressive decrease in response to a given dose after repetitive administration of a pharmacologically or physiologically active substance.

At the end of the experiment, new tissue viability tests were performed on all the rings used, divided into three phases: pre-contraction with 10 µM noradrenaline and/or 20 µM followed by administration of 100 mM acetylcholine; pre-contraction with 20 mM KCl followed by administration of 100 mM acetylcholine; and finally, stimulation with 60 mM KCl.

2.3 Antioxidant activity

Free radical-scavenging activity was evaluated according to the method described by Blois[64]. The absorbance was measured using a Cintra 101 (GBC SCIENTIFIC EQUIPMENT, Melbourne, Australia) UV-Vis spectrophotometer (Figure II.5). For the preparation of the 2,2-diphenyl-1-picrylhydrazyl solution (DPPH) 5.475 mg of DPPH was dissolved in 25 mL of methanol. Then, aqueous extracts were diluted in concentration variation 0.5, 0.6, 0.7, 0.8, 1 and 1.2 mg/mL. For to calibrate the spectrophotometer was prepared a blank (100 mM acetate buffer (1 mL), pH 6.0, and 2 mL of methanol) and the respective control (100 mM acetate buffer (1 mL), pH 6.0, and 1.5 mL of methanol and 500 µM DPPH (0.5 mL)). Then, the aliquots samples were prepared with 0.1 mL of extract and 100 mM acetate buffer (1 mL), pH 6.0, and 1.4 mL of methanol and 500 µM DPPH (0.5 mL) and their respective blank with 0.1 mL of extract and 100 mM acetate buffer (1 mL), pH 6.0, and 1.9 mL of methanol. The reaction mixtures (3 mL) was homogenized and kept for 30 min at room temperature and in the dark. The quantification of the remaining DPPH radicals was recorded at 517 nm. Three separate assays were performed for each concentration of the sample solution which EC_{50} values was determined based on the concentration and the percentage inhibition.

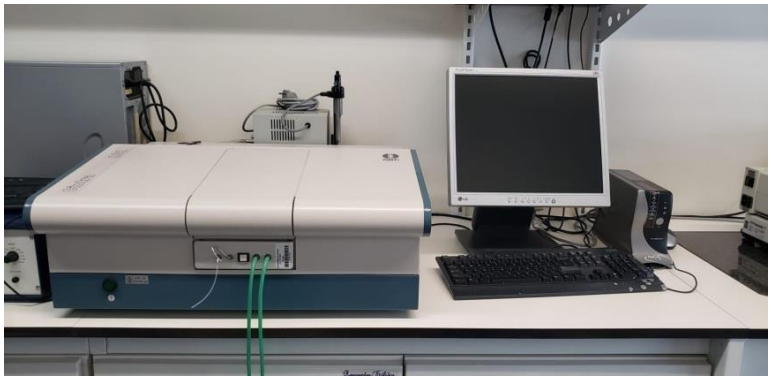

Figure II.4: UV-Vis spectrophotometer used to measure absorbances.

2.4 Statistical Methods

GraphPad Prism® version 5.1 was used for statistical analysis of all assays.

Data is generally presented as mean ± standard error of mean (SEM) of the number of experiments (n) indicated.

In vascular studies, the maximum contraction recorded (E_{max}) represents the intrinsic activity of each compound. The negative logarithm of the extract concentration that induces half the maximal effect (pEC_{50}) expresses the potency of each compound. The pEC_{50} values were obtained by interpolation of each cumulative concentration-response curve on a semi-logarithmic scale (% of maximal contraction vs. logarithm of concentration). Statistical significant differences between two samples were detected by the Student's t-test for independent samples. For three or more samples, the analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used. *P* values lower than 0.05, 0.01 and 0.001 were considered to indicate statistically significant differences.

In the antioxidant activity study we performed three independent tests. For each assay, five concentrations of extract was prepared and the absorbance measures. From the absorbance values for each concentration tested, the percentages of remaining DPPH (% DPPH) were determined and the percentage of antioxidant activity (% activity), i.e the amount of $DPPH^{\bullet}$ consumed.

Next, we built a concentration-absorbance graph, with three lines, which corresponding to the three independent tests. The linear correlation analysis was performed in order to check the significance of each correlation coefficient between variables. The EC_{50} was calculated from the linear regression equation ($Y = mX + b$, where Y is the absorbance; *m* the slope of the line, X the concentration of extract and b the constant of the line) for each test. The EC_{50}, i.e. the concentration of a given substance required to induce half the maximum effect, resulted from the application of the following formula:

$$EC_{50} = \frac{\frac{\text{ABS of sample control}}{2} - b}{m}$$

The final EC_{50} was obtained as the mean of the three independent assays.

The determination coefficient, R^2, was also determined to demonstrate how the response variables were related mathematically and to understand the proportion of the variance (fluctuation) of one variable that is predictable from the other variable.

III. RESULTS AND DISCUSSION

3.1 Antioxidant activity

Cymbopogon citratus were purchased from ERVITAL (Mezio, Castro D'Aire, Portugal) and a voucher specimen was deposited in the herbarium of the University of Coimbra, Faculty of Pharmacy.

From dry leaves infusion of *Cymbopogon citratus*, three extracts were obtained and characterized, by Dr. Artur Figueirinha and the other investigators[8]. The fractioning process providing three major fractions: tannins, phenolic acids and flavonoids.

HPLC-PDA analyses from *Cymbopogon citratus* infusion suggested that the first fraction contain a few compounds that show UV spectrum similar to flavanols, suggesting the probable presence of condensed tannins (proanthocyanidins) in this fraction. The second fraction contains caffeic acid and caffeic and *p*-coumaric acid derivatives, and the third fraction contains flavonoids, mainly, luteolin and apigenin derivatives.

Each fraction representing 4.3, 9, and 6.1% of the infusion weight, respectively.

This polyphenolic chemical composition may contribute to some traditional therapeutic properties attributed to this plant. For example, capacity related with the reactive oxygen species scavenging.

In this work, the reactive oxygen species scavenging capacity was evaluated by DPPH test. Regarding this test, the species used as antioxidant was the dried leaves extract of *Cymbopogon citratus*. Several solutions were prepared with different amounts of extract in test tubes with 0.5 ml methanolic DPPH solution. Then, the absorbance of these solutions was measured at 517 nm. The results of the three independent assays performed for each concentration of prepared sample solution are recorded in the Table III.1.

A decrease of the amount of DPPH radicals and, consequently, decrease in absorbance, with increasing extract present in solution was found. This decrease of DPPH radicals occurs because there are an increasing amount of antioxidant molecules in solution. The absorbance decrease is accompanied by an observable change in the color of the solution from violet to yellow (not colorless, since some of the flavonoids in solution have this color).The absorbances obtained and recorded in Table III.I, were used to construct the graph shown in Figure III.I. Each line was composed of five points, corresponding to the five absorbances obtained in each independent assay respectively, and was obtained by linear regression.

Table III.1: Analysis of the antiradicalar activity of *Cymbopogon citratus* leaf extract using the DPPH test. Concentration of fresh sample – 5 mg/mL.

µL	ABS	DPPH (%)	Activity (%)	Sample (µg)
100	0.782 - -	74.8 - -	25.2 - -	500
120	0.724 0.745 0.798	69.3 68.3 69.9	30.7 31.7 30.1	600
140	- 0.708 0.745	- 64.9 65.2	- 35.1 34.8	700
160	0.621 0.675 0.695	59.4 61.9 60.9	40.6 38.1 39.1	800
180	- 0.583 -	- 53.4 -	- 46.6 -	900
200	0.531 - 0.606	50.8 - 53.0	49.2 - 47.0	1000
240	0.433 0.452 0.476	41.4 41.5 41.7	58.6 58.5 58.3	1200

For each assay, this table contains information on absorbance, the percentages of remaining DPPH (% DPPH) and the percentage of antioxidant activity (% activity). The "-" represents the absorbance values discarded in each test, since to draw each line we use only five points. The discarded values are absorbance's that add more deviation to each line.

