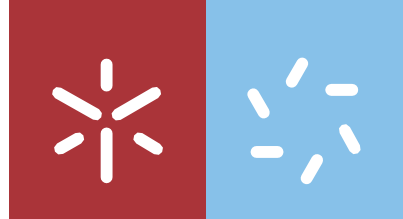




Bruna Gabriela Araújo da Silva

Study of the anti-inflammatory and neuroprotective
activity of plants used in Traditional Chinese Medicine
(TCM)

Universidade do Minho
Escola de Ciências





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**Study of the anti-inflammatory and
neuroprotective activity of plants used in
Traditional Chinese Medicine (TCM)**

Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia
e Bioempreendedorismo em Plantas

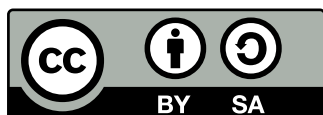
Trabalho efetuado sob a orientação da
**Professora Doutor Alberto Carlos Pires
Dias**

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Study of the anti-inflammatory and neuroprotective activity of plants used in Traditional Chinese Medicine (TCM)

Apocynum venetum é uma planta conhecida pelo seu uso na medicina tradicional chinesa. Neste trabalho, um extrato metanólico de folhas de *A. venetum* foi submetido a uma digestão *in vitro* para realizar uma comparação entre as atividades anti-inflamatórias e antioxidantes do extrato antes e depois da digestão. Os extratos foram submetidos a ensaios *in vitro* incluindo DPPH, atividade sequestrante de radicais ABTS, FRAP e ICA, radical superóxido e NO e atividade de inibitória da COX-2. Os efeitos citoprotetores e citotóxicos de ambos extratos foram também avaliados em células BV-2 e HepG2. As propriedades anti-inflamatórias dos extratos foram avaliadas através da quantificação do NO e avaliação da expressão genética de genes pró-inflamatórios em células BV-2 induzidas por LPS. Os resultados revelaram que ambos os extratos tiveram atividade em todos os ensaios bioquímicos realizados, exceto o extrato digerido nos ensaios ICA e FRAP. Ambos extratos revelaram efeitos protetores contra os danos induzidos pelo t-BHP em células BV-2 e HepG2. A digestão não causou diferenças significativas nas propriedades protetoras do extrato contra os danos induzidos pelo t-BHP. Em relação às atividades anti-inflamatórias, o extrato não digerido foi capaz de diminuir a liberação de NO e inibir a expressão de genes pró-inflamatórios. Enquanto que o extrato digerido só revelou atividade anti-inflamatória através da diminuição da liberação do NO. Estes resultados mostram que *A. venetum* pode ser uma fonte de compostos ativos com propriedades anti-inflamatórias e antioxidante e que estas atividades são mantidas em algum nível mesmo após as modificações que ocorrem durante a digestão.

Palavras-chave: *Apocynum venetum*, atividade antioxidante, digestão *In vitro*, Neuroproteção

ABSTRACT

Study of the anti-inflammatory and neuroprotective activity of plants used in Traditional Chinese Medicine (TCM)

Apocynum venetum is a plant known for its wide use in traditional Chinese medicine. In this work, an *A. venetum* methanolic leaves extract was subject to an *in vitro* digestion to conduct a comparison between the anti-inflammatory and antioxidant activities of the extract after and before the performance of digestion. The extracts were subjected to *in vitro* assays including DPPH, ABTS radical scavenging activity, FRAP and ICA, superoxide anion radical (PMS-NADH system) and NO (Griess method) scavenging activity and inhibition of COX-2 activity. Cytoprotective and cytotoxicity effects of both extracts were also evaluated in BV-2 and HepG2 cells. The intracellular ROS levels were also measured, after the treatment with the extracts and t-BHP in the HepG2 cell line. The anti-inflammatory properties of the extracts were also evaluated through the measurement of NO levels and evaluation of gene expression in LPS-induced BV-2 cells. The results revealed that both extracts had activity in all biochemical assays performed except for the digested extract in ICA and FRAP assays. Results proved that the digestion processes impaired the antioxidant activity in the biochemical analysis. Both extracts did not induce significant cytotoxicity and revealed protective effects against the damages induced by t-BHP in both BV-2 and HepG2 cells, probably due to the decrease of ROS induced by t-BHP, as revealed in HepG2 cells. The digestion did not cause significant changes in the protective properties of the extract against t-BHP-induced damages. Regarding anti-inflammatory activities, the non-digested extract was able to decrease the release of NO and inhibit the gene expression of pro-inflammatory genes. While the digested extract only revealed anti-inflammatory activity through the decrease of NO release.

Our results show that *A. venetum* may be a potential source of pharmacological active compounds related to inflammation and oxidative stress, and that *A. venetum* activities are kept at some level even after the modifications occurring in digestion.

Keywords: Antioxidant activity, *Apocynum venetum*, *In vitro* digestion, Neuroprotection

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ACRONYMS

TM	<i>Traditional Medicine</i>
HPLC-DAD	high Performance Liquid Chromatography with a diode-array detector
AV	<i>Apocynum venetum</i> methanolic leaves extract
dAV	digested of <i>Apocynum venetum</i> methanolic leaves extract
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
DNA	deoxyribonucleic acid
HAT	hydrogen atom transfer
GABA	gamma-amino butyric acid
PCR	polymerase chain reaction
NO	nitric oxide
RNA	ribonucleic acid
TAE	tris-acetate-EDTA buffer
cDNA	complementary DNA
ONOO-	peroxynitrite
GPX	glutathione peroxidase
CAT	catalase
GSH	reduced glutathione
GSSG	oxidized glutathione
SET	single electron transfer
SET	single electron transfer
COX-2	cyclogenase-2

ICOX-2	cyclogenase-2 inhibition
DAMPS	danger associated molecular patterns
PAMPS	pathogen associated molecular patterns
PRRs	pattern recognition receptors
TLRs	toll-like receptors
Nrf2	nuclear factor erythroid 2-related factor 2
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	mitogen-activated protein kinase
AP-1	activator protein-1
NOX	nicotinamide adenine dinucleotide phosphate oxidase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
iNOS	inducible nitric oxide synthase
nNOS	nitric oxide synthase
NOS	nitric oxide synthase
Keap1	kelch-like ECH-associated protein 1
ARE	antioxidant response elements
NDDs	neurodegenerative diseases
AD	Alzheimer's disease
PD	Parkinson's disease
HD	Huntington's disease
CNS	central nervous system
BBB	blood-brain barrier
FST	forced swimming test
5-HT	serotonin
NE	norepinephrine
DA	dopamine

MAO	monoamine oxidase
TST	tail suspension test
LPS	Lipopolysaccharide
D-GalN	D-galactosamine
TNF	tumor necrosis factor
CYP2E1	cytochrome P4502E1
IL-6	interleukin 6
IL-1	interleukin 1
IL-10	interleukin-10
IL-1BETA	interleukin-1BETA
TLR4	toll-like receptor 4
TLR2	toll-like receptor 2
TIR	toll-interleukin-1 receptor
JNK	C-Jun NH2-terminal kinase
BDNF	brain-derived neurotrophic factor
Bax	Bcl-2 Associated X Protein
Bcl-2	B-cell lymphoma 2
CREB	cAMP-response element binding
OGD	oxygen and glucose deprivation
MAPs	microtubule-associated proteins
CUMS	chronic unpredictable mild stress
MDA	malondialdehyde
AGEs	advanced glycation endproducts
mPTP	mitochondrial permeability transition pore
LDH	lactate dehydrogenase
MI/R	myocardial ischemia/reperfusion
Src	proto-oncogene tyrosine-protein kinase

eNOS	endothelium nitric oxide synthase
PI3K	phosphoinositide 3-kinase
EPM	elevated plus maze
TBARS	thiobarbituric acid reactive substance
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	ferric-reducing antioxidant power assay
LDL	low-density lipoprotein
HO-1	heme oxygenase-1
AGEs	advanced glycation end-products
PUFAs	polyunsaturated fatty acids
dwr	dry weight residue
IGF-1	insulin-like growth factor-1
NOi	scavenging of nitric oxide
DPPHr	reduced 2,2-diphenyl-1-picrylhydrazyl
ICA	iron chelating activity
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
FRAP	ferric reducing antioxidant potential
DMSO	dimethyl Sulfoxide
PMS	phenazine methosulphate
NBT	trizma hydrochloride solution
SO	superoxide anion
SOi	scavenging of superoxide anion
NOi	scavenging of nitric oxide
PBS	phosphate buffer
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
t-BHP	Tert-butyl hydroperoxide
SD	Standard deviation

FBS	fetal bovine serum
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
HCl	hydrochloric acid
FeSO₄	ferrous sulfate
SNP	sodium Nitroprusside
APAP	N-Acetyl-p-Aminophenol
NED	naphthylethylenediamine dihydrochloride
Cyt-c	cytochrome-c
PI3K	phosphoinositide 3-kinases
AKt	protein kinase B
ERK1/2	extracellular signal-regulated kinase 1/2
AMPK	AMP-activated protein kinase
mTOR	mammalian target of rapamycin
DMEM	Dulbecco's Modified Eagle Medium
FKN	fractalkine
SYK	spleen tyrosine kinase
p38	mitogen-activated protein kinase
AS	atherosclerosis
TMPD	N,N,N,N -Tetramethyl-p-phenylenediamine
RT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction
AA	arachidonic acid
mRNA	Messenger ribonucleic acid
DCFH	non-fluorescent compound
DCF	2',7'-dichlorodihydrofluorescein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
EC₅₀	statistical estimation of the concentration necessary to reach 50% of the observed effect

IC₅₀ half-maximal inhibitory concentration

CHAPTER 1

General introduction

1.1 *Apocynum venetum*

Apocynum venetum, typically recognized as Luobuma in China is a perennial and semi-shrubby herb [1–3]. Belonging to the Apocynaceae family, *Apocynum venetum* is believed to be native to many countries in northern and South-Eastern Europe and Asia [4]. Commonly found in saline regions and desert margins, *Apocynum venetum* has attracted widespread attention due to their resilience to stress [1, 2]. Despite their stress resistance, *A. venetum* is mostly known due to their long use as a medicinal plant. It is believed that both flowers and leaves from *A. venetum* may be useful for health care. Flowers are a good source of honey that can have benefits for the lungs and heart. While the leaves can be used as a tea and combined with spices and caffeine to make different drinks with different purposes [5]. Recently, several scientific works have confirmed beneficial effects of treatment with *A. venetum* leaves extract, due to its antioxidant, cardiotonic, anxiolytic, and neuroprotective activities and its ability to regulate blood pressure [6–8].

The presence of various flavonoids and phenolic acids has been linked with many pharmacological activities of *A. venetum* [3, 9]. According to reports, the leaves of *A. venetum* are rich in flavonoids such as quercetin, kaempferol hyperoside, and isoquercetin [10]. HPLC analysis also showed that neochlorogenic acid, chlorogenic acid, and other derivatives can be found in the leaves of *Apocynum venetum* [8, 11].

Furthermore, other compounds, such as phloroglucinol phytosterols, have also been identified in *A. venetum* [12]. Apocynosides I and II were isolated for the first time from processed leaves of *Apocynum venetum*. Four apocynin (flavan-3-ols replaced by phenylpropanoid) were also found for the first time in the leaves of *A. venetum*. Moreover, their leaves are also well known for their high content of minerals such as sodium, magnesium, calcium, potassium, and copper [1].

The composition of *Apocynum venetum* flowers has not been fully analyzed, however, the presence of some flavonoids, cardiac glycosides, organic acids, and polysaccharides has already been reported [5].

1.2 Oxidative stress

Oxidative stress is a widely used term defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox (reduction–oxidation) signaling and/or molecular damage". In other words, the body maintains a balance between the production and elimination of reactive oxygen species (ROS)/reactive nitrogen species (RNS), achieving a steady-state redox balance [13, 14]. These species are continually produced in the body and are fundamental to host defense and several other pivotal signal pathways [14–17].

Studies have shown that a decrease in the levels of ROS / RNS can impair physiological processes, increasing susceptibility to the development of diseases. Thus, it is speculated that an increase of ROS/RNS levels in the physiological range may lead to beneficial health effects [18, 19]. However, a further increase can result in damage since these species can interact with various molecules, such as lipids, proteins, and nucleic acids leading to the formation and accumulation of hazardous products. Once the damages induced by these species exceed the reparability of the organism, biological processes such as apoptosis and autophagy can be impaired [17]. In this way, oxidative stress can accelerate aging and multiple diseases progress [14, 20]. To prevent these outcomes, the body has antioxidant systems responsible for maintaining the redox balance in a steady-state, however, in case of excess production of these species or a reduction of cellular antioxidant capacity oxidative stress can occur [13].

1.2.1 SOURCES OF REACTIVE OXYGEN SPECIES/ REACTIVE NITROGEN SPECIES

ROS and RNS can be divided into two types: free radicals and other non-radical reactive derivatives also called oxidants. The first ones (hydroxyl radical ($OH\cdot$), NO, among others) are considered to be more reactive, due to the presence of one or more unpaired electrons in their molecular orbit. In the other hand, non-radical such as singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), and **HOCl** (**HOCl**), are able to oxidize biomolecules and are easily converted into radicals [16, 21, 22].

Mitochondria are a major source of ROS due to the electron transport chain. Under normal conditions, random electrons are leaked during oxidative phosphorylation processes and can further interact with molecular oxygen (O_2). Since O_2 accepts one electron at a time superoxide anion radical ($O_2^{\cdot-}$) can be generated [23–25]. Besides mitochondria, superoxide radical is also produced in large amounts to be used as a defense tool against infectious pathogens [26]. Superoxide radical is also a by-product of other enzymes such as xanthine and cytochrome P450 enzymes [24]. Superoxide radicals can be rapidly converted by the superoxide dismutase (SOD) to (H_2O_2) [23]. Subsequently, H_2O_2 can interact with reduced transition metals (mainly iron and copper), via Fenton reaction producing hydroxyl radical ($OH^{\cdot}$) [27]. This radical is widely accepted as the most reactive species due to its capability to react with nearby biomolecules causing serious damage to them [22, 26].

Throughout the inflammatory response, there is a production of both superoxide anion radical and NO. Nitric oxide is synthesized from L-arginine by three nitric oxide synthase (NOS), endothelium nitric oxide synthase (eNOS), nNOS, and inducible nitric oxide synthase (iNOS). The first one is mostly expressed in endothelial cells, nitric oxide synthase (nNOS) in specific neurons of the brain and the inducible isoform can be induced in cells by a different stimulus. Each one performs essential functions acting as a signaling molecule playing multiple roles in blood pressure regulation, defense mechanisms and other physiological processes [28]. Despite its importance, excess nitric oxide can be cytotoxic. NO can react with superoxide anion radical producing peroxynitrite ($ONOO^-$). Peroxynitrite rapidly protonates, at physiological pH, producing cytotoxic agents that can cause deoxyribonucleic acid (DNA) fragmentation and lipid peroxidation [21, 22, 26].