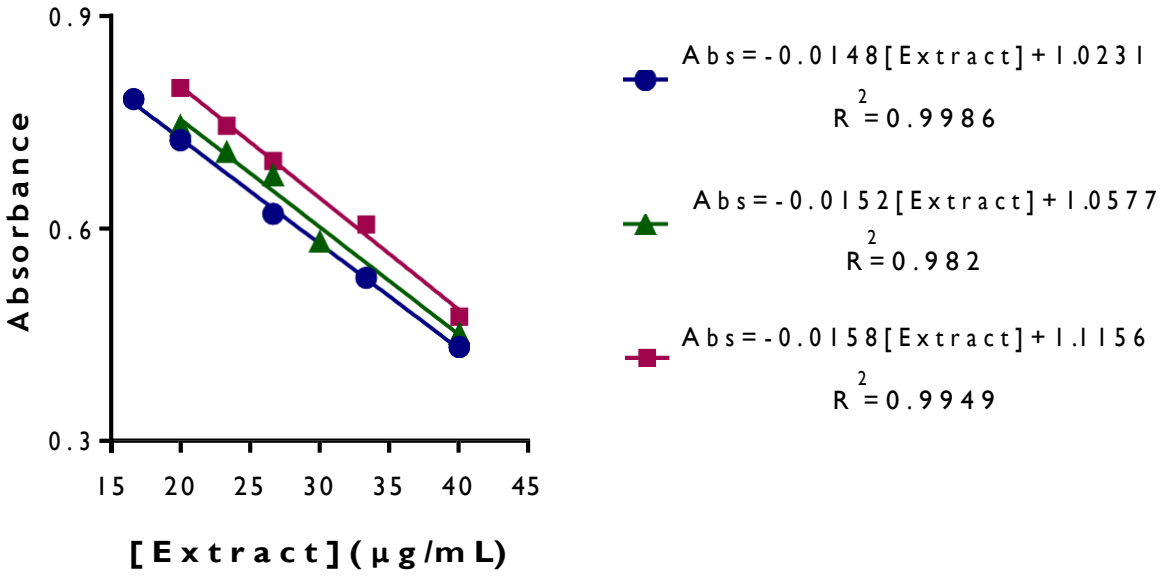

Figure III.1: Anti-radical activity of crude extract of *Cymbopogon citratus* by the DPPH test, in three independent assays. Each line is composed for five points, corresponding to the five absorbances obtained in each test, respectively. Conversion of the equation of the line into an equation of type $Y=mX+b$: Y is the absorbance; m the slope of the line, X the concentration of extract and b the constant of the line. R^2 is the coefficient of determination of the line, that is, it is the measure of adjustment of the linear statistical model in relation to the observed values.

After tracing the linear regression that best fitted the points of each test, we use the parameters of each independent assay and calculated the EC_{50} for each of them. The mean EC_{50} obtained for the crude extract of *Cymbopogon citratus* was 33.98±1.51 µg/ml, corresponding to the average of the three tests performed.

The EC_{50} obtained for the crude extract of dry leaves of *Cymbopogon citratus* corresponds to a higher antioxidant activity than that obtained in others studies using the same extractive process 41.72±0.05 µg/mL[65] and 38.75±1.09 µg/mL.[66]

The compounds that appear to be responsible for the activity of *Cymbopogon citratus* leaf extract are the derivatives of apigenin and luteolin, not ruling out the high potential of the compounds derived from caffeic acid present, since they are the majority fraction[8, 65]. More studies are needed to establish the structure-activity relationship of each compound present in the polyphenolic fraction studied.

3.2 Vascular activity studies in HIMA

For both peripheral and coronary revascularization, autologous vein and artery are clearly the gold standard.[67] The HIMA has long been recognized the gold standard as a model to study vascular physiology, being therefore used in a wide array of studies.[59, 68, 69] The lower mortality and higher patency rates of HIMA compared to other vessel grafts appears to be associated with the striking resistance of this vessel to atheroma, where multiple structural and biological properties of the HIMA could be involved.[69]

Previous studies have shown that HIMA exhibits a lower incidence of perioperative spasm, which increases the mortality and morbidity associated with coronary artery bypass grafting.[68] Structurally, the endothelial layer of HIMA shows few fenestrations, lower intercellular junction permeability, greater anti-thrombotic activity and higher endothelial production of nitric oxide, which are some of the unique ways that make the HIMA resistant to the transfer of lipoproteins, which are responsible for the development of atherosclerosis.[68, 69] The vascular study of HIMA rings was assessed through organ bath experiments. With this method, it is possible to obtain concentration–response curves, through the recording of isometric tension. Despite being the most commonly used method to assess the vascular reactivity of several vascular tissues, this method allows the evaluation of vascular function and not selective evaluation of endothelial function.

Furthermore, the vascular studies in HIMA have associated some limitations, such as hypoxia induced oxidative stress and potential damage or interference because of the harvesting or isolation techniques[68]. All this may have influenced the obtained results.

3.2.1 Evaluation of the viability of HIMA rings

Firstly, to verify the reactivity and viability of the arteries, we used KCl (60 mM), which elicits a vasoconstrictor effect independent of receptor activation. The vascular smooth muscle tonus is highly dependent on the membrane potential, which is essentially determined by the activity of the K^+ channels. Therefore, the addition of KCl to the organ bath, promotes the increase of the extracellular K^+ and, consequently, there is a decrease of K^+ efflux, by the potassium channels, from the smooth muscle cell to the cell membrane. Thus, the intracellular environment becomes less negative and depolarizes.

The depolarization leads to the opening of the voltage-sensitive Ca^{2+} channels, present in the smooth muscle cell membrane, promoting the influx of Ca^{2+}, resulting in contraction, which increases over time until a plateau is reached - maximum contractility. Only the rings that demonstrated (Figure III.2) this behavior were used for experience.

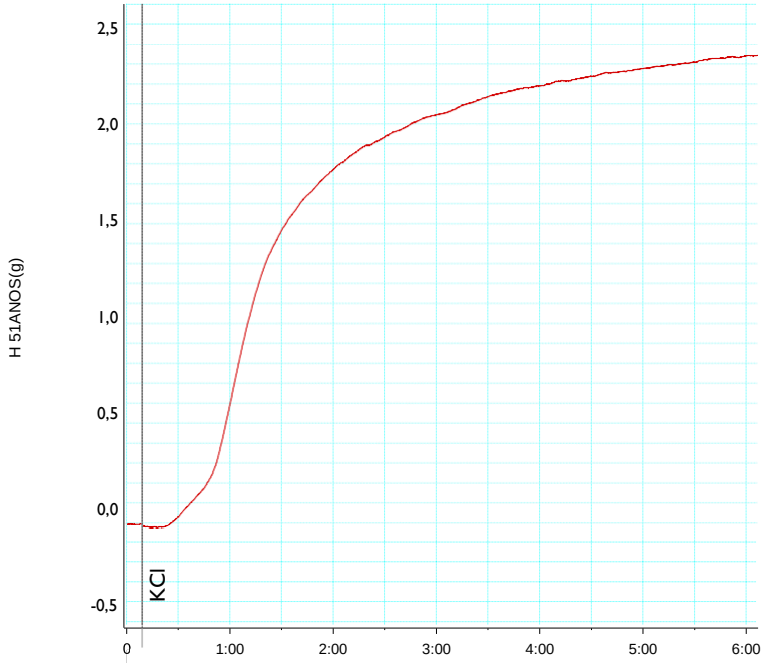

Figure III.2: Type curve of the HIMA response to a stimulation of 60 mM KCl. Graph: vertical scale in grams and horizontal scale in minutes.

3.2.2 Study of vascular tone variation induced by the crude extract and phenolic acids fraction of *Cymbopogon citratus*

The first step in the study of HIMA's vasoactivity began with the evaluation of the intrinsic activity of the crude extract 0.002 to 0.2 mg/mL and the phenolic acid fraction 0.002 to 0.2 mg/mL of *Cymbopogon citratus*.

As shown in Figure III.3, the crude extract and the phenolic acid fraction of *Cymbopogon citratus* induced a significant variation of the vascular tone.

Table III.2 shows the maximum effect recorded (E_{max}), 0.33±0.13 mN to the crude extract and 0.23±0.33 mN to fraction of phenolic acids. In the same table, we presented the potency of each compound, 2.48±0.47 to the crude extract and 3.65±1.88 to phenolic acids.

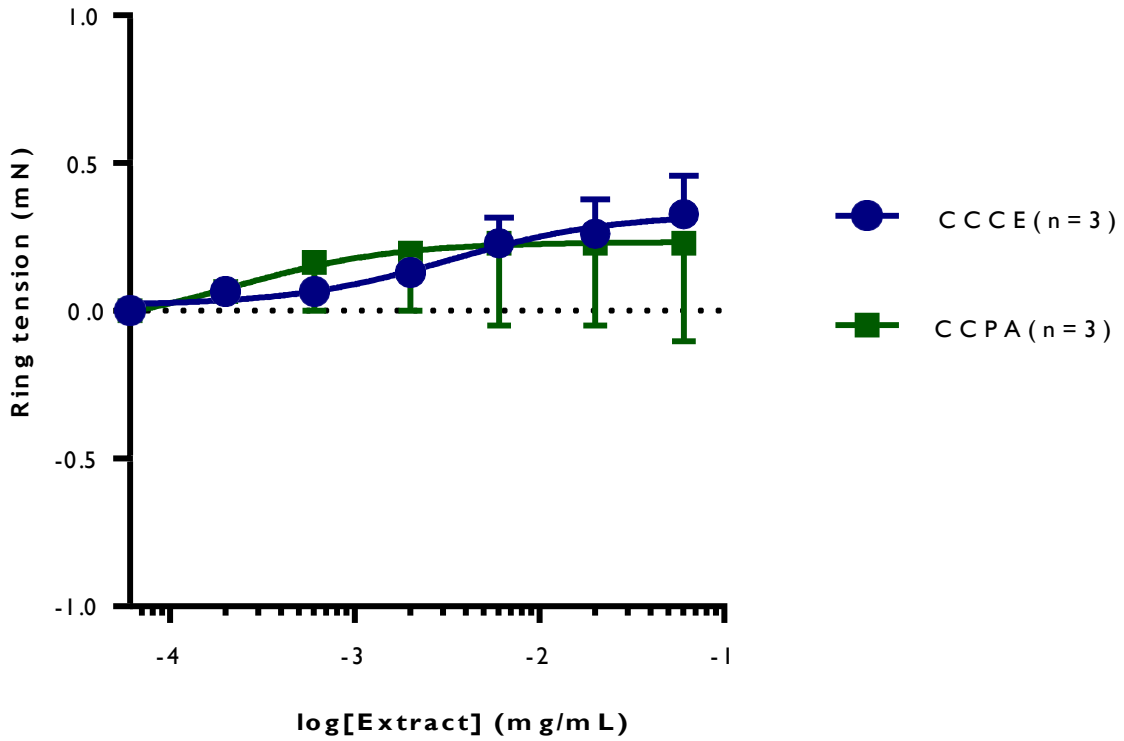

Figure III.3: Concentration-response curve for crude extract (n = 3) and fraction of phenolic acids (n = 3) of *Cymbopogon citratus* in HIMA rings.

Table III.2: Variation of basal tonus induced by the crude extract and fraction of phenolic acids of *Cymbopogon citratus*.

Extract/Fraction	E_{max} (mN)	pEC_{50} (-log[mg/mL])
Crude extract	0.33±0.13	2.48±0.47
Phenolic acid fraction	0.23±0.33	3.65±1.88

For each compound, this table contains information on the maximum contraction (E_{max}, mN) and the potency (pEC_{50}, -log[mg/mL]).

The results demonstrated that the addition of increasing concentrations of the crude leaf extract and the phenolic acid fraction of *Cymbopogon citratus* triggers an increase in HIMA ring tension.

However, more studies are needed to confirm this analysis, namely the increase in the number of assays as well as the range of concentrations.

3.2.3 Study of the interaction with adrenergic system induced by crude extract, phenolic acids, tannins and flavonoids fractions of *Cymbopogon citratus*

In the study of the interaction of extract with adrenergic system, the behavior of the HIMA was observed in response to increasing concentrations of noradrenaline (0.1 to 48 µM). In total, seven successive additions of three different concentrations of noradrenaline were administered to each ring, represented by the dashed vertical lines in Figure III.4. As shown, noradrenaline exerted a total agonistic effect on the adrenergic receptors present in the HIMA, being responsible for its vasoconstriction.

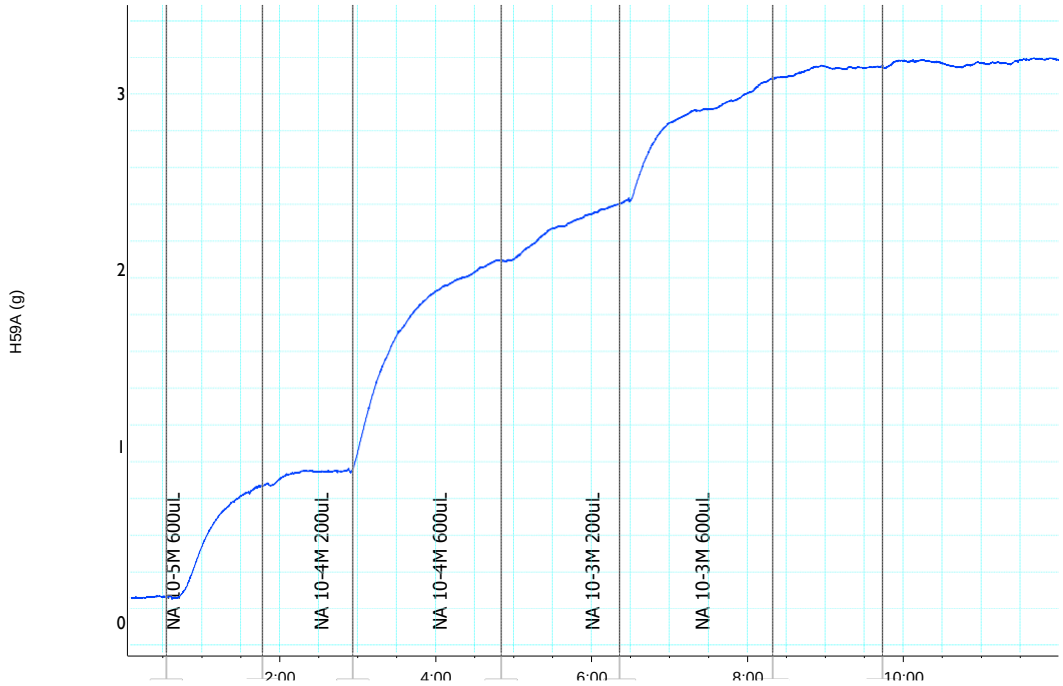

Figure III.4: Contractile response of HIMA to noradrenaline (type curve), with additions of increasing concentrations of noradrenaline. Graph: Vertical scale in grams and horizontal scale in minutes.

The successive addition of doses of noradrenaline triggered an increasing contractile response of the concentration-dependent type. All rings that exhibited this behavior described and visible in Figure III.4 were considered viable rings for the experiment. Vascular smooth muscle cells have α and β adrenergic receptors, for which noradrenaline is a full agonist. The response of the cell to this type of agonist depends on the relative importance of each adrenergic receptor population. However, it is known that, in most vascular tissues, the predominant effect is mediated by the α-adrenergic receptors[53].

There are two subtypes of α-adrenergic receptors (α_1 and α_2), and both are present in vascular smooth muscle cells. However, the contraction of these cells is predominantly mediated by α_1-adrenergic receptors[68]. These α_1-adrenergic receptors are found predominantly in larger vessels of conductance, such as arteries. The arteries have thick walls and a greater amount of elastic tissue, which gives them greater resistance to the pressure exerted by the passage of blood. Although present in the endothelium, these receptors are present in greater abundance in smooth muscle cells[53]. The main effect of the stimulation of α_1-adrenergic receptors by catecholamines, such as noradrenaline, is the increase in contraction force.

The HIMA is not an exception, as several studies have suggested that the noradrenaline-induced contractile response of HIMA is predominantly mediated by α_1-adrenergic receptors[70, 71]. Thus, the vasoconstrictor response shown in Figure III.4 is expected by stimulation with noradrenaline.

Regarding the effect on the noradrenaline-induced contraction of the vascular rings, the pre-incubation with 0.002 mg/mL and 2 mg/mL of crude extract caused a significant potentiation of the contractile response 121.09±26.10% and 137.51±39.33%, respectively to noradrenaline compared to the control, as shown in Table III.3.

Table III.3: Maximum effect and potency of the crude extract of *Cymbopogon citratus* at different concentrations in HIMA arterial rings.

Curves	E_{max} (%)	pEC_{50} (-log[M])
Control	100	5.57±0.05
0.0002 mg/mL	41.80±26.37	5.90±0.81
0.002 mg/mL	121.09±26.10*	6.01±0.41*
0.02 mg/mL	117.35±13.70	5.87±0.15
0.2 mg/mL	92.08±10.10	5.57±0.14
2 mg/mL	137.51±39.33*	5.64±0.37*

For each concentration, this table contains information on the maximum contraction (E_{max} %) and the potency (pEC_{50} -log[M]). The data were analyzed with repeat-measures one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. *$p<0.05$ vs. control.

Potentiation of the contractile response induced by the concentration of 0.002 mg/mL was expected, since this concentration is included in the increasing range of concentrations used in the previous vascular tonus effect evaluation test, in which the response was also of stimulation of contraction of HIMA.

Table III.3 also shows that all other concentrations had no significant effect on maximal contraction to noradrenaline after incubation of the arterial rings for 30 minutes.

Figure III.5 represents the effect of different concentrations of *Cymbopogon citratus* in the contractile response of HIMA rings to noradrenaline.