1.2.2 OXIDATIVE DAMAGE TO BIOMOLECULES

As earlier mentioned, when the levels ROS/RNS overload the antioxidant system, structure modifications and function modulation in nucleic acids, lipids and proteins can occur [14]. Oxidative stress can be critical for DNA since damage to it can impair the transcription and replication of significant genes which can ultimately lead to unfavorable outcomes. Unlike

other macromolecules, DNA can not be replaced thereby relying on many repair mechanisms to counteract oxidative DNA lesions [29, 30].

The oxidation of the nucleoside guanosine by $OH\cdot$ is the most prevailing purine base oxidation and has highly mutagenic potential [29, 30]. Moreover, ROS can react with deoxyribose causing the loss of DNA-bases that can then cause a break in the single or double DNA strand(s), contributing to genome instability cell cycle disruption and cell death [14].

Proteins are a major target for oxidants and can be oxidized both at the protein backbone and on the amino acid side-chains, leading to the formation of carbonyl groups (aldehydes and ketones) [31]. The most common carbonyl products are formed after the cleavage of peptide bonds by the alpha-amidation pathway or by the oxidation of glutamyl residues. These carbonyl groups can then be introduced into proteins by secondary reactions, which eventually leads to the formation of advanced glycation end-products (AGEs). Furthermore, the introduction of carbonyl groups on proteins can also occur with aldehydic products derived from lipid peroxidation [32]. Protein carbonylation is a major hallmark for protein oxidation since they are normally considered irreversible post-translational modifications [30]. The abstraction of hydrogen ions from the thiol group of cysteine can cause the formation of disulfide bonds which can impair the folding of protein causing abnormal protein folding. These changes may alter the conformation - due to the different characteristics of these residues - causing denaturation and protein aggregation [14, 31].

Lipid peroxidation is a process by which radical species, such as peroxy and hydroxyl radicals, can remove electrons from lipids generating reactive intermediates, starting a chain that consequently results in oxidative deterioration of lipids. Cellular and organelle membranes are particularly susceptible to this phenomenon due to their high content in polyunsaturated fatty acids (PUFAs) [33]. The peroxidation of lipids in the membranes may cause a decrease in its fluidity and permeability and inactivation of receptors. Furthermore, the oxidative attack forms many reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal, that can later be attached to proteins as previously mentioned [30]. The quantification of these by-products can be used to estimate lipid peroxidation which can be assessed by thiobarbituric acid reactive

substance (TBARS) assay. Besides causing damage to proteins and therefore its function, lipid peroxidation has been associated with many inflammatory diseases and can also act as a cell death signal inducing programmed cell death [33].

1.2.3 ANTIOXIDANT SYSTEMS

Given the toxic effects of ROS/RNS overload, living creatures have evolved many defense mechanisms to neutralize these species to achieve redox balance. Antioxidants are molecules that possess the ability to delay or prevent the oxidation of macromolecules [34–36]. The antioxidants system concerns antioxidants whose goal is to suppress the generation of free radicals and prevent oxidation propagation and repair enzymes such as lipases, and proteases, responsible for restoring the damage caused by ROS/RNS overload [34].

There are three major types of enzymatic antioxidants in the body SOD, glutathione peroxidase (GPX), and catalase (CAT) which act as the first line of defense. Superoxide dismutase scavenges superoxide radicals and converts them to water, thereby avoiding the formation of peroxynitrite from superoxide radical [36]. Both glutathione peroxidase and catalase can then remove H_2O_2 converting it to water. The first enzyme is the most abundant and is present in both mitochondria and cytosol. Glutathione peroxidase uses the reduced reduced glutathione (GSH) as a substrate to transfer electrons to peroxides, while oxidized glutathione (GSSG) is produced [20, 37]. Apart from that GPX catalyzes the reduction of peroxide radicals to alcohols and oxygen. Thus, preventing the formation of lipid peroxyl radicals [38, 39]. Additionally, transition metals such as iron and copper can be sequestered by iron-binding protein, for instance, to avoid the generation of reactive free radicals [20].

Besides enzymatic, non-enzymatic antioxidants are part of the antioxidant system and can be obtained from the diet. Ascorbic acid (vitamin C), for instance, is present in many aliments and can rapidly be distributed to all tissues [20]. It effectively scavenges much relevant ROS/RNS causing the generation of ascorbyl radicals, which can then be regenerated to ascorbic acid. Additionally, ascorbic acid plays a distinguished role in restoring small molecules

such as alpha-tocopherol and urea so they can continue acting as antioxidants [40]. Vitamin C also possesses the ability to maintain the metal ions present in the active center of enzymes in a reduced state acting as a co-factor. Nonetheless, the reduction of these metal ions can result in a pro-oxidant effect, due to the intervention on Fenton reactions causing ROS generation [41]. Vitamin E (alpha-Tocopherol) has good solubility in lipid and can break the cycle of lipid peroxidation by reacting with peroxy before it can attack lipid molecules [42]. Growing evidence has shown that phenolic compounds can also contribute to the regulation of ROS RNS levels and intervene in oxidative stress. The major defense lines and pro-oxidant reactions are represented in figure 1.

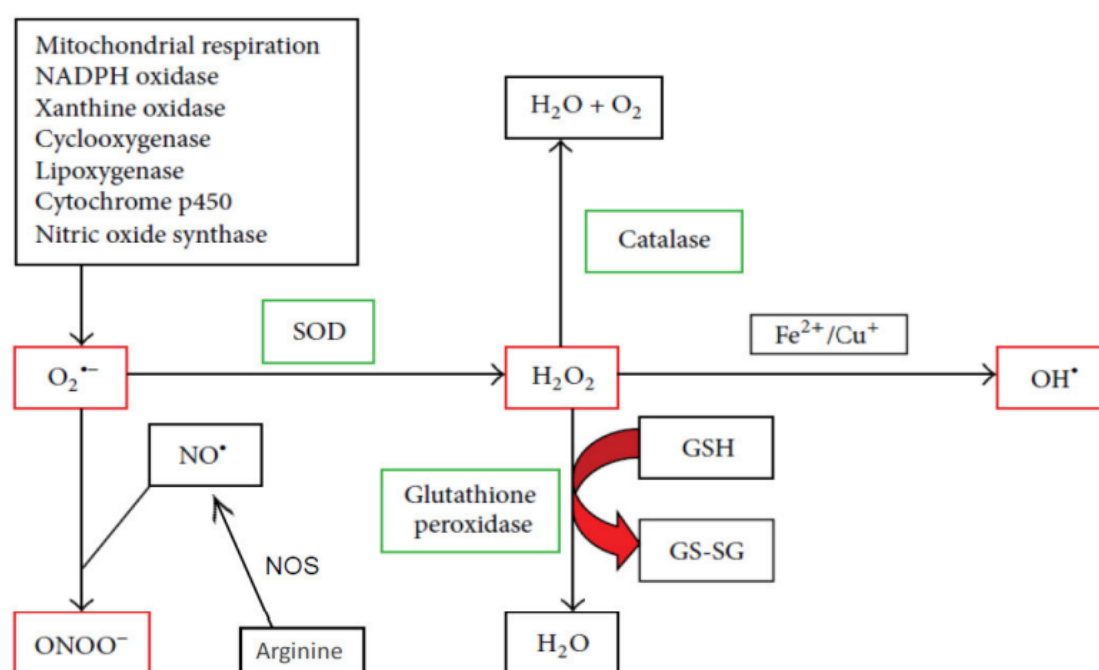


Figure 1: Representations of the major pro-oxidant and antioxidant reactions in a biological system. Several sources can produce superoxide which can rapidly react with nitric oxide (NO) to produce peroxynitrite. Superoxide dismutase (SOD) can avoid this pathway through the catalyzation of superoxide to produce hydrogen peroxide. Which can then be neutralized by catalase or glutathione peroxidase. However, in presence of transition metal ions (iron and copper), hydroxyl free radicals may be produced from superoxide via the Fenton reaction. Reactive species are shown in red and antioxidant enzymes are shown in green boxes. SOS: superoxide dismutase; GSH: reduced glutathione; GS-SG: oxidized glutathione; NOS: nitric oxide synthase. Adapted from Biswas et al, 2016 [43].

1.3 Phenolic compounds

Phenolic compounds are secondary metabolites that are widely spread across plants, playing relevant roles in their existence [44, 45]. These compounds perform different functions, most of them are related to plants' defense against pathogens, including bacteria and viruses, and abiotic stresses like drought and salinity. Also, contributes to its characteristics such as flavor, and color, among others. Phenolic compounds are mostly found attached to other molecules such as sugars and proteins, the existence of its free forms is less common in plants [46, 47]

Bearing one or more aromatic rings with two or more hydroxyl groups as represented in figure 2, these compounds can be divided into two main classes: flavonoids and non-flavonoids [48, 49]. The last ones comprise phenolic alcohols, hydroxybenzoic and hydroxycinnamic acids, and their derived esters and glycosides as well as other minor classes such as stilbenes [44, 45].

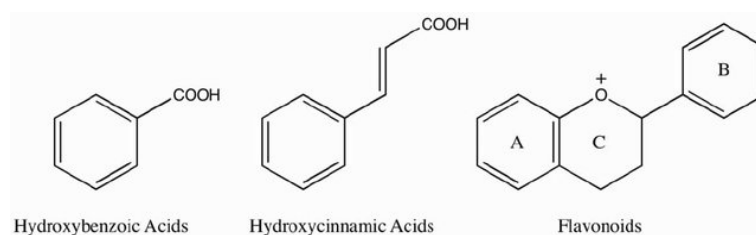


Figure 2: Basic structures of phenolic acids. Adapted from Khoddami et al., 2013 [50].

Two aromatic rings enclosing a heterocyclic six-membered ring with oxygen are the base structure of flavonoids. The differences found on the pyran ring and the different patterns of methylation and hydroxylation on the rings allow the classifying of flavonoids into six groups: flavanones, flavonols, flavones, anthocyanins, and isoflavones [44, 45] (see figure 3).

The molecular structures of these phenolic compounds confer the capacity of inactivating free radicals. The ability to scavenge free radicals, donating hydrogen atoms, electrons, or chelate metal cations allows these compounds to protect the human body from some hazards such as oxidative stress [52]. This ability seems to allow compounds to intervene in several biological processes such as anti-inflammatory, anti-mutagenic, anti-carcinogenic, and antioxidant properties [44, 53]. Besides the direct inactivating of free

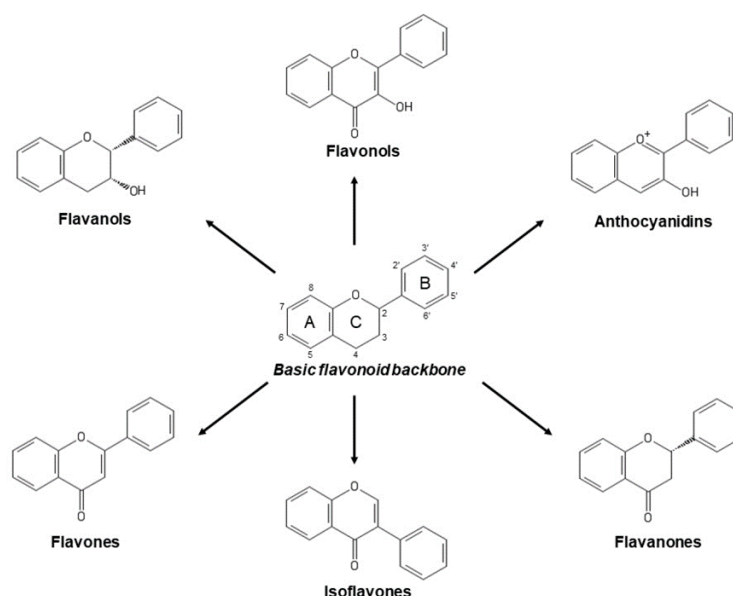


Figure 3: Flavonoid structure and main types of flavonoids. Adapted from Cosme et al., 2020 [51].

radicals, phenolic compounds can favor indirect antioxidant mechanisms, by modulating the activity of several antioxidant enzymes, mostly by the mediation of the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [54].

Nrf2 is a positive regulator of a set of antioxidants and detoxication enzymes. At normal conditions kelch-like ECH-associated protein 1 (Keap1) promotes degradation of Nrf2 preventing their translocation to the nucleus where it binds to antioxidant response elements (ARE) in the promoter region. When oxidation occurs Keap1 loses the ability to degraded Nrf2. In this case, Nrf2 can easily target the nucleus leading to the activation of ARE, resulting in a cascade of reactions leading to ultimate anti-inflammatory and antioxidant effects (see figure 4) [54–57].

Furthermore, flavonoids may also inhibit key ROS/RNS-producing enzymes such as xanthine oxidase, NOS and cyclooxygenase-2 (COX-2) [58]. Recent studies have also revealed a connection between phenolic compounds and other inflammatory-pathways through the mediation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling [55, 59, 60].

Despite the valuable connection between phenolic compounds and anti-inflammatory and antioxidant activities found in several scientific works, there is still controversy on whether

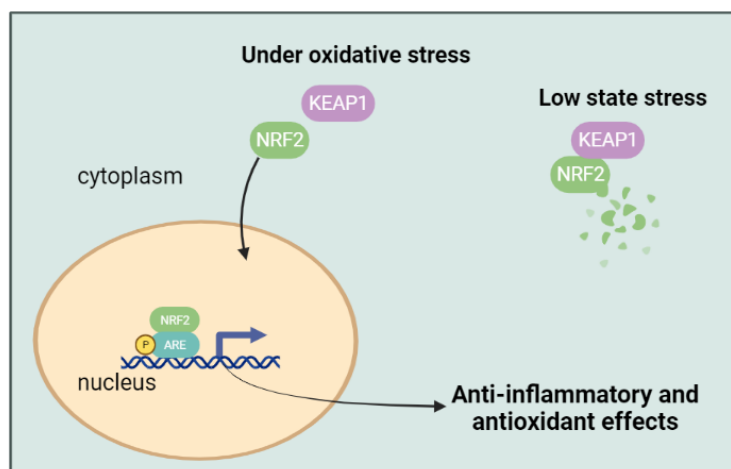


Figure 4: Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) in cytoplasm during low stress states. Under oxidative stress situations Keap1 loses the ability to degrade Nrf2 which can then be translocated to the nucleus, where it binds to antioxidant response elements (ARE) leading to cytoprotective antioxidant gene transcription. As a result, enhanced antioxidant and anti-inflammatory activity. Created with BioRender.com.

these compounds may have similar effects on the human body. Mainly due to the difficulty to know if the compounds responsible for those bioactivities can reach the targets at levels capable of inducing the desired effects. But also due to the sensibility of these compounds to the modifications that occur in the digestion processes [61, 62].