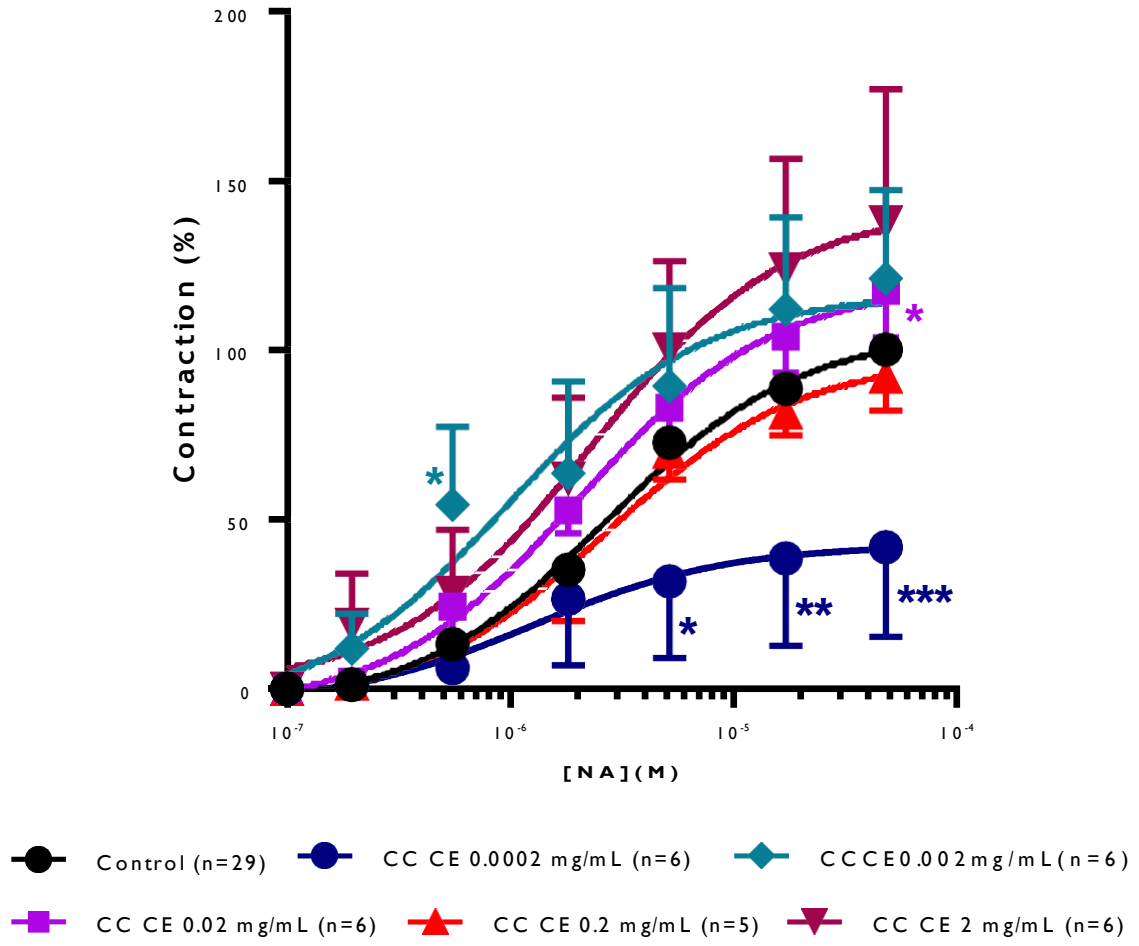

Figure III.5: Concentration-response curves for crude extract of *Cymbopogon citratus* before (control) and after the incubation of different concentrations prepared. Values are expressed as mean±SEM. The data were analyzed with multiple t test using the Bonferroni- Dunn method. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. Control.

In HIMA rings, noradrenaline was able to induce contractions that were strongly inhibited by crude extract of *Cymbopogon citratus* in the concentration of 0.0002 mg/mL (maximal inhibition of 41.80±26.37%, $p<0.001$ vs. control). However, in some concentrations, the crude extract potentiated noradrenaline-induced contraction in HIMA rings. The contractions significantly potentiated by concentrations of 0.002 and 0.02 mg/mL, 121.09±26.10% ($p<0.05$ vs. control) and 117.35±13.70% ($p<0.05$ vs. control), respectively.

The study of interaction of extract with adrenergic system was extended to three available of the fractions of *Cymbopogon citratus*: flavonoids, tannins and phenolic acids.

The fraction of phenolic acids has been shown to have a significant potentiation effect on the response to HIMA ring contraction when stimulated with the successive increasing dose regime of noradrenaline.

When compared to the control curve, i.e. the noradrenaline curve before incubation, the concentration of 1 mg/mL significantly potentiated the vasoconstriction effect of noradrenaline from 100% to 127.05±34.44%. And, the concentration of 2 mg/mL also potentiated the noradrenaline-induced contraction from 100% to 132.37±60.89% (Table III.4).

Table III.4: Maximum effect and potency of the phenolic acids of *Cymbopogon citratus* at different concentrations of HIMA arterial rings.

Curves	E_{max} (%)	pEC_{50} (-log[M])
Control	100	5.72±0.08
0.2 mg/mL	100.98±17.63$^{\#}$	5.47±0.21$^{\#}$
1 mg/Ml	127.05±34.44*	6.01±0.45*
2 mg/mL	132.37±60.89	5.61±0.53

For each concentration, this table contains information on the maximum contraction (E_{max}, %) and the potency (pEC_{50}, -log[M]). The data were analyzed with repeat-measures one-away ANOVA followed by Bonferroni's multiple comparisons test. *$p<0.05$ 1mg/mL vs. Control and $^{\#}p<0.05$ 0.2 mg/mL vs. 1mg/mL.

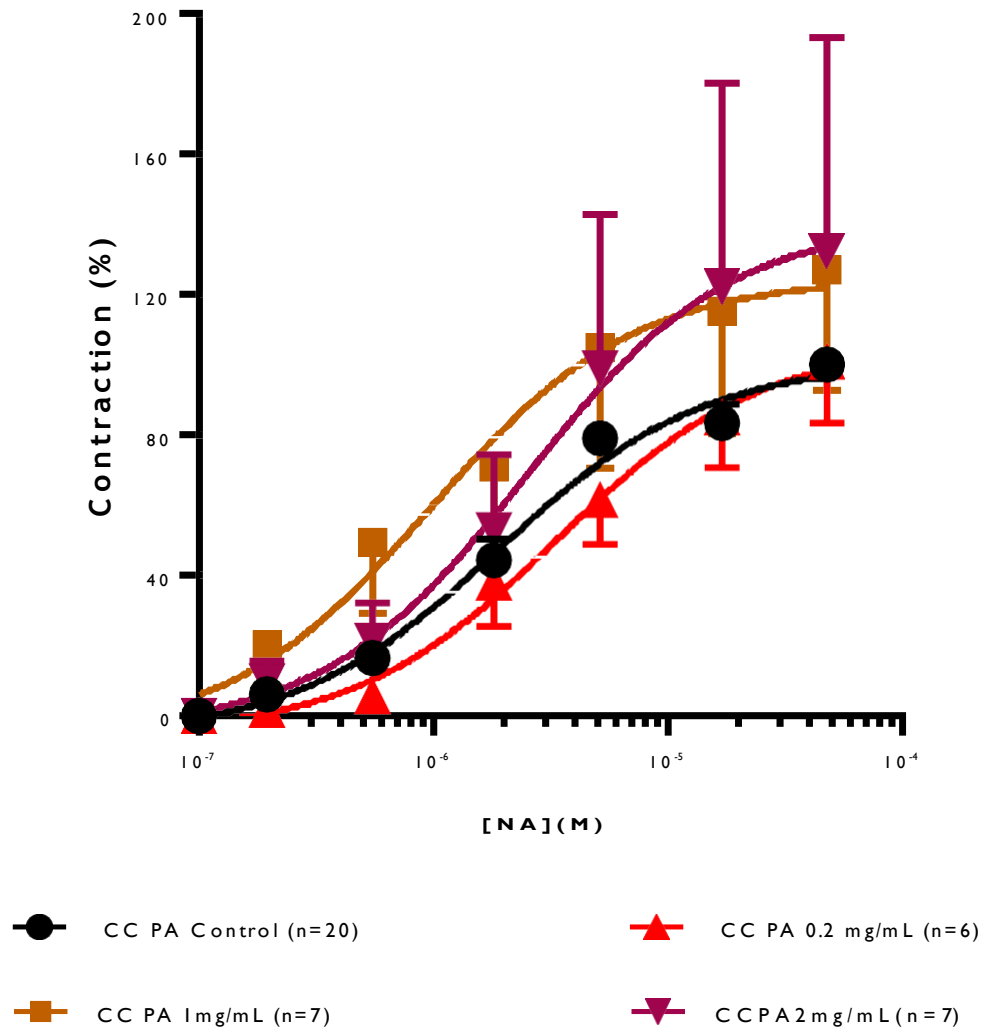

Figure III.6: Concentration-response curves for phenolic acids before (control) and after the incubation of different concentrations prepared. Values are expressed as mean±SEM.

Still by statistical analysis of Table III.4, and as we see in the Figure III.6, the concentration of 1 mg/mL was more effective than the concentration of 0.2 mg/mL (127.05±34.44 and 100.98±17.63%, respectively), and it was more potent (6.01±0.45 vs. 5.47±0.21).