Bioaccessibility is a term that refers to the first step to making an ingredient bioavailable, in other words, is the step where compounds/ ingredients are released from the food matrix to make them available for absorption. The compounds are the target of several modifications during digestion so that can be absorbed and enter the bloodstream which can be distributed to tissue to exercise its bioactivities. All these steps are part of the bioavailability [63–65].

During digestion are several changes that may alter the bioactivities of the initial food or compound when compared with the resulting metabolite. First, in the mouth, some bacteria present there together with saliva and chewing movements can cause the deglycosylation of some ingredients and phenolic compounds [64, 65]. While others reach the stomach unmodified. Amylase, the most abundant enzyme in saliva, has no great impact on phenolic compounds. The stomach pH impairs the stability of phenolic compounds as well as other compounds. Some articles propose that some phenolic compounds can be absorbed

into the stomach through transporters in the stomach wall, but that is a small percentage. In the small intestine, some phenolic compounds may undergo hydrolysis becoming aglycones and due to their polarity, aglycones can easily pass through the portal vein to the liver where they undergo further reactions. Furthermore, the enzymes secreted by the pancreas and bile can contribute to the absorption of a phenolic compound through the formation of water-soluble micelles helping the digestion of apolar components. For instance, isoflavonoids and flavonoids can also be micellarized increasing their bioavailability [61, 62, 64–66]. In the colon, phenolic acids can be absorbed or modified by esterases and colon microbiota yielding absorbable metabolites [62].

The need to study the bioavailability of phenolic and other compounds, to understand the changes in their metabolite bioactivities, is thus highlighted through these modifications. There are several *in vitro* digestion protocols available to evaluate the final metabolites of the compounds and determine their bioavailability. Most protocols include the simulation of physiological conditions of the digestive process in the mouth, stomach, small intestine and, in some cases, intestinal fermentation. These procedures mainly consider factors such as digestive enzymes, pH, digestion time and saline concentrations [63].

1.4 Inflammation and neurodegeneration

The inflammatory response is a protective mechanism that aims to restore the normal physiology of the organism through the regeneration of damaged tissue/cell and/or removal of the pathogen that initiated the injurious stimuli [35, 67, 68]. The host cell can recognize the nature of the stimulus due to the presence of danger associated molecular patterns (DAMPS) and pathogen associated molecular patterns (PAMPS) that are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) a family of cell-surface receptors. After the recognition of the stimulus, each TLRs activates distinct transcription factors that enters the nucleus to bound to the promoter's region activating a series of genes. For instance, lipopolysaccharides (LPS) a major component of the cell walls of gram-negative bacteria (an endotoxin) activates specifically toll-like receptor

4 (TLR4) which recruits its downstream adaptors through interactions with the toll-interleukin-1 receptor (TIR) activating NF-KB and/or AP-1 which then expresses antimicrobial components to put an end in the initial stimulus (see figure 5) [67, 68]. Transcription factor AP-1 is induced by the activation of MAPK pathways and can induce an inflammatory response

The major signalling pathway that involved in TLRs recognition is NF-KB. Normally, this transcription factor is inhibited by *IkB*, which is degraded, after TLRs activation, allowing NF-KB to be translocated to the nucleus. There, NF-KB up-regulates the expression of genes encoding for pro-inflammatory cytokines such as interleukin 1 (IL-1) interleukin 6 (IL-6), tumor necrosis factor (TNF) (to increase vascular permeability among other functions), COX-2 (aiming the production of prostaglandins to promote further recruitment of inflammatory cells) and iNOS and nicotinamide adenine dinucleotide phosphate oxidase (NOX) (NO and ROS are used to phagocytosed microbes) [38, 68–70].

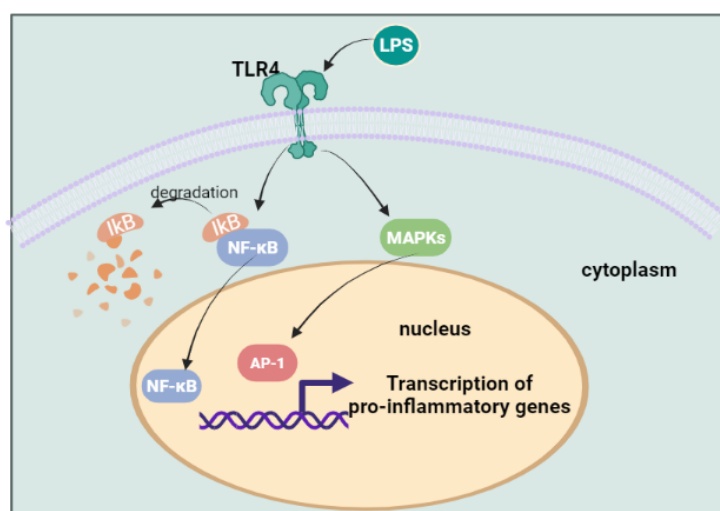


Figure 5: Lipopolysaccharides (LPS) inflammatory response. LPS is recognized by toll-like receptor 4 (TLR4) which activates inflammatory pathways. *IκB*, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) inhibitor are destroyed allowing NF-KB to be translocated to the nucleus. Simultaneously, mitogen-activated protein kinase (MAPK) pathway can be activated, resulting in the phosphorylation and activation of activator protein- 1 (AP-1). NF-KB and AP-1 controls inflammatory responses through the induction of inflammatory cytokines. Created with BioRender.com.

The production of NO and ROS during inflammatory response is a major clear-cut example of the mutual connection between redox processes and inflammation [35].

Furthermore, inflammasome a multiprotein complex responsible for processing inactive precursor forms of pro-inflammatory cytokines is activated by ROS and ROS itself can activate NF- κ B activation generating a vicious cycle [71–73]. Therefore, maintaining the control of inflammatory response is crucial to avoid an overload of both oxidative stress and inflammatory status [74, 75]. The term “oxinflammation” has been proposed as a pre-pathological condition due to the above-described relationship. Characterized by a chronic inflammatory status which is coupled with an oxidative stress situation. This occurs when, after an initial stimulus the inflammatory response is not properly ended due to persistent stimulus or due to a failure in normal resolution mechanisms, resulting in a continuous vicious circle [72, 76, 77].

To date, several pathologies have been associated with this vicious cycle created by a mutual combination of chronic inflammatory state and abnormal redox balance. This includes not only genetically determined diseases, such as Rett and Down’s syndrome but also more common metabolic disorders, such as diabetes, cardiovascular disease, cancer, and neurodegenerative diseases (NDDs) [72, 76].

Neuronal death underlies the symptoms of many NDDs, including, among others, Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington’s disease (HD). The progressive loss of neurons, at specific regions of central nervous system (CNS), leads to a gradual loss of cognitive and/or motor skills, resulting in dementia, debilitation and premature death [78, 79]. Depending on which group of neurons begins to undergo pathological changes, a clinical condition of the particular neurodegenerative disease phenotype will emerge. Thus, there is a wide variety of clinical courses and pathological manifestations in the different NDDs even so, an overlap of symptoms can also occur [80, 81]. Despite the variety of clinical courses, there are common features in the different NDDs such as programmed cell death, mitochondrial dysfunction, and accentuating inflammatory status [79, 82].

The brain is considered one of the most vulnerable organs in the body to oxidative stress mainly due to its high oxygen consumption rate and the presence of large amounts of polyunsaturated fatty acids, which are highly susceptible to lipid peroxidation [83]. Also, iron

is easily found throughout the brain, since many enzymes require it for its proper functioning. This high iron content can promote the ROS generation contributing to oxidative stress [84]. Another critical aspect of the brain lies in the fact that it is mostly composed of terminally differentiated neurons and glia thus, its potential to replace damaged cells is too little [83]. Moreover, the neurons possess relatively low levels of glutathione, a relevant antioxidant [85]. All the features mentioned above make the brain highly susceptible to oxidative stress, which can then cause direct damage and initiate many processes related to cell apoptosis, mitochondrial dysfunction, and inflammation [35, 86, 87]. Furthermore, a crucial role of inflammation has been highlighted in some NDDs such as AD PD and HD, since has been proven that proteins such as amyloid-beta or alpha-synuclein can bind to toll-like receptor 2 (TLR2) present in both microglia and astrocytes. Thus, promoting the phagocytic activity to remove the protein aggregates. However, at the same time, inflammatory signaling pathways are being activated, which may create an "Oxinflammation" status. Furthermore, those proteins can themselves activate inflammasomes promoting the activation of interleukins contributing to neuroinflammation. Despite the inhibition of this inflammatory status may not resolve the NDDs, the reduction of neuroinflammation may result in clinical benefits, therefore is important to unveil how an inflammatory response may be controlled in microglia [70, 88–90].

Microglia, are CNS's primary resident immune cells, have a myeloid origin and are able to self-renewal independent of hematopoietic stem cells [91–93]. Microglia can adapt to different phenotypes to respond to changes in their surroundings and two classic phenotypes of activated microglia are well characterized. The pro-inflammatory M1-like that releases destructive mediators and the anti-inflammatory M2-like phenotype, that produces beneficial mediators such as interleukin-10 (IL-10) and interleukin-4. Thus, a balance in these phenotypes is fundamental for immune homeostasis in the brain [94].

Three major roles can be associated with these cells (see figure 6): 1) housekeeping, functions that contribute to the maintenance of neurons and their well-being; 2) sentinel which includes the constant evaluation of changes in their environment; and lastly 3) defense function to protect against injurious agents [92].

The housekeeping functions include the elimination of many products in the brain such as synaptic elements, dying or dead cells, or abnormally accumulated proteins. Additionally, microglia can release neurotrophic factors such as insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF) which contributes to the protection of neurons [92, 95, 96].

The microglia's sentinel function is essential for its good performance in its housekeeping and defense functions [97]. Their defense function is accomplished due to the presence of PRRs that respond to a variety of molecular patterns. Activation of these receptors leads to the production and of anti- and pro-inflammatory mediators including interleukins, free radicals, proteases (matrix metalloproteinases) among others [92, 93, 98]. The release of matrix metalloproteinases has been linked to the disruption of blood-brain barrier (BBB) by degrading junctional complex proteins which can contribute to the recruitment of circulating leukocytes [99, 100]. This aims to help the neuro-immune system end with the initial insult, however, this can impair BBB permeability resulting in brain infarction [101]. Activated microglia can also induce neuronal apoptosis through the release of cathepsins and can cause neuronal excitotoxic through the direct release of glutamate [92, 102]. In healthy brains, microglia can perform all the mentioned functions aiming at the protection of the CNS and the end of the initial stimulus. However, dysregulation of any of these functions can initiate or propagate an ongoing neurodegeneration situation [92].

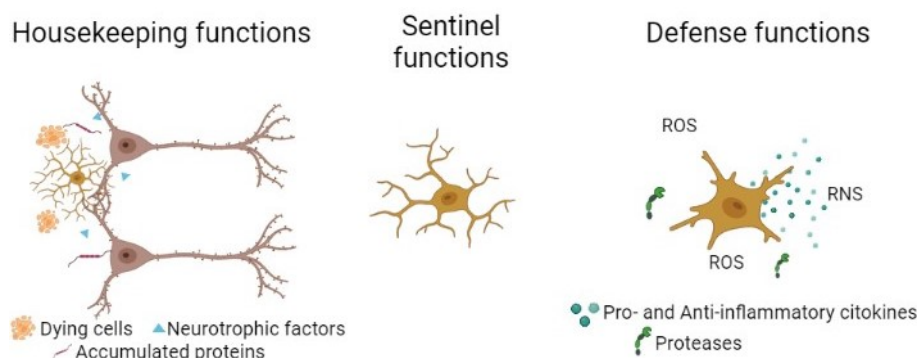


Figure 6: Three major roles associated with microglia: housekeeping, functions that contribute to the maintenance of neurons and their well-being; sentinel which includes the constant evaluation of changes in their environment; defense function to protect against injurious agents Sentinel. Created with biorender.

Bearing this in mind, several neuroprotective strategies have arisen, aiming at the defense of the nervous system against neuronal injury and/or death when exposed to trauma or damages caused by both acute and chronic neurodegenerative disorders [103–105]. It is believed that the early reduction of neurodegeneration hallmarks, such as oxidative stress, immune dysfunction related to protein aggregation and apoptosis, can delay neuronal dysfunction, resulting in the late onset of NDDs and decrease of the symptoms associated with these diseases [104]. Thereby, some neuroprotective strategies involve, among others: 1) antioxidant activity either by scavenging activity or by activation of key antioxidant enzymes in the brain; 2) reduction of neuroinflammation by down-regulation of pro-inflammatory cytokines and transcription factors; 3) ability to decrease NO production by inhibitory action on neuronal and inducible nitric oxide synthase activity; 4) capability to modulate signaling pathways, such as MAPK cascade and cAMP-response element binding (CREB); and 5) potential to regulate autophagy [83, 106, 107].

1.5 Objectives and work-plan

Apocynum venetum L. (Apocynaceae) has a long history as Chinese traditional medicine and in recent years many studies have confirmed its beneficial effects [108–111]. Nonetheless, further evidences are needed to understand the neuroprotector mechanisms promoted by *A. venetum* extracts and more studies addressing *Apocynum venetum* safety and regarding the effects of digestion on *Apocynum venetum* activities are necessary.

Neurodegenerative diseases represent a large group of syndromes related to the nervous system and are one of the most challenging health problems worldwide [82]. Their debilitating outcomes cause impairments at the medical, social, and financial levels in human society. Recent studies have associated some NDDs to a genetic origin or even as consequences of metabolic processes or as a result of microbial infections, however, some origins are still unknown [80]. Moreover, researchers have found some shared hallmarks between the different NDDs such as mitochondria dysfunction, oxidative stress, and immune dysregulation among others [20, 77, 79, 82, 87]. The early reduction of these hallmarks can delay the onset of

NDDs and may cause a reduction of its symptoms [90]. Based on this premise, several plants have been screened to identify potential therapeutics for the treatment of NDDs [104, 107, 112].

Taking everything into account, the major goal of this work is to study the antioxidant and anti-inflammatory effects of *Apocynum venetum* leaves methanolic extract as well as understand the effect of *in vitro* digestion on those activities. For that, the following tasks were performed: a) simulate digestion of the extract in the oral, gastric, and small intestine phases; b) biochemistry analysis of scavenging activity of the non-digested and digested extract; c) evaluation of the ability of extracts to inhibit COX-2 activity d) perform a phytochemical analysis of the extracts. e) cytotoxicity evaluation of the extracts in both BV-2 (murine microglia) and HepG2 (human hepatoma cell); f) cytoprotection effects of the extracts towards oxidative damages induced by tert-butyl hydroperoxide (t-BHP) in both cell lines; g) evaluation of redox state of HepG2 cell line after the pre-treatment with the extracts against the effects induced by t-BHP; h) evaluation of extract's anti-inflammatory potential, through the measurement of NO in the medium and the levels of gene expression of pro-inflammatory genes.

CHAPTER 2

Systematic review of the pharmacological activities

2.1 Introduction

Apocynum venetum is a small perennial herb commonly found in salt marshes. Widely distributed in central and northwestern China, *A. venetum* has attracted great interest due to its resilience to stress and its resources such as kenaf and white hemp [1, 2, 113–115]. Furthermore, flowers and leaves of the *A. venetum* plant, are also of great interest due to their pharmacological properties and their use for treating health conditions [116, 117].

A. venetum was firstly discovered in the 1950s in the Luopu plains in Xinjiang and was firstly named ‘Luobu hemp’ [2]. Nevertheless, its use as food and tea was, already recorded at the beginning of the 15th century in an ancient Chinese herbal book [118]. *A. venetum* was then listed in the Pharmacopoeia of the People’s Republic of China as a primary treatment for hypertension in the year 1977 [2]. Ever since other medical uses have been associated with *A. venetum*, including promoting diuresis, treatment of insomnia and troubles in the the liver [116, 117].

It is believed that the effects of this plant on promoting health benefits are related to its composition. Up to date, researchers have found several flavonoids, phenolic acids, glycoside conjugates as well as polysaccharides and minerals in its composition [9, 10, 113]. Some of these compounds such as quercetin derivatives have been described as anti-inflammatory and antioxidant and have demonstrated anti-hypertensive, neuroprotective, and cardioprotective effects [112, 119, 120]. Furthermore, phenolic acids have been related to protection against cancer and anti-diabetic effects [121, 122].

Currently, *A. venetum* leaf tea is becoming popular as a healthy beverage, acting as an anti-aging nutritional supplement [123]. This has increased the interest in this plant leading to the need for research to confirm *A. venetum* biological activities and composition to avoid possible side effects. Following this need *A. venetum* has been subjected to several phytochemical and pharmacological studies to validate its traditional use, and to understand the mechanisms behind its beneficial results [1, 124].

This chapter aims to give an overview of what has been done and published concerning the pharmacological properties of *A. venetum*.

2.2 Methods

2.2.1 SEARCH STRATEGY

The literature in this systematic review covered all the published studies until January 3, 2022, and was performed using "*Apocynum venetum*" as a keyword in the following databases: PubMed, ScienceDirect, and web of science. Reviews were excluded in the first step. The accessible literature on *A. venetum* written in English, obtained from database searches, was grouped and duplicate articles were removed. The remaining articles were analyzed by title and by abstracts. Studies that did not meet the inclusion criteria (see section 2.2.2) were excluded. Lastly, the full text of the remaining articles was analyzed and those that did not satisfy inclusion criteria were excluded. The EPPI reviewer software (Digital Solution Foundry and EPPI-Centre (2022), EPPI-Mapper, Version 2.1.0. EPPI-Centre, UCL Social Research Institute, University College London).

2.2.2 INCLUSION AND EXCLUSION CRITERIA

In this review, only articles reporting pharmacological properties of *A. venetum* were included. Papers reporting other subjects such as chemical composition, chemistry analysis, ecologic, genomics and metabolic were excluded. Reviews or publications that did not have abstract or full text available, or were written in a language other than English were excluded.

2.3 Results

The search of keywords in the different databases returned the following number of articles: PubMed (n=127), Web of Science (n= 179) and ScienceDirect (n= 167). The resulting papers were screened for duplicates (n= 148), 23 non-English articles were eliminated, remaining a

total of 302 articles. Then title, abstract and full text were analyzed to understand if they fit the inclusion criteria. Finally, 53 articles were reevaluated for eligibility and lastly, 51 articles met all inclusion criteria (figure 7) After the selection process, 51 articles referring

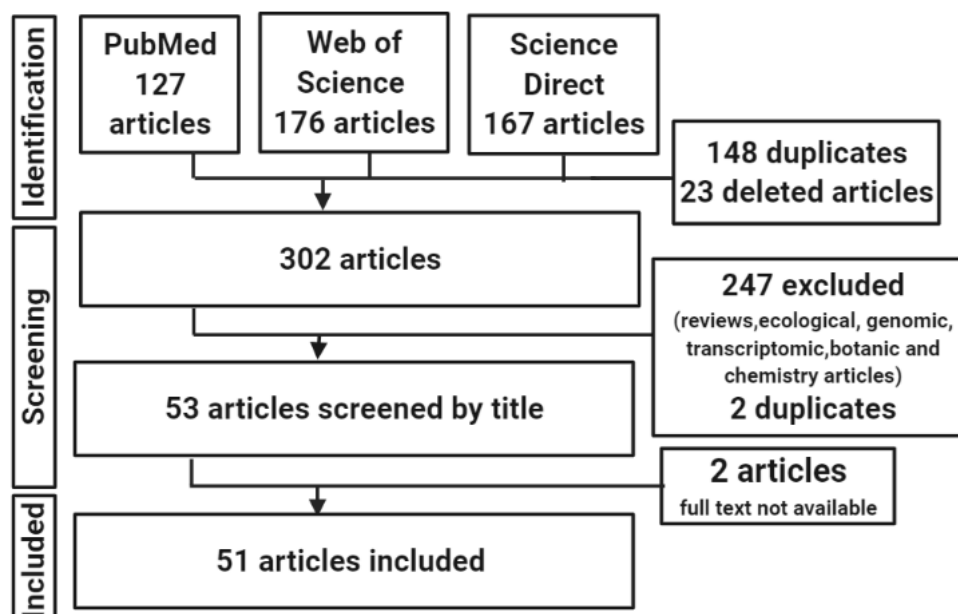


Figure 7: Flow diagram: Search strategy and analyzed data.

to bioactivities were selected. The pharmacological activities found in the literature included in this review are the antioxidant (22%), cardioprotective (13%), anti-hypertensive (12%), hepatoprotective (12%), Neuroprotective (10%), antidepressant (16%), anxiolytic (8%) and others (7%) as represented in figure 8. Nonetheless, 45% of the articles analyzed mentioned antioxidant activities of *Apocynum venetum*.

2.3.1 ANTIOXIDANT PROPERTIES

The pharmacological properties of *A. venetum* such as anti-hypertensive, anxiolytic, hepatoprotective, and antidepressant activities that will be discussed here are mostly related to general mechanisms of antioxidation activities of *A. venetum*. Several articles regarding the evaluation of *A. venetum* antioxidant activities in biochemical analysis such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

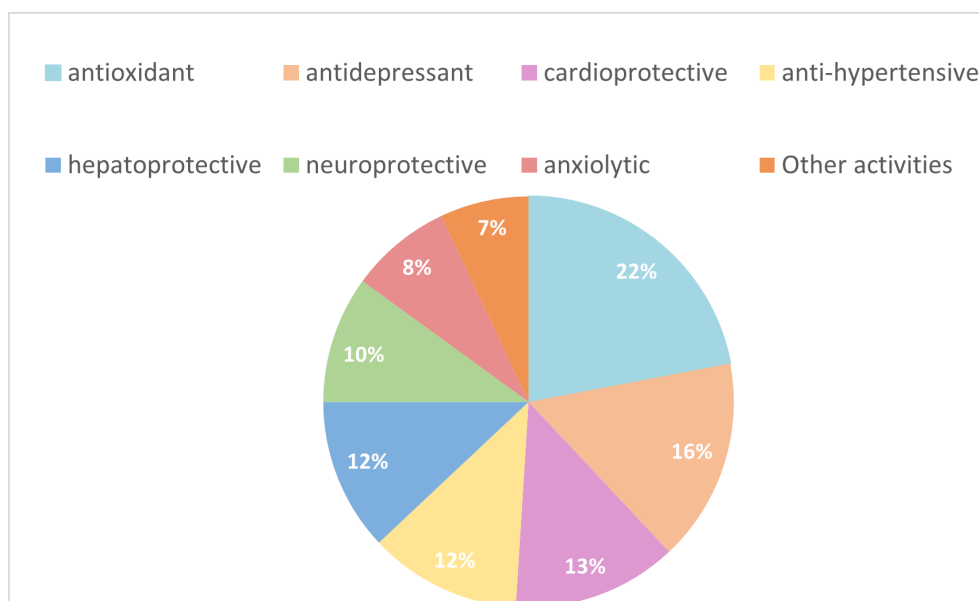


Figure 8: Percentage of categorized pharmacological activities found in the literature of *Apocynum venetum* until January 3, 2022.

(ABTS) among others [10, 125–130], will not be discussed here because those analyses are not always translated into bioactivities in the human body.

An *A. venetum* leaves extract protected against damage H_2O_2 -induced damages in 293T cells through the increment of GSH, SOD and CAT expression [131]. Another study using an aqueous extract from *A. venetum* leaves revealed an inhibitory effect on TBARS formation indicating that *A. venetum* can rise low-density lipoprotein (LDL) resistance to oxidation, which can help to prevent atherosclerosis (AS) and cardiovascular diseases [115].

Two studies [8, 132] using d-Galactose-induced aging in mice/d-Galactose-induced oxidative stress prove that treatment with *A. venetum* tea and polyphenol extract, respectively, protects against d-Galactose-induced damages. Pathological observations revealed that polyphenol extract [132] could inhibit oxidative damage to the skin, liver and spleen of mice induced by D-galactose. Besides the reduction of GSH, SOD after the treatment with *A. venetum* an up-regulation of Nrf2 expression levels, was also reported in this study. The same article also reported for the first time, an effect on a stress protein expression, heme oxygenase-1 (HO-1) related to anti-inflammatory and antioxidant processes. The results achieved by *A. venetum* treatment was stronger than others caused by vitamin C a well-known antioxidant. Li et al. [8] prove that the *A. venetum* extract can alleviate

hepatocyte edema and inflammatory infiltration. The tea administration not only up regulated GSH, SOD and CAT but also cause an increase of anti-inflammatory factors (IL-10) along with a down-regulation of pro-inflammatory factors (IL-6, TNF). In particular, the treatment could regulate the weight of the major organs and diminished the generation of NO and malondialdehyde (MDA) levels. Both works proved that *A. venetum* exhibited antioxidant effects and can have multiple beneficial effects. Nonetheless, further clinical studies are required to better comprehend its mechanisms.

2.3.2 ANTIDEPRESSANT PROPERTIES

Butterweck and colleagues [133], encouraged by the content of hiperoside and isoquercetin present in *A. venetum* leaves decided to assess the antidepressant capacity of *A. venetum*. The forced swimming test (FST), commonly used for antidepressant assessment essays, revealed that *A. venetum* leaves extract markedly shortened the immobility time of male rats and its effects were similar to those induced by the antidepressant imipramine. Then, the same authors tried to establish a relationship between imipramine and *A. venetum* through the analysis of regional levels of serotonin (5-HT), norepinephrine (NE) and dopamine (DA). However, both differences and similarities were found, nonetheless, these were the first evidence of the effects of *A. venetum* on monoamine levels [134]. Since then, many authors have been trying to find the mechanism by which *A. venetum* can influence monoamine levels. For instance, Zhou and colleagues [135], evaluated the effect of *A. venetum* leaves on the monoamine oxidase (MAO) an important enzyme in the monoamine neurotransmitters metabolism. Their findings showed that the aqueous phase of ethanolic extracts of *A. venetum* significantly inhibited the activity of MAO, however, the ethanolic extract was not able to cause a significant inhibition.

The antidepressant properties of flavonoids isolated from *A. venetum* were evaluated by Yan and colleagues [136], revealing that quercetin, kaempferol-3-O- β -D glucose and quercetin-3-O- β -D-glucose possesses antidepressant activity. This may be due to the increase of NE, DA and 5-HT as well as reduction of 5-HT metabolism in mice brain and CNS.

Another study [137] also reported an increase of NE and DA concentration, along with their metabolites, after the administration of *A. venetum* leaves extract. The antidepressant effects of the extract on FST and tail suspension test (TST) were prevented by the pre-treatment with dopamine D1 and D2 receptor antagonists, indicating that the antidepressant effect of *A. venetum* is dependent on the interaction with dopaminergic systems.

A study on PC12 cell lines [138], under the premise that the antidepressant effects of *A. venetum* are attributable to the presence of hyperoside in the extract evaluated the properties of this compound. The antidepressant effects of hyperoside from *A. venetum* reported may be related to its cytoprotective action. This includes the increase of gene expression related to antidepressant activity after chronic treatment, such as BDNF and CREB and the decrease of intracellular Ca^{2+} . Those cytoprotective actions were also verified after fluoxetine administration, a commonly used antidepressant.

Decrease of intracellular Ca^{2+} was also reported by Zheng and colleagues [139] as a neuroprotective effect caused by *A. venetum* leaves extract against corticosterone-induced neurotoxicity in PC12 cells an *in vitro* model of depression. The up-regulating of BDNF and microtubule-associated proteins (MAPs) may also contribute to its neuroprotective effects. The administration of fluoxetine, which increases serotonin levels also up-regulates BDNF. Previous work also revealed a linkage between the increase of serotonin and BDNF [140]. However, according to Butterweck et al. [134] and Zheng [137] *A. venetum* leaves extract administration did not cause an increase of serotonin, so another mechanism must be involved in the up-regulation of BDNF provoked by the extract.

Animal studies further illuminated the protective effects of *A. venetum* in a chronic unpredictable mild stress (CUMS) of depression. Changes caused by CUMS were significantly mitigated after extract administration in two different studies [141, 142]. According to Wu and colleagues [141], after four weeks of administration, *A. venetum* leaves significantly reversed apoptosis induced by CUMS in both the hippocampus and cortical areas. The down-expression of cytochrome-C, caspase-3, and caspase-9 proteins along with the normalization of Bcl-2 Associated X Protein (Bax)/B-cell lymphoma 2 (Bcl-2) ratio seems to

be responsible for the decrease of apoptosis. Furthermore, the administration led to a significant increase of BDNF and CREB expressions.

Multiple reports [140–142] have noted the effects of *A. venetum* on BDNF expression. Despite the upstream signaling pathway-regulated expression remaining unclear [142], some evidence has linked the increment in oxidative stress to the decrease of BDNF expression in neurodegenerative disorders [143]. Moreover, the reduction of BDNF mRNA and protein levels has been prevented by the treatment with vitamin E, through its antioxidant activities [144]. Thus, probably the antioxidant effects of *A. venetum* may cause an indirect effect of BDNF expression. Nonetheless, the direct effect on the modulation of BDNF is also a topic worthy of study.