The fraction of tannins presented the same behavior as the phenolic acid fraction.

Incubation of the 1 mg/mL concentration in the 30 minute period triggered a potentiation of the vasoconstriction in the HIMA rings, with a maximum effect recorded of 125.79±30.11% (Table III.5).

As observed in Figure III.7 and register in Table III.5, the concentration of 0.2 mg/mL did not show a significant variation in the contractile response triggered by noradrenaline.

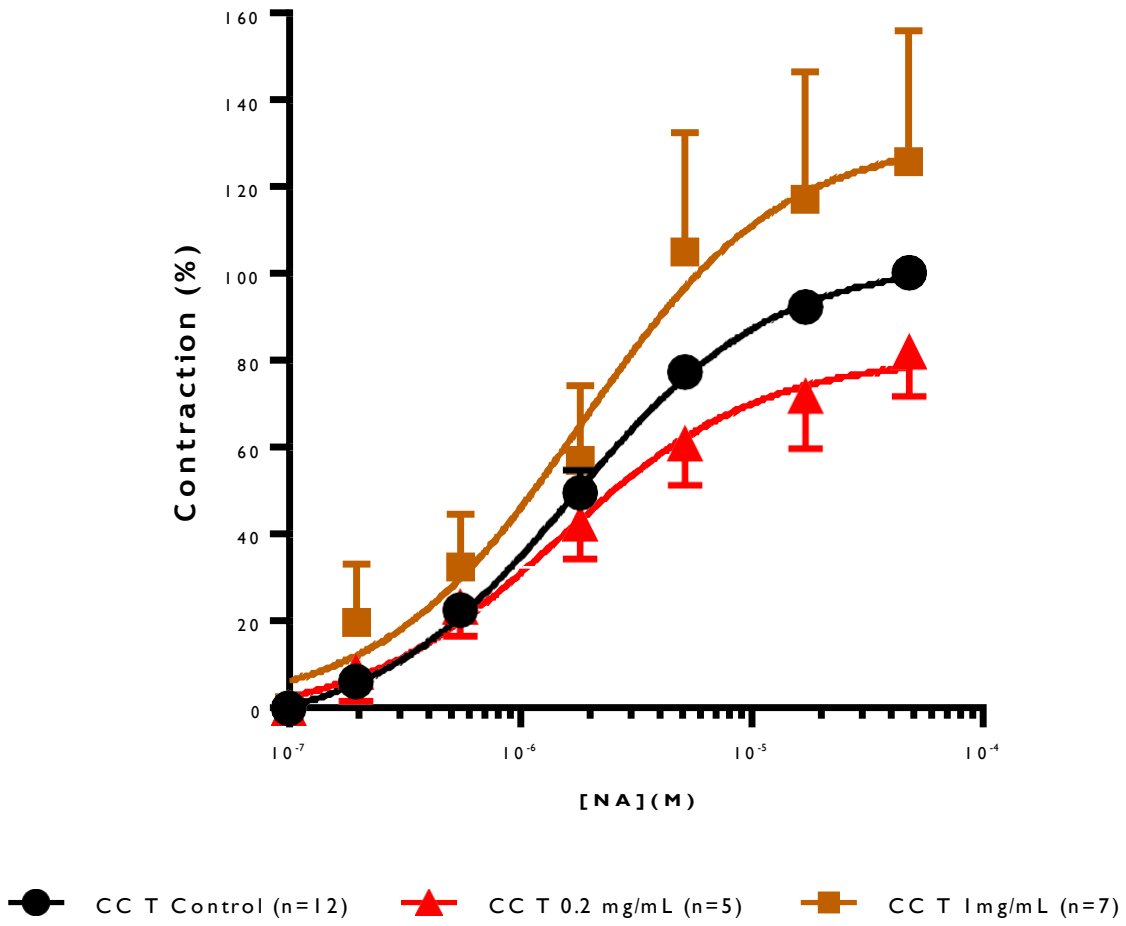

Figure III.7: Concentration-response curves for tannins fraction before (control) and after the incubation of different concentrations prepared. Values are expressed as mean ± SEM.

Table III.5: Maximum effect and potency of the tannins fraction of *Cymbopogon citratus* at different concentrations of HIMA arterial rings.

Curves	E_{max} (%)	pEC_{50} (-log[M])
Control	100	5.77±0.06
0.2 mg/mL	81.94±10.32	5.83±0.21
1 mg/mL	125.79±30.11*	5.74±0.34*

For each concentration, this table contains information on the maximum contraction (E_{max}, %) and the potency (pEC_{50}, -log[M]). The data were analyzed with repeat-measures one-away ANOVA followed by Bonferroni's multiple comparisons test. *p<0.05 1mg/mL vs. Control.

The fraction of flavonoids presented an antagonistic behavior in relation to the remaining fractions.

Table III.6: Maximum effect and potency of the flavonoids fraction of *Cymbopogon citratus* at different concentrations of HIMA arterial rings.

Curves	E_{max} (%)	pEC_{50} (-log[M])
Control	100	5.62±0.08
0.2 mg/mL	86.67±13.34*	5.10±0.22*
1 mg/mL	76.34±12.27	5.54±0.23

For each concentration, this table contains information on the maximum contraction (E_{max}, %) and the potency (pEC_{50}, -log[M]). The data were analyzed with repeat-measures one- away ANOVA followed by Bonferroni's multiple comparisons test. *p<0.05 0.2 mg/mL vs. control.

It should be noted that incubation of the 0.2 mg/mL concentration attenuated the vasoconstrictor activity caused by stimulation of the HIMA rings with noradrenaline.

At this concentration, there was a decrease in the maximal effect caused by noradrenaline from 100% to 86.67±13.34% was recorded (Table III.6). As also observed in Figure III.8, noradrenaline induced contraction in HIMA rings is significantly inhibited by the same 0.2 mg/mL concentration (maximal inhibition=38.92±12.84%, p<0.05 0.2 mg/mL vs control).

Unlike the other fractions, the concentration of 1mg/mL of the flavonoid fraction did not trigger significant changes in the vasoconstrictor activity described by the noradrenaline in the HIMA rings.

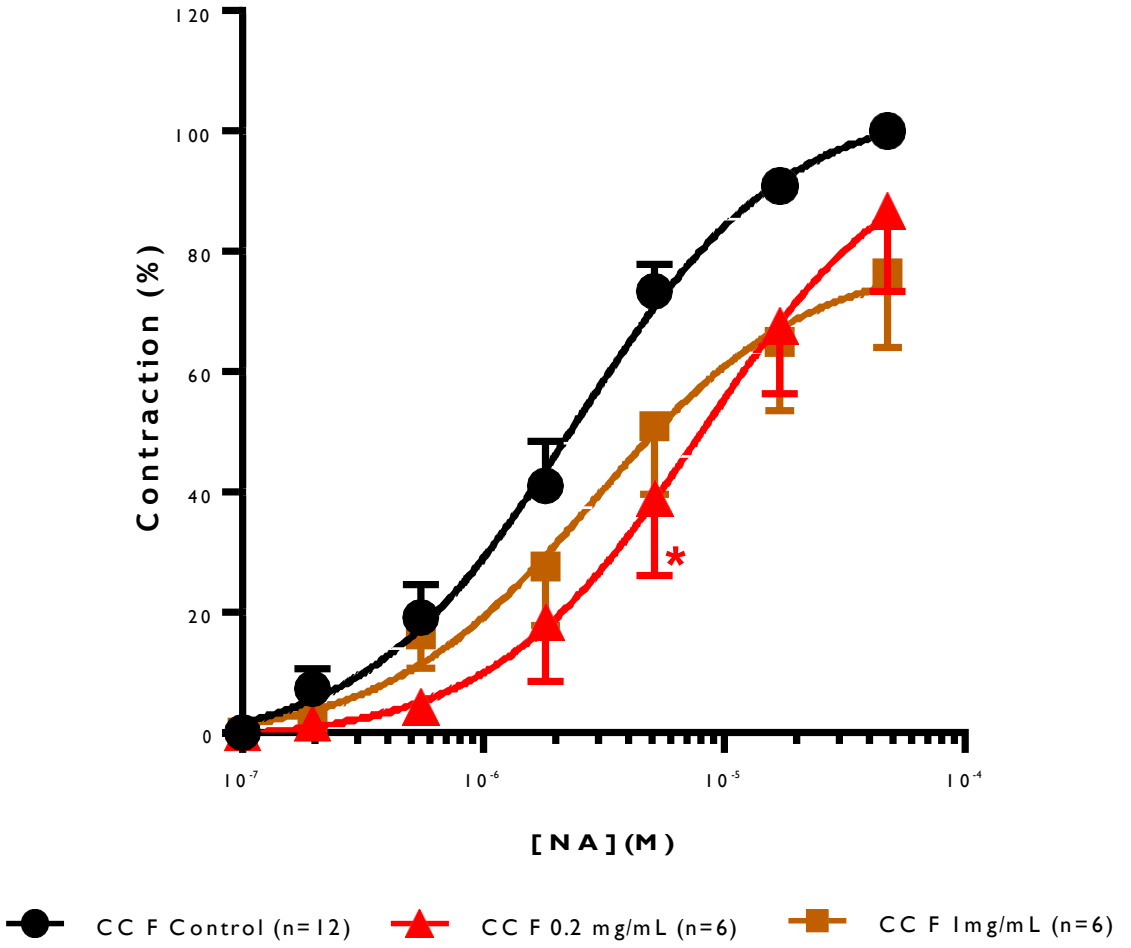

Figure III.8: Concentration-response curves for flavonoids fraction before (control) and after the incubation of different concentrations prepared. Values are expressed as mean SEM. *p<0.05 0.2 mg/mL vs. Control

3.2.4 Study of the vasorelaxant activity induced by crude extract, phenolic acids, tannins and flavonoids fractions of *Cymbopogon citratus*

In order to complete and conclude the vasoactivity study of the crude extract and fractions of *Cymbopogon citratus*, we evaluated its vasorelaxant activity.