The search for the main antidepressant compounds in *A. venetum* extract along with the scientific works on which ones can pass through BBB is also necessary. The MAPs seems to be related to several pathologies such as Alzheimer's disease, so understanding the effects of *A. venetum* on increasing the expression of these microtubules may be relevant for the treatment of other diseases. Finally, studies are needed to understand the influence of *A. venetum* extract on the monoamine systems 5-HT, NE, and DA involved in clinical depression.

2.3.3 ANTI-HYPERTENSIVE PROPERTIES

Traditional Chinese medicine has labeled the dried leaves of *A. venetum* as “anti-hypertensive tea”. Kim et al. [115] administered *A. venetum*' leaves extract at a dose of 70 mg/rat per day to a hypertensive animal model. The study showed that the treatment was able to decrease blood pressure and the observed effects were associated with an increase in glomerular filtration. Nonetheless, the decreasing effects were weaker than captopril, a common drug used in hypertension.

Another study performed by Kwan et al.[145] proved that *A. venetum* leaves extract caused vascular relaxation effects in both rat aorta and superior mesenteric artery. The vasorelaxation effects in the rat aorta were completely reversed by using a NOS inhibitor and a K^+ channel blocker proving the relevant roles of NO and K^+ channels in this effect.

Nonetheless, the effects were not specifically confined to one type of K^+ ion channel. The authors further discarded the possibility of *A. venetum* acting on endothelial muscarinic receptors through the use of atropine, an inhibitor of those receptors which did not inhibit the observed effects. The same study also reported that the effects persisted even after a brief exposure and subsequent prolonged washing, suggesting a great affinity between the compounds found in the extract *A. venetum* and in the generating system of NO. Another explanation for these prolonged effects would be related to the inhibition of the superoxide anion or effects on its elimination, which prevents the formation of peroxynitrite and, thus, increases the bioavailability of NO allowing a prolonged vasorelaxation effect [145].

Lau et al. [146], explore the second hypothesis using angiotensin II and phenylephrine, which not only causes vasoconstriction via receptor coupled calcium signaling pathway but also via the production of ROS - due to the activation of NOX. The results proved that *A. venetum* could counteract these effects through the inhibition of superoxide anion radical formation and the release of NO. The researchers also proposed that the inhibition of angiotensin II contractility and superoxide production may be related to the ability of *A. venetum* to down-regulated NOX subunit (p67 phox and gp 91 phox). Additionally, *A. venetum* proved to be a potent inhibitor of vasoconstriction through the stimulation of vascular receptors, such as alpha-adrenergic and angiotensin II in both endothelium-intact rat aortic rings.

Another article studied the mechanisms by which *A. venetum* can induce NO release [147]. Firstly, the authors confirmed the NO-mediated vasorelaxation induced by *A. venetum* through the measurement of total NO metabolites in human endothelial cells and isolated rat aortas. The enhancement provoked by *A. venetum* treatment was blocked by the presence of proto-oncogene tyrosine-protein kinase (Src) kinase and phosphoinositide 3-kinases (PI3K) inhibitors. The increment of NO and the protein expression levels of phospho-protein kinase B (AKt) and phospho-eNOS caused by the treatment were also abolished by the use of those inhibitors. Polyphenols are known to cause NO-mediated endothelium-dependent relaxation through the activation of PI3K/Akt pathway leading to eNOS activation and increase of endothelial NO generation [148]. Quercetin, a major compound found in *A. venetum* leaves protected against reduced NO production in diabetic mice via activation of PI3K/Akt signalling pathways [149]. In this way, quercetin among other compounds can be responsible for the relationship

between Src/PI3K signalling pathways and *A. venetum* properties. These findings settled the anti-hypertensive effects of *A. venetum*, unraveling the linkage between Src/PI3K signalling pathways and the effects of *A. venetum* [147].

Irie and colleagues [150] further evaluated the cardiogenic effects of different extracts of this plant in isolated guinea pig right atria. The extracts were able to induce a contractile response of the isolated atria and increased the pulse at a concentration of 1 mg/ml, a higher concentration than the concentration reported by kwan [145]. The vasorelaxation effects provoked by *A. venetum* extracts were not inhibited by propranolol, proving that the observed effects were not related to beta-receptors. The authors hypothesized a possible association between the observed effects and phosphodiesterase-3. However, more studies regarding the possible linkage between phosphodiesterase-3 and *A. venetum* properties are required. Long-term NO stimulation has been associated with the modulation of phosphodiesterase-3 expression in endothelial cells, therefore this could be also occurring after *A. venetum* treatment [151].

The anti-hypertensive effects described by the ingestion of *A. venetum* tea agrees with the studies herein analyzed. Probably, *A. venetum* can activate Src/PI3K/Akt signalling pathway, causing an activation of eNOS leading to the increase of NO generation. Moreover, their vasoprotective effects may also be related to the increase of NO bioavailability and probably due to its superoxide scavenging activities.

2.3.4 HEPATOPROTECTIVE PROPERTIES

A water extract of *A. venetum* was firstly reported [152] as hepatoprotective against two injury models: CCL_4 and D-galactosamine (D-GalN) / LPS. The treatment with the extract was not able to reduce the production of TNF but was able to inhibit TNF-induced cell death. These results suggest that *A. venetum* hepatoprotective effects occur after the production of TNF. In the same work, the authors proved that all fifteen flavonoids isolated from *A. venetum* demonstrated inhibitory effects on TNF-induced cell death with different intensities. Since it

is known that flavonoids have scavenging activity, it is presumed that by scavenging ROS, they can interfere with signal cascade initiated by TNF, causing the inhibition of cell death [152].

Zhang and colleagues [124] also revealed that total flavonoids from *A. venetum* (25, 50, and 100 µg/mL) exert a protective effect on carbon tetrachloride CCL_4 -induced hepatocyte damage both *in vitro in vivo*, mainly due to their antioxidant effects such as decreasing the MDA formation and increasing antioxidant enzymes activities in liver tissue. Further analysis proved that besides the ability to modulate the activity of antioxidant enzymes activities the treatment with *A. venetum* also decreases the expression levels of cytochrome-C and suppresses caspase-3 and caspase-8 cleavage. N-Acetyl-p-Aminophenol (APAP) is a toxic metabolite involved in liver injury, through oxidative stress responsible for mitochondrial dysfunction, caspase-3 activation, DNA fragmentation, and finally necrotic/apoptotic cell death. The pre-treatment with *A. venetum* leaves extracts significantly inhibited nuclear DNA damages, normalized the levels of GSH, hepatic MDA, and reestablished the activities of GPX and SOD in mice [153]. The same authors [154] further confirmed that hyperoside a major flavonoid found in *A.venetum* had hepatoprotective effects. Again, APAP was used to cause liver damage. Like the *A. venetum* extract, hyperoside was able to restore the activity of antioxidant enzymes and attenuated the effects caused by APAP. Hyperoside accelerated the detoxification of APAP through the enhancement of detoxification enzymes expression and activities, as well as the inhibition of cytochrome P4502E1 (CYP2E1) which is responsible for the formation of APAP reactive intermediate. These activities appear to be related to its ability to modulate the translocation of Nrf2 which is involved in inducing the expression of antioxidant enzymes and phase II enzymes.

Isoquercetin is another major compound present in *A. venetum*, demonstrated similar hepatoprotective effects to the hyperoside when tested in rats with damages induced by APAP. The decrease iNOS, TNF, and IL-6 production proved the anti-inflammatory effects of isoquercetin against the inflammation caused by APAP. In line with these results, the authors studied the relationship of isoquercetin and MAPK pathways, revealing that the pre-treatment with the compound suppressed APAP-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase (p38), C-Jun NH2-terminal kinase (JNK), which activates MAPK pathway [111].

Further study by Jiang and colleagues [155] proved that *A. venetum* extract and its major compounds showed hepatoprotective effects against APAP-induced liver injuries, through the decreased Cytochrome P450 levels. This study correlates the positive effects of *A. venetum* with its major compounds and shows its positive effects. Further assessment should be done to understand the influence of *A. venetum* in the MAPK pathway to better understand its hepatoprotective properties. The influence of *A. venetum* anti-inflammatory properties in its hepatoprotective effects should also be a target of the study. In short, although the underlying mechanisms are not fully known, *A. venetum* appears to have good hepatoprotective effects and clinical studies should analyze this potential.

2.3.5 CARDIOPROTECTIVE PROPERTIES

An article regarding the activity of *A. venetum* tea against the formation of advanced glycation end products (AGEs) revealed protective effects [156]. AGEs formation is promoted by the presence of ROS, and besides being highly connected with diabetes, AGEs are involved in aging-related organ damage, cardiovascular diseases and neurodegenerative diseases such as AD [157]. Thereby, avoiding these end products can avoid some of these conditions [156]. The ability of *A. venetum* to inhibit AGEs may be related to its radical scavenging activity [158]. However, more research is needed to establish whether *A. venetum* attenuates the AGEs's in vivo. A study using a polysaccharide-rich extract [159] from *A. venetum* showed significant anti-hypoglycemic effects and the ability to modulate gut microbiota in diabetes rats. The treatment led to a significant decrease in fasting blood glucose levels, total cholesterol, triacylglycerols, and low-density lipoprotein cholesterol.

A study using a cardiotoxicity rat model further shed a light on the protective effects of *A. venetum* leaves extract on the injuries induced by pirarubicin [160]. The molecular mechanisms behind the injury provoked by pirarubicin are not fully understood, nonetheless, ROS and oxidative stress seem to play a major role. The experiment found out that the pre-treatment decreased cardiac markers for myocardial necrosis and MDA levels as well as increased SOD activity. Additionally, an increase in Bcl-2/Bax ratio and reduction of

cleaved-Caspase-3 protein were also reported in the same study. The article also suggested for the first time the ability of the *A. venetum* extract to reduce the release of cytochrome-C and to reverse the opening of mitochondrial permeability transition pore (mPTP) caused by pirarubicin revealing anti-apoptotic properties [160]. The opening of this pore has been linked to mitochondrial depolarization which can be followed by progressive mitochondrial swelling speeding up apoptosis processes [161]. The mPTP has been proposed as a promising target for the treatment of PD due to its importance on mitochondrial dysfunction and excessive cell death, so further studies on the mechanism behind these effects may be relevant [162].

Another study [163], confirmed the ability of *A.venetum* to suppress the release of cytochrome-C and the closure of mPTP revealing cardioprotective effects. The antioxidant activities of *A.venetum* were partially responsible for the observed cardioprotective effects as well as the decrease of cleaved caspase-9 and cleaved caspase-3 expression implying that *A. venetum* may inhibit the activation of apoptotic pathways, through Akt/Bcl-2 signalling pathway [164]. Hyperoside a compound found in *A.venetum* also revealed anti-apoptotic effects against H_2O_2 -induced apoptosis on endothelial cells through the decrease of intracellular Ca^{2+} concentration and cleaved caspase-3. Additionally, effects on Bcl-2/Bax ratio were observed and suggests the regulation of p38 and a possible association with MAPK revealing anti-apoptotic properties and suggesting association with MAPK signalling pathway .

In a myocardial ischemia/reperfusion (MI/R) model, treatment with *A. venetum* leaves extract also led to a reduction of cardiac markers. The authors associated the positive effects with the direct reduction of oxidative stress through the increment of SOD activity and decrease of MDA content [165]. However, these effects were blocked by the use of inhibitors of ERK1/2 and PI3K, implying a linkage between *A. venetum* and Akt/ERK1/2 signaling pathways. At the same time, the authors confirmed a previous hypothesis theorized by Lau et al. [146] that *A. venetum* treatment could significantly reduce gp91(phox) expression, a critical subunit of NOX inhibiting superoxide generation.

Quercetin, a compound found in *A. venetum*, was able to down-regulate the expression of p47phox, p67phox, two NOX components, exhibiting anti-hypertensive and proving to be

a preventive compound against atherosclerosis (AS) [119, 166]. Apocynin, also found in *A. venetum* is a well-known nicotinamide adenine dinucleotide phosphate (NADPH) inhibitor [167] and signaling research found that it was able to restore endothelial dysfunction in streptozotocin-diabetic rats due to the down-regulation of p22(phox) and gp91(phox) expression, two subunits of NOX [168]. So, further research on the influence of these compounds on the observed effects after the treatment should be done.

Another work explores the effects of *A. venetum* treatment with atherosclerosis. A reduction in total cholesterol and triglyceride concentration was observed after the treatment with *A. venetum* which suppressed the progression of the diseases [7]. Furthermore, the treatment inhibited the excessive collagen synthesis in the aorta and the over-proliferation of smooth muscle cells. The authors associated these effects with the inhibition of AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway.

Zhang et al. [116] further enlighten the effects of *A. venetum* on AS progression on rats. The treatment with *A. venetum* extract reduced blood lipid levels and suppressed the progression of the disease. The authors indicated that the reduction of MDA levels and serum inflammatory factors such as IL-6 as well as the increase of SOD levels are the mechanisms behind the observed effects. Additionally, the experiment found a significant reduction of the protein expression of fractalkine (FKN), spleen tyrosine kinase (SYK), and p38, a pivotal signaling pathway in the occurrence and development of AS.

To sum up, all articles herein analyzed show that the antioxidant and anti-apoptotic activities induced by *A. venetum* seem to be relevant to its cardioprotective properties. This indicates that *A. venetum* could be a promising complementary treatment for cardiovascular diseases, especially in preventing diseases and promoting well-being. Nonetheless, more research is needed to enlighten the relationship between *A. venetum* and the different signaling pathways herein discussed. Furthermore, the study of the relationship between *A. venetum* and mPTP could be a promising field for the treatment of other diseases.

2.3.6 NEUROPROTECTIVE PROPERTIES

A study by Xiang et al., [169] found that *A. venetum* leaves extract has neuroprotective effects against cell death induced by oxygen and glucose deprivation (OGD) on an *in vitro* model of ischemia-reperfusion. The reduction of neuronal death and down-regulation of apoptosis due to the inhibition of caspase-3 activation and modulation of Bax Bcl-2 ratio are the possible mechanisms underlying these effects [169].

The modulation of Bcl-2/Bax proteins and inhibition of caspase-3 cleavage by *A. venetum* leaves extract was also reported in a study whose goal was to analyze the protective effect of the extract against H_2O_2 -induced oxidative stress in PC12 cells. Besides this, the extract decreased the production of ROS and increased autophagic activity [110]. Several antioxidants can reduce oxidative stress by increasing autophagic activity, through the activation of the PI3K-Akt-mTOR pathway. Although the relationship between the AMPK/mTOR pathway and *A. venetum* have been reported, more studies are required to further shine a light on this relationship in the nervous system [7]. Furthermore, there is the need to confirm the effects of *A. venetum* on autophagic activity using primary cells and/or *in vivo* animal models to further confirm its effects.

Other protective effects against neurological injuries, such as strokes, have been also linked to *A. venetum* leaves extract. *A. venetum* leaves extract alleviated BBB morphological damage and attenuated its permeability after cerebral ischemia-reperfusion. These effects may be due to the inhibition of matrix metalloproteinase-9 expression and activity [109]. Although the mechanisms underlying this event are still unknown, it is known that neuroinflammation and oxidative stress contribute to the activity of these enzymes [170]. In this way, the antioxidant and anti-inflammatory effects of *A. venetum* can be accounted for by the inhibition of these proteins, but further studies are needed. Furthermore, apocynin a phenolic compound also found in *A. venetum* [152], has been described as a neuroprotector, proving to be able to decrease the ROS generation and prevent inflammatory response in several animal disease models through the down-regulation of iNOS expression and inhibition of NF-KB pathways.

The compound was also able to improve body weight loss and normal behavior PD-marmosets [171, 172].

A study by Kuo and colleagues [108] demonstrated that *A. venetum* leaves inhibits voltage-gated Na^+ channels and voltage-gated K^+ channels, to a lesser extent in mouse neuroblastoma N2A cells. Ionic channels have been linked to neuroprotection since changes in Na^+ and K^+ homeostasis have been associated with different stages of the apoptotic progression. Therefore, further studies are needed to understand if the modulation of ion channels may contribute to the anti-apoptotic activities herein reported [173]. Due to the anti-hypertensive effects of *A. venetum*, previously discussed here, the authors evaluated the influence of NO release in mouse neuroblastoma N2A cells. The reported effects were not influenced by the use of a potent inhibitor of NO release, proving that the block of Na^+ is independent of NO release [108]. The blockade of Na^+ channel activities has been linked to anxiolytic activities and may also be involved in antidepressant activities [174].

2.3.7 ANXIOLYTIC PROPERTIES

Anxiolytic activities of *A. venetum* have been also evaluated by elevated plus maze (EPM). EPM test revealed that oral administration of an extract prepared from the leaves of *A. venetum* can induce an anxiolytic effect in mice since it markedly increased the number of entries and the time spent on open arms. The anxiolytic effects were equal to the ones induced by benzodiazepine diazepam and the 5-HT_{1A} agonist buspirone. However, the effects of *A. venetum* extract could only partially be blocked by the unspecific 5-HT receptor and unspecific GABA receptor antagonist. Indicating that anxiolytic effects caused by *A. venetum* administration can be mediated via the GABA-receptor complex and 5-HT_{1A}-receptor can also be involved [6]

The same authors further evaluated the anxiolytic effect of one flavanol found in *A. venetum*: Kaempferol. This compound revealed anxiolytic-like properties that were only significantly antagonized by the administration of unspecific gamma-amino butyric acid (GABA) receptor antagonist [175]. Thus, kaempferol may contribute to the anxiolytic

effects of *A. venetum*, nonetheless other compounds may also contribute to its anxiolytic activities.

High concentrations of *A. venetum* has been proven to be well tolerated in rodents, which encourages the study of its use in humans for anxiolytic purposes [134]. Furthermore, a recent investigation shows that extracts from leaves of *A. venetum* does not induce Cytochrome P450 3A4 or P-glycoprotein in rats [176]. Unlike St John's wort which is used to treat anxiety but causes an induction of Cytochrome P450 3 which can significantly increase hepatotoxicity of other compounds [177]. As such, *A. venetum* can be a viable alternative to anxiolytic-purposed treatments.

2.4 Conclusion

Articles herein reported validated the traditional uses of *A. venetum* in numerous pathologies. Pharmacological activities of *A. venetum* are mainly explained by the presence of various flavonoids, which have been proved to have activities when isolated from *A. venetum*. Nonetheless, the specific chemical composition of the *A. venetum* needs to be elucidated and the direct relationship between *A. venetum* pharmacological effects and its composition needs to be enlightened. Furthermore, research to confirm the potential interaction between *A. venetum* treatment and different pathways- such as PI3K/Akt, MAPK, Nrf2 AMPK/mTOR - is required. In this work, several articles reported neuroprotective effects of *A. venetum*, so this plant can be tested to be used as a way of protection against neurodegenerative diseases. Also, studies analyzed here indicate a possible relationship between *A. venetum* and anti-inflammatory processes, so it is necessary to investigate whether this plant does have direct effects on these pathways.

One advantage of *A. venetum* is that quality control protocols are being created and preliminary studies revealed that recommended doses of *A. venetum* would not influence hepatic cytochrome P450 and P-glycoprotein in rats. Nevertheless, more detailed studies on other cytochrome and transporters should be done in the future. In addition other bioavailability, pharmacokinetics and physiological research of *A. venetum* needs augmented research.

CHAPTER 3

Preparation of the extracts and phytochemical characterization

3.1 Introduction

The prevalence of diseases such as cancer, cardiovascular, diabetes, and degenerative disorders is increasing, partly due to the extension of lifespan [178]. The lack of fully reliable early diagnostic tools and resolute therapies is a common element in these different diseases. This led to the search for preventive approaches to avoid these pathological conditions. Following this view, a new diet-health paradigm has emerged, where food has now assumed the status of 'functional' food [179, 180]. The presence of many phytochemicals - such as phenolic acids, flavonols, carotenoids, and vitamins - makes functional foods able to offer more than basic nutrition. Many of those phytochemicals have been described as immune-modulators, anti-inflammatory and antioxidants and have been related to the prevention of several diseases [181–183].

According to epidemiologists, the intake of antioxidants through fruits and vegetables can significantly decrease the risk of developing diseases such as cancer, and cardiovascular diabetes. Plant phenolics such as phenols, phenolic acids, flavonoids and stilbenes are thought to be responsible for the reported antioxidant activities due to the reactivity of aromatic rings of these compounds. The number and relative position of the hydroxyl groups in this fraction as well as the glycosylation of the free compounds confers different stabilities causing different levels of antioxidant activity [122, 184].

Although the correlation between antioxidant activities and the structure of phenolic compounds is still unclear, it is still relevant to look for possible relationships between them [122, 184]. Quercetin, for instance, is a common flavonol found in plants and has several beneficial effects for the human body, acting as an anticancer, and anti-inflammatory among other activities [112, 120, 185]. However, the biological properties may be diminished in its glycosylated form, as is the case with rutin (glycosylated quercetin) and the anti-inflammatory activities of quercetin [185]. In addition, the biological properties of these compounds are also influenced by their bioavailability, which comprises all processes such as absorption, digestion and metabolism until the compound enters the circulatory system [51]. Therefore, in this work, an *in vitro* digestion containing oral, gastric, and small intestine phases, was conducted

to understand how the modifications occurring during digestion can change the compounds present and their possible bioactivities.

Considering the association between the ingestion of these compounds and the reduction of disease risk, great efforts have been made to carry out efficient methods of extracting phytochemicals [181]. Therefore, this chapter aims to perform a phytochemical analysis of the extracts under study.

3.2 Material and methods

3.2.1 CHEMICALS AND REAGENTS

For high Performance Liquid Chromatography with a diode-array detector (HPLC-DAD) analysis and extraction methanol and formic acid both 99.8% purity obtained from VWR, (Carnaxide, Portugal) were used. For the *in vitro* digestion assay, alpha-amylase from human saliva, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were purchased from Sigma, (Darmstadt, Germany). Hydrochloric acid (HCl), dimethyl Sulfoxide (DMSO) (>99.7%) were purchase from Fisher, (Hampton, New Hampshire) diethyl ether, sodium hydrogenphosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and potassium chloride (KCl) were bought from Panreac, (Barcelon, Spain). Sodium bicarbonate ($NaHCO_3$) **NaOH!** (**NaOH!**)), sodium chloride (NaCl), were bought from VWR, (Portugal).

3.2.2 PREPARATION OF EXTRACT

Apocynum venetum leaves were obtained from China (Xinjiang province). The lyophilized powder of leaves was subjected to a methanolic extraction. For each gram of biomass, 10 ml of 70% methanol were added. This mixture was left to be macerated for one week in the dark, at room temperature, and then it was filtered through a filter. The remaining biomass

was subjected to a second methanolic extraction using the same conditions and protocol. Thereafter, both organic phases were joint and concentrated with the aid of a rotavap (Buchi RII) at 40°C. Finally, the extracts were evaporated to dryness in a lyophilizer (Christ Alpha 2-4, B. Braun). The resulting powder was suspended in DMSO and stored at -4°C until further analysis.

3.2.3 *In vitro* DIGESTION OF *Apocynum venetum* LEAVES EXTRACT

The study of digestion effects on the bioactivities and phytochemical composition is an area that has seen a continuous increase in interest in recent years [186]. There are several protocols available to simulate digestion, which is cheaper and simpler than performing *in vivo* experiments. Here, an *in vitro* digestion including a mouth, gastric and intestinal phase was performed. The *in vitro* digestion of AV was conducted according to the methodology described by Magalhaes et al., [187].

First, 3 g of the extract was mixed with 9 ml of artificial saliva solution containing 2.38 mg/ml N_2HPO_4 , 0.19 mg/ml KH_2PO_4 , 8.0 mg/ml NaCl and alpha-amylase (1.5 mg/ml). The pH was adjusted to 6.75 at 37°C with HCl 1 M (pH calibrator, Hanna instruments). To simulate the chewing movements of the oral phase, the mixture was shaken at 80 rpm for 2 min (incubator shaker, infors HT multitron Pro). Next, the pH was adjusted to 1.2 with HCl 5 M to initiate the gastric phase and 9 ml of artificial gastric fluid (3.2 mg/ml pepsin in 0.03 M NaCl) was added. For the next 120 min. the mixture was incubated on a shaker at 150 rpm, at 37 ° C. Finally the pH was adjusted back to 6.0 with $NaOH$ 5 M, and 1.5 ml NaCl (120 mM), 1.5 ml of KCl (5 mM) and 9 ml of artificial intestinal fluid (10 mg/ml of pancreatin and 11 mg/ml of bile extract in 9 ml of 0.1 M $NaHCO_3$) were sequentially added and incubated at 37 ° C at 150 rpm. After 1 hour of incubation, the extract obtained was centrifuged (Centrifuge falcons Sigma 2-16K) at 5000 rpm for 5 minutes, the pellet was discarded and the supernatant frozen at -80°C (Deep freezer Sanyo and Panasonic) and subsequently lyophilized. Following this protocol, we obtained a second extract: digested of *Apocynum venetum* methanolic leaves extract (dAV).

3.2.4 EXTRACT ACIDIC HYDROLYSIS

To confirm the compounds identified in the extract, an acidic hydrolysis of glycosides was conducted. Briefly, to 1 ml of the AV extract at a concentration of 20 mg dry weight residue (dwr)/ml in methanol 70%, 1 ml of HCl 2 M was added. The mixture was then incubated for 2 hours at 80 °C in a water bath (Gran). Next, 1 ml of H_2O was added, followed by the addition of 2 ml of diethyl ether. After that, two phases appeared in the flask, the top layer was discarded and this protocol was performed twice. Finally, the rest of the diethyl ether was left to be evaporated in the hood. To the obtained residue 2 ml of methanol 70% was added, achieving a final concentration of 10 mg/ml.

3.2.5 HPLC-DAD ANALYSIS

Apocynum venetum leaves extract (AV) and the hydrolysed AV were analysed by High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD). HPLC-DAD analysis was conducted according to Dias and colleagues [188].

Apocynum venetum methanolic leaves extract was dissolved in 200 µl of DMSO and 800 µl of methanol 70% were added achieving a final concentration of 10 mg dwr/ml. Both AV and hydrolyzed AV were filtrated and injected in the HPLC-DAD equipment (HITACHI, LabChrom Elite, Japan) controlled by the elite EZChrome software (Agilent technologies, California, United States).

The detection occurred in the range between 250 nm and 600 nm. Chromatograms were recorded at 260, 280 and 350 nm for qualitative analysis of the compounds present in the extract. A column LichroCART (Lichrospher 100. Rp-18e. 5 µm, Merck, German) was used in this analysis. The mobile phase used solvent A (0.1% of formic acid in methanol) and solvent B (0.1% formic acid in ultrapure water). Elution was performed at a flow rate of 0.8 ml/min using a gradient developed for the analysis of the extracts described in table 1. The identification of phytochemical compounds present in *Apocynum venetum* extract was done through the

comparison of their UV spectra and the retention time to standard reference compounds. Quercetin and chlorogenic acid were used as external standards to quantify the compounds present in the extracts. The quantification of compounds was performed according to Wang et al.,[189]. The values of the peak area obtained for standards and its known concentration were used to determine the response factor (peak area/concentration of standard). Next, the peak area of the compound found in the extract, under study, was divided by the response factor of the standard (area of peak/response factor). Finally, the obtained value was divided by the concentration of extract (10 mg/ml) to know the concentration of the compound in 1 mg of extract.

Table 1: Elution gradient for the analysis of non-digested *Apocynum venetum* leaves extract

Time (min)	Solvent A (%)	Solvent B (%)
0	5	95
5	5	95
30	50	50
40	90	10
45	90	10

3.3 Results and discussion

The analysis of the phytochemical composition of plants is fundamental for conducting quality control protocols and also to look for possible correlations between bioactive properties of plants and their composition [49, 190]. HPLC-DAD analysis is a wide use technique to analyze and identified the compounds present in a sample and is based on a stationary phase and a mobile phase. HPLC-DAD uses a column often packed with reversed-phase C18 column material (stationary phase), a pump responsible for moving the mobile phase through the column, and a detector that shows the retention times of the molecules [49]. Each compound present in a sample has different molecular structures and functional groups that give them different degrees of affinity with the mobile and stationary phases. The higher the affinity to

the stationary phase the slower would be the movement of that specific compound and the higher would be its retention time.

The UV-detector further allows detecting the solute as they elute from the column [191, 192]. In this way, each compound has a distinct migration rate and can be identified through the chromatogram generated by the electrical signals recorded by the HPLC-DAD detector.