In the study of vasorelaxant activity, we evaluated the behavior of HIMA when we administered an increasing range of extract concentrations from 0.002 to 0.2 mg/mL, after inducing pre-contraction with noradrenaline (20 µM).

In total, seven successive additions of different concentrations of extract were administered in each ring.

From the analysis of the Table III.7 we conclude that the crude extract of *Cymbopogon citratus* showed a significant intrinsic HIMA relaxation activity 6.46±2.40% after a maximal pre-contraction induced by noradrenaline.

However, Devi *et al.*[58] had already demonstrated that methanolic extracts of leaves of *Cymbopogon citratus* at doses ranging from 0.00624 mM to 6.24 mM, induced a significant relaxation on vascular tension of endothelium-intact rat aortic rings on phenylephrine- induced in spontaneously hypertension rats (66.76±6.75%) and in normotensive rats - Wistar Kyoto rats (64.92±6.15%), most significant that our results.

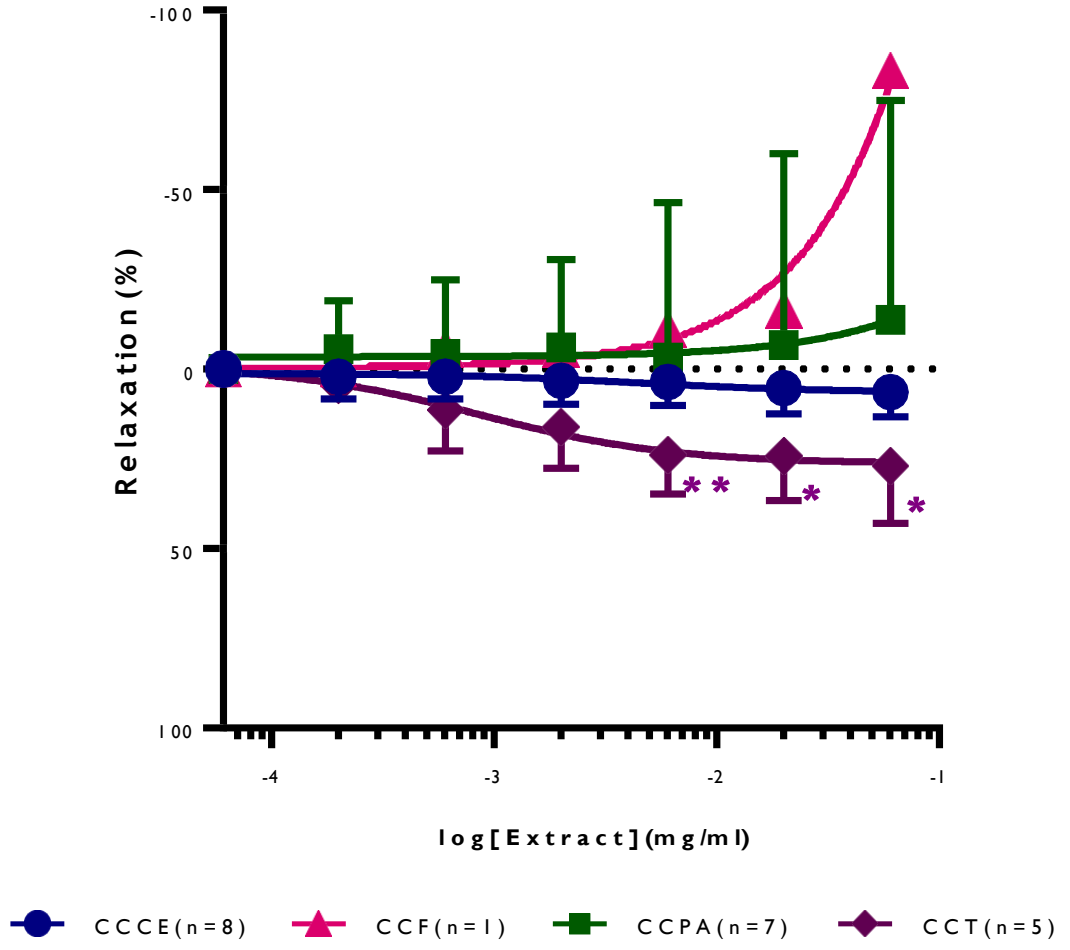

Figure III.9: Dose-response curves for vasorelaxation effect of crude extract, phenolic acids, tannins and flavonoids fractions of *Cymbopogon citratus* on noradrenaline-induced contraction in HIMA rings. Values are expressed as mean±SEM. The data were analyzed with multiple t test using the Bonferroni-Dunn method. *p<0.05 tannins fraction vs. crude extract.

The tannins fraction also produced a significant relaxant response 26.91±7.05% after pre-contraction with noradrenaline (20 μM) and being much more effective than the crude extract. As can be seen in Figure III.9, the vasorelaxant action of the tannins fraction is

highlighted, especially in the last three additions. These additions corresponding to: 0.0012 mg/mL, 0.004 mg/mL and 0.012 mg/mL of tannins fraction.

Thus, the tannins fraction presents a vasorelaxant effect significantly higher than the crude extract in all these additions 23.98±4.80% ($p<0.01$ vs. crude extract), 24.01±5.47% ($p<0.05$ vs. crude extract) and 26.91±7.05% ($p<0.05$ vs. crude extract), respectively.

Table III.7: Maximum relaxation and potency of crude extract, phenolic acids, tannins and flavonoids fractions of *Cymbopogon citratus* in HIMA arterial rings after pre-contraction with noradrenaline.

Extract	R_{max} (%)	pEC_{50} (-log[mg/mL])
Crude extract	6.46±2.40	2.35±0.81
Tannins	26.91±7.05*	3.07±0.35*
Phenolic acids	-13.83±23.00	0.08±41.08

For each concentration, this table contains information on the maximum relaxation (R_{max},%) and the potency (pEC_{50}, -log[mg/mL]). The data were analyzed with repeat-measures one-away ANOVA followed by Bonferroni's multiple comparisons test. *$p<0.05$ phenolic acids vs. tannins.

The fraction of phenolic acids does not present any evidence of vasorelaxant agent. Technically, after pre-contraction with 20 µM of noradrenaline, the artery reaches its maximum contractility. However, the fraction of phenolic acids exhibits vasoconstrictor behavior, as verified in the study of vascular tonus variation, stimulating a contraction of 13.83±23.00%, even after 20 µM noradrenaline.

The flavonoids fraction seems to have the same behavior of phenolic acids fraction. However, the number of experiments (n) is not enough to conclude something with evidence and certainty.

However, the fraction of phenolic acids and flavonoids presented opposite results. The phenolic acid fraction significantly potentiated HIMA contraction 13.83±23.00% even after pre-contraction with 20 µM noradrenaline.

In the same way, as shown in the Figure III.9, the flavonoid fraction appears to describe the same behavior as the phenolic acid fraction. However, the number of experiments (n) is insufficient, not allowing us to draw a credible conclusion about the result obtained.

In order to study the vasorelaxant effects of *Cymbopogon citratus*, several studies have been carried out with some of the different constituents of this plant.

For example, Bastos J. *et al.*[57] studied the vasorelaxant effects of citronellol, an essential oil extracted from *Cymbopogon citratus*, in isolated mesenteric rat artery rings. Their results demonstrated that citronellol induced relaxations, in rings of rat mesenteric artery, with or without endothelium. In endothelium-denuded rings, citronellol strongly inhibited the contraction induced by $CaCl_2$. In the same way, in mesenteric rings under Ca^{2+}-free solution, citronellol inhibited transient contractions induced by phenylephrine or by caffeine.