The results obtained by HPLC-DAD revealed the presence of phenolic acids and flavonoids derivatives in the AV as shown in figure 9. Four major compounds were identified as derivatives of caffeoylquinic acid and quercetin due to their UV-Visible spectrum, retention time and wavelength of maximum absorption. Quantification of compounds is indicated in table 2. The

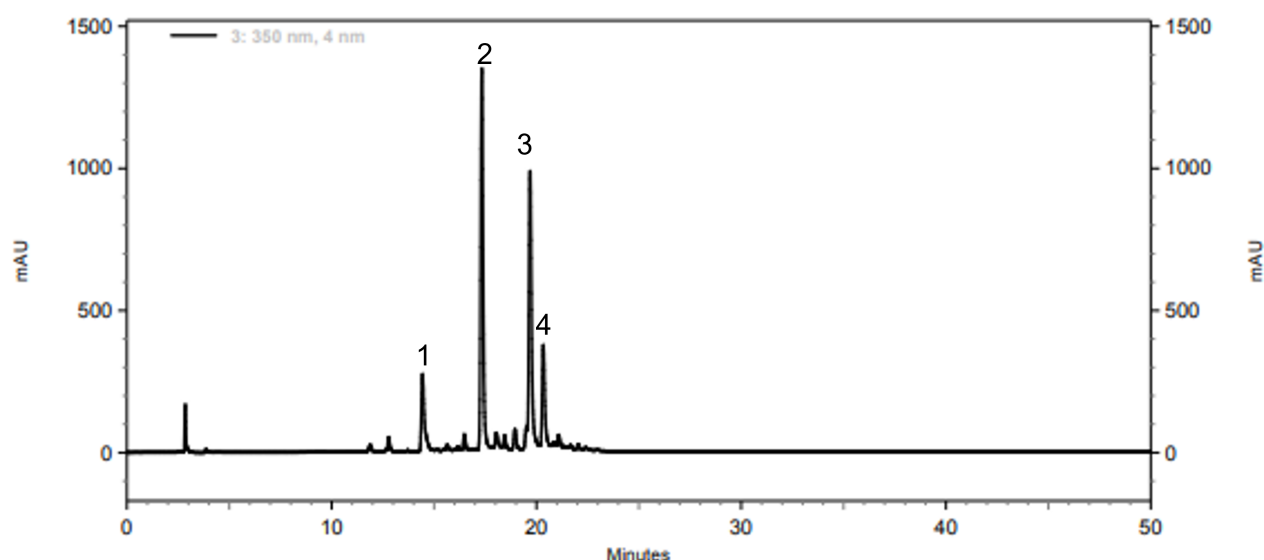


Figure 9: HPLC-DAD Chromatogram (350 nm) of: non-digested *Apocynum venetum* leaves extract (10 mg/ml); Peaks: 1:caffeoylquinic acid derivatives; 2,3,4:quercetin derivatives.

results show that quercetin derivatives are the major compounds found on the AV followed by derivatives of caffeoylquinic acid. The results are in concordance with those found in the literature which also reckoned flavonoids as the main compounds of AV. Nonetheless, other authors were able to identify other flavonoids such as kaempherol, and hyperoside in *A.venetum* leaves extract [9, 135, 143, 193]. Some of these compounds have been linked to antioxidant activity. [52].

Table 2: Phenolic quantification by external standard.

Compounds found in AV	Peak	Quantification through the external standards (ug/mg AV)
Caffeoylquinic acid derivatives	1	9.1
	2	22.8
Quercetin derivatives	3	15.1
	4	10.1

To ensure that the comparison with UV-Visible spectrum, retention time and wave-length of maximum absorption was a reliable method for the identification of the compounds acidic hydrolysis of the extract was performed to identify the free forms [50]. Flavonoids are often extracted from herbal samples with methanol, ethanol, acetone, water, or mixtures of these solvents [50]. The extraction may depend on the time, active compounds and the type of plant. Flavonoids are often found in plants bound to some sugar, creating its derivatives. To obtain the flavonoids aglycone is necessary to follow a protocol that subjects the extract to acidic hydrolysis at high-temperature conditions. Hydrolysis of flavonoid glycosides of *A. venetum* methanolic leaves extract was carried out in 2 M HCl at 80 ° C for 2 h. These conditions completely hydrolyze 3-O-glucosides (such as hyperoside, isoquercitrin and rutin) within a few minutes while flavonol with glucose attached to the 7-hydroxyl is distinguished to more resistant to acid hydrolysis [194, 195]. The HPLC-DAD analysis are showed in figure 10. Only quercetin was found in this analysis. Quercetin was not identified in the original AV HPLC-DAD analysis, confirming the identification of quercetin glycosides in AV. Caffeoylquinic acid derivatives were not identified in this analysis, due to their degradation in these acidic conditions as previous described by Biesaga et al., [196]. This analysis also proves that glycosides are cleaved in acidic conditions, which might occur in stomach digestion. According to the literature, the conditions occurring in digestion may impair the structure of phenolic compounds. Amylase found in saliva does not have great effects on phenolic compounds and they seem to be stable under gastric conditions due to the low pH that protects them from degradation [197–200]. However, according to some sources some free forms of phenolic compounds can be

absorbed such as phenolic acids and hydrolysis and deconjugation can also occur in the gastric conditions, depending on the phenolic compounds class [64, 65, 201]. The literature describes the condition of the small intestine as the most critical for phenolic compounds' stability. The alkaline conditions as well as the presence of pancreatic esterase and bile acid cause degradation of these compounds [64, 197–200].

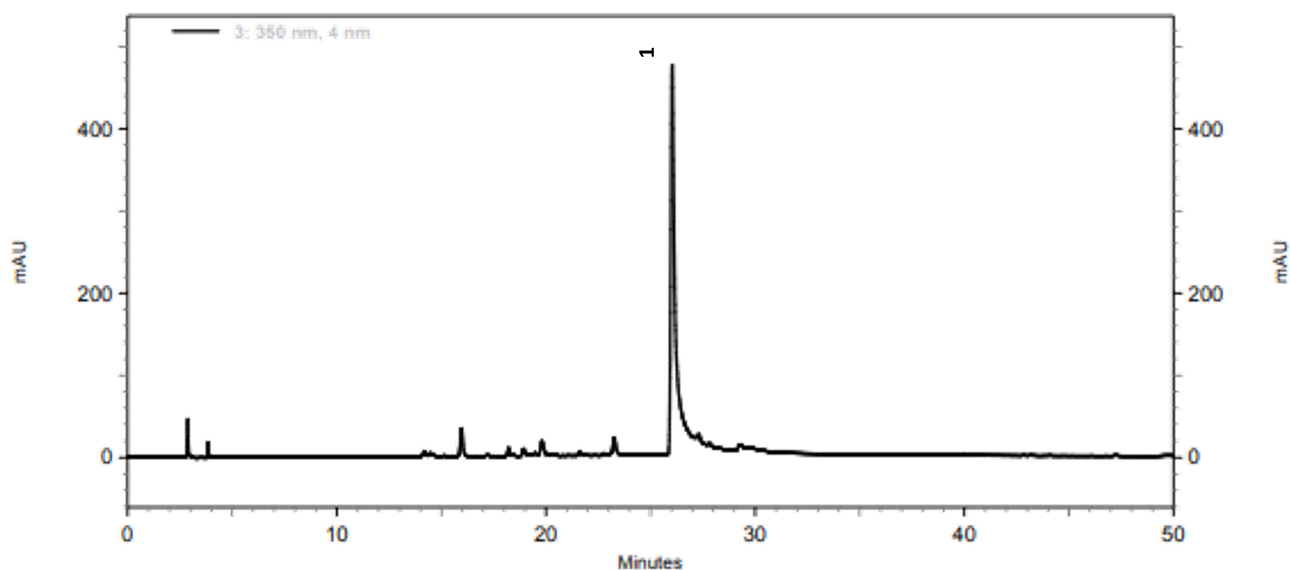


Figure 10: HPLC-DAD Chromatogram (350 nm) of: hidrolised *Apocynum venetum* methanolic leaves extract (10 mg/ml); Peaks: 1:quercetin.

Nevertheless, there are also studies reporting significant losses of phenolic compounds in oral and stomach phases and others reporting higher stability of those compounds in small intestine conditions. For instance according to [197] only 3% of the total loss of rutin occurred in the intestinal phase, while 44% to 95.7 % of the total loss of chlorogenic acid, took place in the small intestinal phase. The results obtained by the different articles regarding the effects of *in vitro* digestion are sometimes contradicted and challenging to analyze due to the different protocols performed. Furthermore, the food matrix seems to be a critical factor in the effects of digestion [63, 197]. Thus, more efforts should be made to arrange a reliable uniform protocol of the modifications occurring in the digestion. Furthermore, a phytochemical analysis of the digested extract needs to be conducted, to analyze the effects of the *in vitro* protocol conducted on the composition of the extract.

3.4 Conclusions

The study of bioavailability and metabolism of phenolic compounds has been increasing steadily during the past years. This information is crucial to a proper analysis of its potential health effects. Only by understanding which metabolites and at what concentrations can reach tissues and cells, is possible to truly evaluate the beneficial effects of a phenolic compounds-rich diet.

The phytochemical analysis and the hydrolysis performed showed that AV is mainly composed of flavonoid derivatives and phenolic acids which are known to have antioxidant properties. Quercetin derivatives such as rutin, hyperoside, and isoquercitrin were the most abundant compounds herein analyzed, and are related to antioxidant and anti-inflammatory properties.

CHAPTER 4

Biochemical *in vitro* analysis of the
antioxidant and anti-inflammatory properties

4.1 Introduction

The rising acceptability and accessibility of *Traditional Medicine* (TM) have encouraged many researchers to study the properties of several traditional medicinal plants [202, 203]. At present, for instance, many traditional Chinese medicinal plants are under scientific evaluation to assess their bioactive efficacy and safety [203–205]. Many of those studies focus on the evaluation of the properties of phytochemicals classes such as alkaloids, phenols, tannins, steroids, cardiac glycosides, diterpenes, and saponins, found in those plants. The evaluation of biological and chemical properties of those compounds can be a valuable source to help the development of effective drugs [206].

A relevant bioactivity to be explored is the antioxidant capacity, which can be exercised in several ways: oxidizing agents of free radicals and/or non-radicals, chelating agents of transition metals, compounds that inhibit the generation of oxidants and/or that can stimulate the production of endogenous antioxidant compounds [207]. Several biochemical assays have been developed to evaluate these different antioxidant activities. These assays are normally related to the generation of different radicals acting through a variety of mechanisms [208–210]. Usually, they are based on a spectrophotometric determination as is the case of DPPH or ABTS assay. Due to the variety of the protocols many standard compounds, such as quercetin and Trolox, are used to allow comparisons [211].

Besides the evaluation of antioxidant properties of extracts under study, anti-inflammatory properties were also target of study here. Since NO intervenes in inflammatory situations, the NO scavenging properties evaluated by Griess of the extracts can be considered an anti-inflammatory property [35]. The ability of the extracts to inhibit the activity of COX-2 was also evaluated in this work. COX-2 is one of the most significant enzymes since its inhibition is the pharmacological mechanism of non-steroidal anti-inflammatory drugs. Thus, finding new safer, and perhaps more efficacious COX-2 inhibitors is an important area of research. The COX-2 is an inducible isoform of cyclooxygenase which are responsible for the production of prostaglandins [212]. The first step of COX-2 is the addition of molecular oxygen to arachidonic acid (AA) to form the unstable prostaglandin G2. Then, prostaglandin G2 is

converted to endoperoxide by the peroxidase function of COX-2, which is then non-enzymatic converted to prostaglandin E2. There are three ways to measure COX-2 activity: measurement of the final product of the reaction, prostaglandin E2, by ELISA; measurement of the oxygen uptake that occurs during the first step of the reaction using an oxygen sensor; and the third is to measure the second reaction spectrophotometrically. For this work, the last method was chosen [213].

Considering all this, this chapter aims to present the biochemical methodologies carried out to determine the antioxidant/anti-inflammatory activities of AV and the digested extract (dAV) by radical scavenging activity (DPPH and ABTS), FRAP method, ICA, superoxide anion radical (PMS-NADH system) and nitric oxide (Griess method) scavenging activities and inhibition of COX-2) as well as to discuss the results obtained.

4.2 Material and methods

4.2.1 CHEMICALS AND REAGENTS

For the biochemical assays, DMSO 99.7% purity, quercetin, (95% purity), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4'-disulfonic acid sodium salt, 97% purity) all purchased on Sigma, (Darmstadt, Germany). Nicotinamide adenine dinucleotide (*NADH*) was purchased at Roche Diagnostics, (Penzberg, Germany) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (98% purity) to FLUKA, (Buchs, Switzerland). Phenazine methosulfate phenazine methosulphate (PMS) 99% purity and Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97% purity) was purchased from Acros Organics, (Geel, Belgium). Persulfate solution, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) and ethanol were bought from Merck, (Darmstadt, Germany). Trizma hydrochloride solution and hydrochloric acid (HCl) were purchased from Fisher, (Hampton, New Hampshire).

Iron (III) chloride hexahydrate (97% purity), sodium nitrite, aluminium chloride hexahydrate, sulfanilamide, naphthylethylenediamine dihydrochloride (NED) (98% purity), trizma hydrochloride solution (NBT), Iron(II) sulfate heptahydrate, ferrous sulfate $FeSO^4$, iron(II) sulfate heptahydrate, ABTS, 1-butanol, hematin porcine, AA (85% purity), cyclooxygenase 2 (human), COX-2 inhibitor (*Methyl[5 – methylsulfonyl – 1 – (4 – chlorobenzyl) – 1H – 2 – indolyl]carboxylate*) and N,N,N,N -Tetramethyl-p-phenylenediamine (TMPD) (99% purity), were purchased from Sigma, (Darmstadt, Germany). Acetate buffer was purchased to Panreac (Barcelon, Spain) and phosphoric acid 85% to Riedel-de Haen, (Portugal).

4.2.2 DPPH ASSAY

The antiradical activity of extracts was determined using DPPH, a violet free radical. This method is based on the change of color from violet to yellow after DPPH reduction, resulting in a decrease in absorbance. The change observed is proportional to the radical scavenging activity of the tested sample (see figure:13) [209].

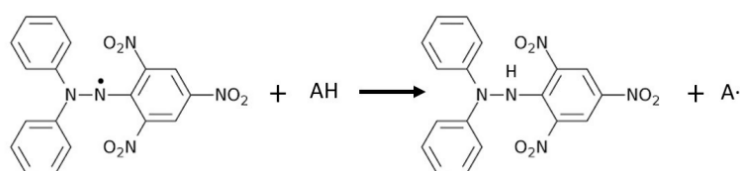


Figure 11: DPPH-scavenging reaction scheme. Adapted from; Alam et al 2013 [214].

The following assay was performed according to the method described by Silva and colleagues [215], with slight modifications. Firstly, a stock solution of DPPH (0.4 mM in ethanol) was prepared, kept protected from the light, and stored at $-4^{\circ}C$ until necessary. To perform this protocol several dilutions of the extracts were prepared as follows: 10 μ l of extract, at different concentrations, and 140 μ l of a DPPH previously prepared solution, for a final range of extract concentrations between 1 mg dwt/ml to 0.0625 mg dwt/ml for both AV and dAV. For 60 minutes, the absorbance was continuously monitored at 515 nm using a SpectraMaxPlus

microplate reader, to ensure the steady-state of the reaction was reached. The DPPH solution alone was used as control and a blank for each concentration was performed using 140 μ l of ethanol instead of DPPH solution.