These results suggested that citronellol may interfere with calcium influx, blocking the voltage-operated calcium channels and with the mobilization intracellular calcium stores, blocking the sensitive receptors to inositol 1,4,5-trisphosphate (IP_3) and caffeine.

Devi et al,[58] studied the effect of methanolic extracts of leaves, stems and roots of *Cymbopogon citratus* and citral, the biggest constituent of this plant, in isolated thoracic rat aorta. In this study they used two types of rats: male spontaneously hypertensive rats and male normotensive Wistar Kyoto rats. In this study, the authors concluded that both citral and extracts of leaves, stems and roots of *Cymbopogon citratus* caused relaxation of vascular smooth muscle. However, citral only caused a significant relaxation in spontaneously hypertensive rats, while extracts of leaves and roots caused a significant relaxation in both types of rats. The stem extract did not elicit a significant relaxation. Based on these results, the authors hypothesized that the relaxation induced by citral and extracts of leaves and roots may derive from an inhibition of voltage-operated calcium channels and/or a mobilization of intracellular calcium stores. However, citral also seems to induce vasorelaxation through endothelial production of nitric oxide.

In regard to the effect of leaves extract, the same study concluded that the leaves may contain both vasoconstrictor and vasorelaxant agents, with the relaxing effect being dominant, possibly through PGI_2-mediated vascular smooth muscle relaxation, after activation of the muscarinic receptors.[58]

IV. CONCLUSION

Currently, cardiovascular disease is still the leading cause of death in the world. Despite the scientific advances already achieved, there are still many barriers to the treatment of CVD.

Endothelial dysfunction is common in several cardiovascular disease conditions. It is characterized by a failure of the blood vessel to mediate acetylcholine-induced vasorelaxation.

It is evident that agents which promotes the production of vasorelaxant compounds, such as NO, prostanoids, EDHF, and/or inhibits vasoconstrictors as free radicals, may result in better vascular health.

It is believed that plant-based polyphenols are likely to possess such beneficial actions at the vascular endothelial cell level.

Thus, during the 2nd year of the Master's in Applied Pharmacology, Faculty of Pharmacy, University of Coimbra, we studied the vascular effects and antioxidant activity of *Cymbopogon citratus*.

Regarding the study of antioxidant activity, the total extract of *Cymbopogon citratus* with an EC_{50} = 33.98±1.51 µg/ml seems to be a very potent antioxidant. This is because a compound is considered to be very potent antioxidant when EC_{50} < 50 mg/L[72].

Regarding vascular studies in HIMA:

- Both the crude extract and the phenolic acids fraction showed to have a contractile effect, in the range of concentrations used.
- The crude extract and tannins fraction appear to have vasorelaxant capacity, since they reversed the maximum contraction reached by noradrenaline. Meanwhile the phenolic acids fraction had an antagonistic effect.
- Noradrenaline induced dose-dependent contractions, in the range of concentrations used, showing to have intrinsic activity for existing HIMA receptors.
- For crude extract, only the incubations with the concentrations of 0.0002 and 0.2 mg/mL reduced the contractile effect of noradrenaline.
- The tannins fraction demonstrated to be able to attenuate the contractile effect of noradrenaline with an incubation of 0.2 mg / mL concentration. However, with the increase in incubation concentration this effect did not occur.

- The flavonoids fraction was shown to have a vasorelaxant behavior, attenuating noradrenaline-induced contractions, at both concentrations used in the incubations.
- The phenolic acids fraction was coherent, potentiating the contraction induced by the noradrenaline in all concentrations prepared and used in the incubation.

These results suggest that the presence of flavonoids, namely compounds derived from apigenin and luteolin, seem to be the main responsible for the inhibitory effect observed in HIMA rings, reversing the action caused by noradrenaline.

In sum, it is concluded that *Cymbopogon citratus* may be a candidate as an antioxidant and vasodilator agent. However, the scarcity of raw materials and the lack of time to proceed with the fractionation and isolation of compounds, limited the experiments.

In the future, it would be important to characterize the signaling pathways involved in the effects observed in our study. Furthermore, it would be crucial to extend this study to subfractions and isolated compounds of *Cymbopogon citratus*.

V. BIBLIOGRAPHY

1. VEERESHAM C. - *Natural products derived from plants as a source of drugs.* J Adv Pharm Technol Res, 3 (2012) 200-1.
2. NEWMAN D.J. ; CRAGG G.M. - *Natural products as sources of new drugs over the 30 years from 1981 to 2010.* J Nat Prod, 75 (2012) 311-35.
3. DIAS D.A. ; URBAN, S. ; ROESSNER, U. - A historical overview of natural products in drug discovery. Metabolites, 2 (2012) 303-36.
4. RUCHIKA, J.N. - Ashutosh Pandey *Synthetic Metabolism and Its Significance in Agriculture*, en *Current Developments in Biotechnology and Bioengineering* (2019) 365-391.
5. *DIRECTIVA 2001/83/CE DO PARLAMENTO EUROPEU E DO CONSELHO.* (2001) Artigo 1°.
6. SIMÕES, C. [et.al.] - *Farmacognosia do produto natural ao medicamento*, 1ª, 2017
7. SHAH, G. [et.al.] - *Scientific basis for the therapeutic use of Cymbopogon citratus, stapf (Lemon grass).* J Adv Pharm Technol Res, 2 (2011) 3-8.
8. FIGUEIRINHA, A. [et.al.] - *Cymbopogon citratus leaves: Characterization of flavonoids by HPLC–PDA–ESI/MS/MS and an approach to their potential as a source of bioactive polyphenols.* Food Chemistry, 110 (2008) 718-728.
9. KHANUJA [et.al.] - *Essential oil constituents and RAPD markers to establish species relationship in Cymbopogon Spreng. (Poaceae).* Biochemical Systematics and Ecology, 33 (2005) 171-186.
10. EKPENYONG C.E. ; AKPAN E. ; NYOH A. - *Ethnopharmacology, phytochemistry, and biological activities of Cymbopogon citratus (DC.) Stapf extracts.* Chin J Nat Med, 13 (2015) 321-37.
11. *FERN K. (2014). Useful Tropical Plants Database.* Acedido a: 13 de junho de 2019 em *http://tropical.theferns.info/viewtropical.php?id=Cymbopogon+citratus.*
12. HILGERT N.I. - *Plants used in home medicine in the Zenta River basin, Northwest Argentina.* Journal of Ethnopharmacology 76 (2001) 11-34.
13. SCARPA G.F. - *Medicinal plants used by the Criollos of Northwestern Argentine Chaco.* J Ethnopharmacol, 91 (2004) 115-35.
14. MACIA M.J. ; GARCIA E. ; e VIDAURRE P.J. - *An ethnobotanical survey of medicinal plants commercialized in the markets of La Paz and El Alto, Bolivia.* J Ethnopharmacol, 97 (2005) 337-50.
15. COELHO-FERREIRA M. - *Medicinal knowledge and plant utilization in an Amazonian coastal community of Maruda, Para State (Brazil).* J Ethnopharmacol, 126 (2009) 159-75.
16. DI STASI L. [et.al.] - *Medicinal plants popularly used in the Brazilian Tropical Atlantic Forest.* Fitoterapia, 73 (2002) 69-91.

17. DE ALBUQUERQUE U.P. [et.al.] - *Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: a quantitative approach.* J Ethnopharmacol, 114 (2007) 325-54.

18. AU D.T. [et.al.] - *Ethnobotanical study of medicinal plants used by Hakka in Guangdong, China.* J Ethnopharmacol, 117 (2008) 41-50.

19. MESIA G.K. [et.al.] - *Antiprotozoal and cytotoxic screening of 45 plant extracts from Democratic Republic of Congo.* J Ethnopharmacol, 115 (2008) 409-15.

20. CANO J.H. ; VOLPATO G. - *Herbal mixtures in the traditional medicine of eastern Cuba.* J Ethnopharmacol, 90 (2004) 293-316.

21. TENE V. [et.al.] - *An ethnobotanical survey of medicinal plants used in Loja and Zamora- Chinchipe, Ecuador.* J Ethnopharmacol, 111 (2007) 63-81.

22. AGYARE C. [et.al.] - *An ethnopharmacological survey and in vitro confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima- Kwanwoma area, Ghana.* J Ethnopharmacol, 125 (2009) 393-403.

23. TICKTIN T. ; DALLE, S.P. - *Medicinal plant use in the practice of midwifery in rural Honduras.* J Ethnopharmacol, 96 (2005) 233-48.

24. KHARE C.P. - *Indian Medicinal Plants An Illustarted Dictionary*, 2007.

25. POONAM K. ; SINGH G.S. - *Ethnobotanical study of medicinal plants used by the Taungya community in Terai Arc Landscape, India.* J Ethnopharmacol, 123 (2009) 167-76.

26. ZUMSTEG I.S. ; WECKERLE C.S. - *Bakera, a herbal steam bath for postnatal care in Minahasa (Indonesia): documentation of the plants used and assessment of the method.* J Ethnopharmacol, 111 (2007) 641-50.

27. ANDRADE-CETTO A. - *Ethnobotanical study of the medicinal plants from Tlanchinol, Hidalgo, Mexico.* J Ethnopharmacol, 122 (2009) 163-71.

28. CASTILLO-JUAREZ I. [et.al.] - *Anti-Helicobacter pylori activity of plants used in Mexican traditional medicine for gastrointestinal disorders.* J Ethnopharmacol, 122 (2009) 402-5.

29. GIOVANNINI P. ; HEINRICH, M. - *Xki yoma' (our medicine) and xki tienda (patent medicine)–interface between traditional and modern medicine among the Mazatecs of Oaxaca, Mexico.* J Ethnopharmacol, 121 (2009) 383-99.

30. SHRESTHA P.M. ; Dhillion S.S. - *Medicinal plant diversity and use in the highlands of Dolakha district, Nepal.* Journal of Ethnopharmacology, 86 (2003) 81-96.

31. COE F.G. - *Rama midwifery in eastern Nicaragua.* J Ethnopharmacol, 117 (2008) 136-57.

32. AJAIYEOBA E.O. [et.al.] - *Cultural categorization of febrile illnesses in correlation with herbal remedies used for treatment in Southwestern Nigeria.* Journal of Ethnopharmacology, 85 (2003) 179-185.

33. AJIBESIN K.K. [et.al.] - *Ethnobotanical survey of Akwa Ibom State of Nigeria.* J Ethnopharmacol, 115 (2008) 387-408.
34. NOVAIS M.H. [et.al.] - *Studies on pharmaceutical ethnobotany in Arrabida Natural Park (Portugal).* J Ethnopharmacol, 93 (2004) 183-95.
35. OSTRAFF M.A. [et.al.] - *Traditional Tongan cures for morning sickness and their mutagenic/toxicological evaluations.* Journal of Ethnopharmacology 71 (2000) 201-209.
36. KUMAR S. ; PANDEY A.K. - *Chemistry and biological activities of flavonoids: an overview.* ScientificWorldJournal, 2013 (2013) 162750.
37. BABU P.V.A. ; Liu D. - *Flavonoids and Cardiovascular Health.* 371-392.
38. RUNNIE I. [et.al.] - *Vasorelaxation induced by common edible tropical plant extracts in isolated rat aorta and mesenteric vascular bed.* J Ethnopharmacol, 92 (2004) 311-6.
39. WOODMAN O.L. ; MEEKER W. F. ; BOUJAOUDE M. - *Vasorelaxant and Antioxidant Activity of Flavonols and Flavones: Structure – Activity Relationships.* J Cardiovasc Pharmacol, 46 (2005) 302-309.
40. SIENIAWSKA E. ; BAJ T. - *Tannins.* Medical University of Lublin, Lublin, Poland, Chaper 10 (2017) 199-232.
41. PIETTA P.M. ; MINOGGIO M. ; BRAMATI L. - *Plant Polyphenols: Structure, Occurrence And Bioactivity* Studies in Natural Products Chemistry, 28 (2003) 257-312.
42. OGWURU N. ; Adamczeski M. - *Bioactive Natural Products Derived From Polygonum Species Of Plants: Their Structures And Mechanisms Of Action* Studies in Natural Products Chemistry, 22 (2000) 607-642.
43. CHUNG K.T. [et.al.] - *Tannins and human health: a review.* Crit Rev Food Sci Nutr, 38 (1998) 421-64.
44. SERRANO [et.al.] - *Tannins: Current knowledge of food sources, intake, bioavailability and biological effect.* Mol.Nutr.FoodRes, (2009) 310.
45. TONG W. Y. [et.al.] - *Inhibiting enzymatic starch digestion by hydrolyzable tannins isolated from Eugenia jambolana.* LWT - Food Science and Technology, 59 (2014) 389- 395.
46. ACTIS-GORETTA L. [et.al.] - *Cytotoxic effects of digalloyl dimer procyanidins in human cancer cell lines.* J Nutr Biochem, 19 (2008) 797-808.
47. HELENO S.A. [et.al.] - *Bioactivity of phenolic acids: metabolites versus parent compounds: a review.* Food Chem, 173 (2015) 501-13.
48. FERNÁNDEZ DE CÓRDOVA M.L. ; MEDINA A.R. - *Analytical Methods for Determination of Polyphenols in Beer.* (2014) 289-299.
49. VINCENTE A.R. [et.al.] - *Nutritional Quality of Fruits and Vegetables* (2014) 69-122.

50. ANDRADE N [et.al.] - *Knowledge about cardiovascular disease in Portugal.* Rev Port Cardiol, 37 (2018) 669-677.
51. WHO (2017). Cardiovascular diseases (CVDs). In.: World Health Organization.
52. Eurostat (2018). Cardiovascular diseases statistics. Statistics Explained
53. FONSECA D.A. ; ANTUNES P.E. ; COTRIM M.D. - *The Morphology, Physiology and Pathophysiology of Coronary Microcirculation.* (2016).
54. SITI H.N. ; KAMISAH Y. ; KAMSIAH J. - *The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review).* Vascul Pharmacol, 71 (2015) 40-56.
55. WALDEN R. ; TOMLINSON B. - *Cardiovascular Disease.* Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition., (2011).
56. KUMAR V.R. [et.al.] - *Protective effect of lemongrass oil against dexamethasone induced hyperlipidemia in rats: possible role of decreased lecithin cholesterol acetyl transferase activity.* Asian Pac J Trop Med, 4 (2011) 658-60.
57. BASTOS J.F. [et.al.] - *Hypotensive and vasorelaxant effects of citronellol, a monoterpene alcohol, in rats.* Basic Clin Pharmacol Toxicol, 106 (2010) 331-7.
58. DEVI R.C. ; SIM S.M. ; ISMAIL R. - *Effect of Cymbopogon citratus and Citral on Vascular Smooth Muscle of the Isolated Thoracic Rat Aorta.* Evid Based Complement Alternat Med, 2012 (2012) 539475.
59. FONSECA D.A. [et.al.] - *Histomorphometric analysis of the human internal thoracic artery and relationship with cardiovascular risk factors.* PLoS One, 14 (2019) e0211421.
60. HANSSON G.K. - *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 352 (2005) 1685-95.
61. FONSECA D.A. [et.al.] - *Hyperthermia Severely Affects the Vascular Effects of MDMA and Metabolites in the Human Internal Mammary Artery In Vitro.* Cardiovasc Toxicol, 17 (2017) 405-416.
62. SAJJA L.R. ; MANNAM G. - *Internal thoracic artery: anatomical and biological characteristics revisited.* Asian Cardiovasc Thorac Ann, 23 (2015) 88-99.
63. GAO Y.J. [et.al.] - *Perivascular adipose tissue modulates vascular function in the human internal thoracic artery.* J Thorac Cardiovasc Surg, 130 (2005) 1130-6.
64. BLOIS, M.S. - *Antioxidant Determinations by the Use of a Stable Free Radical.* Nature, 181 (1958) 1199-1200.
65. TAVARES F. [et.al.] - *Cymbopogon citratus industrial waste as a potential source of bioactive compounds.* J Sci Food Agric, 95 (2015) 2652-9.

66. SOUSA R. S. G. - *Desenvolvimento e otimização de formas farmacuticas orais contendo Cymbopogon citratus.* Dissertação de Mestrado em Tecnologias do Medicamento. Faculdade de Farmácia - Universidade de Coimbra (2016) 1-98.

67. KONIG G. [et.al.] - *Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery.* Biomaterials, 30 (2009) 1542-50.

68. FONSECA D.A., ANTUNES, P.E., e COTRIM, M.D. - *Endothelium-dependent vasoactivity of the human internal mammary artery.* Coron Artery Dis, 25 (2014) 266-74.

69. OTSUKA F. [et.al.] - *Why is the mammary artery so special and what protects it from atherosclerosis?* Ann Cardiothorac Surg, 2 (2013) 519-26.

70. BEVILACQUA M. [et.al.] - *Alpha 1 adrenoceptor subtype mediates noradrenaline induced contraction of the human internal mammary artery: radioligand and functional studies.* Cardiovasc Res, 25 (1991) 290-294.

71. GIESSLER C. [et.al.] - *Noradrenaline-induced contraction of human saphenous vein and human internal mammary artery: involvement of different alpha-adrenoceptor subtypes.* Naunyn Schmiedebergs Arch Pharmacol, 366 (2002) 104-109.

72. HASIN [et.al.] - *Potential of lemongrass leaves extract (Cymbopogon citratus) as prevention for oil oxidation* Journal of Chemical and Pharmaceutical Research, 7 (2015) 55-60.