Quercetin was used as a positive control. All measurements were done in triplicate and three independent assays were performed. Radical scavenging activity was expressed by reduced 2,2-diphenyl-1-picrylhydrazyl (DPPHr) percentage which was calculated according to the formula present in the figure 12.

$$\% \text{ DPPHr} = \frac{A_c - (A_s - A_b)}{A_c} \times 100\%$$

A_c absorbance of DPPH without extract

A_s absorbance of DPPH and extract

A_b absorbance extract without DPPH

Figure 12: Formula used for calculation of radical scavenging activity of DPPH.

The percentage of the DPPH reduction was also used to estimate EC_{50} (concentration that caused 50% inhibition of DPPH radicals) through non-linear regression analysis.

4.2.3 ABTS ASSAY

The ABTS assay is one of the most commonly used methods to measure the total antioxidant activity of a compound or extract [208]. The method is based on the generation of the ABTS radical a blue/green chromophore after the reaction of ABTS and potassium persulfate. The addition of antioxidants to the pre-formed radical cation reduces it, causing a decrease of absorbance (see figure 13) [210]. The protocol performed was done according to Ree et al., [210], with slight modifications. To perform this assay 88 μ l of a potassium persulfate solution (148 mM in ultrapure water) was added to 5 milliliters of ABTS solution (7 mM in ultrapure

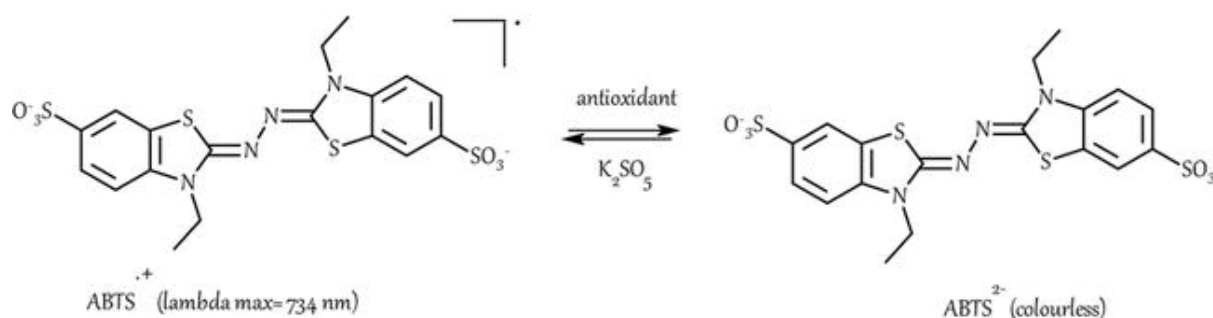


Figure 13: Reaction of ABTS radical with antioxidant compounds. Adapted from; Alam et al 2013 [214].

water) and let at room temperature and dark conditions for 12–16 hours before use. A working solution was then prepared, using 500 μl of the previous solution and 44 ml of sodium acetate buffer solution (20 mM, pH 4.5). For this assay 12 μl of the extract at different concentration (16,66 $\mu\text{g dwr/ml}$ to 833 $\mu\text{g dwr/ml}$ in phosphate buffer (PBS)) were added to 188 μl of the working solution reaching a final concentration range between 50 $\mu\text{g dwr/ml}$ to 1 $\mu\text{g dwr/ml}$. After 30 minutes of incubation at room temperature and dark conditions, the absorbance was measured using the SpectraMaxPlus microplate reader at 734 nm. All measurements were done in triplicate and three independent assays were performed. The same procedure was used for the control and blank, however, the extract and the working solution were replaced with the equivalent amount of PBS respectively.

Trolox was used as a standard and the percentage of ABTS scavenging activity was calculated according to the formula represented in figure 14. Then, the EC_{50} the concentration of sample necessary to decrease the absorbance by 50%– was obtained as previously mentioned.

$$\% \text{ ABTS scavenging activity} = \frac{A_c - (A_a - A_b)}{A_c} \times 100\%$$

A_b absorbance of blank of the sample (extract, PBS)

A_a absorbance of sample (working solution and extract)

A_c absorbance of control (working solution, without extract)

Figure 14: Formula used for calculation of ABTS scavenging activity percentage of the extracts.

4.2.4 SUPEROXIDE SCAVENGING ACTIVITY

The superoxide anion scavenging activity was measured according to Valentão and colleagues' method with slight modifications [216]. To generate superoxide radicals, a phenazine methosulphate - nicotine adenine dinucleotide reduced form (PMS-nicotinamide adenine dinucleotide (NADH)) system, a non-enzymatic method was used. The generated superoxide radicals can react with NBT reducing it into formazan, a blue compound having an absorption maximum at 560 nm. The elimination of superoxide radical, by antioxidants, decreases the reduction of NBT causing a decrease in formazan formation. Thus, by the difference in absorbance, it is possible to quantify the superoxide anion scavenging activity of the tested sample (see figure 15). For this protocol a NADH solution (1.97 mM) in sodium

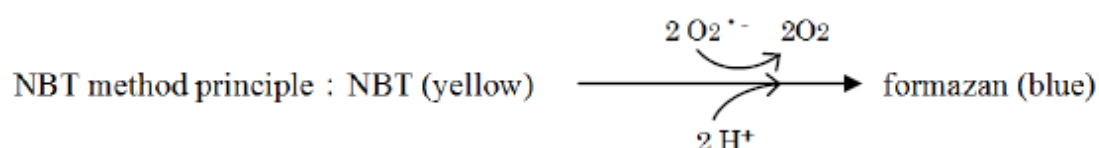


Figure 15: Superoxide scavenging assay, non-enzymatic system, principle. Adapted from Valentão. et al, 2002 [216].

PBS (19 mM in deionized water, pH 7.4) was prepared, as well as a solution of PMS (16 μM) and NBT (81.5 μM) both in PBS. To evaluate AV and dAV chelating ability a dilution series were prepared as follows: 50 μl of extract at different concentrations (0.375 mg dwr/ml-6 mg dwr/ml inPBS), was added to 50 μl of NADH, 150 μl of PMS and 50 μl of NBT, reaching a final range of extract concentration of 0.0625 mg dwr/ml to 1 mg dwr/ml for both extracts under study. The control and blank were made following the same volumes, however, PMS and the extract were replaced by buffer in the blank and in the control, respectively. After 10 minutes of incubating at room temperature, the absorbance was measured at 562 nm using SpectraMaxPlus microplate reader.

Quercetin was used as a positive control as a chemical reference in comparison to the superoxide scavenging activity capacities of both extracts.

All measurements were done in triplicate and three independent assays were performed. The percentage of SOi was calculated according to the formula represented in figure 16. The

$$\% \text{ SOi} = \frac{A_c - (A_s - A_b)}{A_c} \times 100\%$$

A_c absorbance of NADH, NBT and PMS without extract

A_s absorbance of NADH, NBT and PMS and extract

A_b absorbance of NADH, NBT and extract

Figure 16: Formula used for calculation of the percentage of SOi.

EC_{50} - the concentration of sample necessary to decrease the absorbance by 50%- was obtained as previously described.

4.2.5 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

The FRAP of *A. venetum* was assessed according to the method described by Torres et al.,2015 [217] , with several alterations. This assay measures the ability of antioxidants to reduce the complex formed by TPTZ and $FeCl_3 \cdot 6H_2O$, which generates an intense blue-colored ferrous complex at low pH (see figure:17). Thus, by the difference in absorbance, it is possible to quantify FRAP of the extracts under study. For this protocol stock solutions of HCl (40

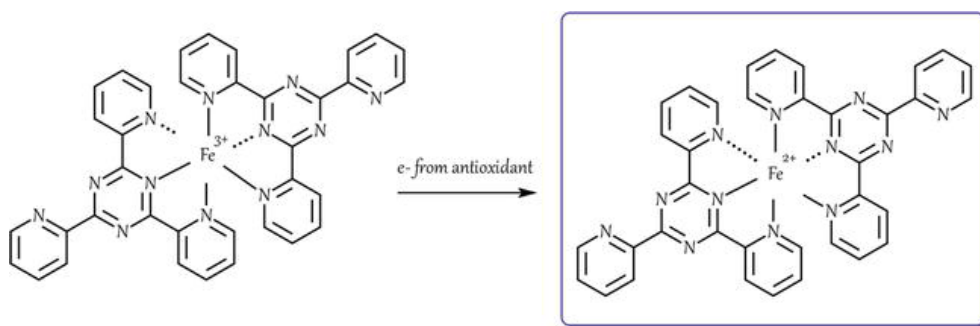


Figure 17: Reaction of the FRAP assay in the presence of an antioxidants. Adapted from; Santos-Sanchez et al.,2019 [218].

mM), TPTZ (10 mM in HCl 40mM), $FeCl_3 \cdot 6H_2O$ (20 mM in ultrapure water), $FeSO^4$ (100 mM in ultrapure water) and sodium acetate buffer (300 mM pH 3.6) were previously prepared.

A working solution with sodium acetate buffer, TPTZ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a proportion of 10:1:1, was prepared daily to be used in this protocol. To perform the assay 75 μl of pre-heated working solution (37°C) were added to 10 μl of each concentration (0.213 mg dwr/ml - 8.5 mg dwr/ml in ultrapure water) to obtain a final range of extract concentrations between 0.025 mg dwr/ml to 1 mg dwr/ml for both extracts under study. The same procedure was used for the control and blank, however, the extract and ferrozine were respectively replaced by the equivalent amount of PBS. After an incubation period of 5 minutes, the absorbance was measured at 593 nm using a SpectraMaxPlus microplate reader. Standard curve of FeSO^{2+} ferric reducing was performed. The FeSO^{2+} concentration of each tested concentration was determined by replacing the absorbance value in the linear equation obtained on the calibration curve.

4.2.6 IRON CHELATION ACTIVITY (ICA)

ICA can be estimated through the measurement of the ability of antioxidants to chelate Fe^{2+} , (Iron II chloride-4-hydrate). This assay is based on the ability of ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulphonic acid sodium salt) to form a purple complex in the presence of iron. However, when chelating agents are present the formation of this complex is interrupted causing a color shift [219]. Thus, by the difference between the absorbance of the control (complex formed by Ferrozine and iron) and the absorbance of the sample (ferrozine, iron, and extract), the chelation activity of *Apocynum venetum* can be quantified.

The chelating ability of *Apocynum venetum* was evaluated according to Russo and colleagues' method [220]. At first, stock solutions of FeSO^4 (0.12 mM in ultrapure water) and Ferrozine (0.6 mM in ultrapure water) were prepared and kept protected from the light until used. To evaluate *Apocynum venetum* ability to chelate iron a dilution series were prepared as follows: 50 μl of extract at different concentrations (0.75 mg dwr/ml - 3 mg dwr/ml in ultrapure water), 50 μl of FeSO^4 stock solution and 50 μl of Ferrozine stock solution reaching a final range of extract concentrations of 0.25 mg dwr/ml to 2 mg dwr/ml.

The control and the blank were performed following the same conditions, however the extract and ferrozine, were replaced by the equivalent amount of ultra-pure water.

Na_2EDTA was used as a positive control and as a chemical reference in comparison to the iron-chelating activity capacities of the extracts. After 15 minutes under dark conditions and at room temperature, the absorbance was measured at 515 nm, with a SpectraMaxPlus microplate reader. All measurements were done in triplicate and three independent assays were performed. The percentage of ICA was then estimated through the equation represented in figure 18. Due to the fact that EC_{50} was not achieved using the higher concentration of

$$\% ICA = \frac{Abs_c - (Abs_s - (A_b))}{A_c} \times 100\%$$

A_c ferrozine absorbance with iron, without extract

A_s ferrozine absorbance with iron and extract

A_b absorbance of the extract without ferrozine

Figure 18: Formula used for calculation of the ICA percentage.

2 mg dwr/ml of *A. venetum*, the EC_{25} was determined. The EC_{25} (concentration that caused chelation of 25% of the iron added) was estimated, after plotting the % ICA against tested concentrations.

4.2.7 NITRIC OXIDE SCAVENGING ACTIVITY

The ability of *Apocynum venetum* to scavenge nitric oxide production was measured according to the method of Colle et al., 2012 [221]. This assay is based on the fact that sodium Nitroprusside (SNP) decomposes in an aqueous solution at physiological pH producing nitric oxide radical. This radical can react with oxygen and generate stable products such as nitrate and nitrite, that can be quantified using Griess reagent. The presence of antioxidants inhibits

the production of nitric oxide radical generated from SNP which can then be quantified by Griess reaction (represented in figure:19) [214].

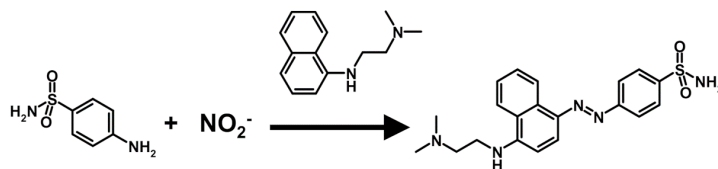


Figure 19: Scheme of the Griess diazotization reaction. Adapted from : Brizzolari et al., 2021 [222].

For this protocol a stock solutions of PBS (19 mM in deionized water, pH 7.4), phosphoric acid (5% in ultrapure water), SNP (20 mM in ultrapure water, protected from the light), sulfanilamide (1% in 5% phosphoric acid) and NED (0.1% in ultrapure water) were prepared.

For this assay 50 μ l SNP were added to 50 μ l of the different concentrations of the AV (3 μ g dwr/ml to 150 μ g dwr/ml in PBS) to obtain a range of concentrations between 0.5 μ g dwr /ml to 10 μ g dwr/ml. Following this the samples were incubated for 60 minutes at room temperature. Then, 50 μ l of Griess reagent (1% sulfanilamide in 5% phosphoric acid 0.1% NED in ultrapure water with 1:1 porportion) were added.

After 15 minutes at dark conditions and room temperature, the absorbance was read at 546 nm using a SpectraMaxPlus microplate reader. The same procedure was used for the control and blank, however, the extract and Griess reagent were, respectively, replaced with the equivalent amount of PBS. A blank for the control was also performed, where the extract and Griess were replaced by PBS.

Quercetin was used as a positive control. All measurements were done in triplicate and three independent assays were performed. The percentage of NOi was calculated according to the formula presented in figure 20. The EC_{25} value was determined as previously described.

4.2.8 PEROXIDASE ASSAY FOR MEASUREMENT OF COX-2 ACTIVITY

COX-2 plays a crucial role in the development of inflammatory status. COX-2 is an inducible isoform capable of converting arachidonic acid (AA) to prostaglandin E2 which is the precursor

$$\% \text{ NOi} = \frac{(A_c - A_{bc}) - (A_a - A_b)}{(A_c - A_{bc})} \times 100\%$$

A_b absorbance of blank of the sample (SNP, extract)

A_a absorbance of sample (SNP, extract and Griess)

A_c absorbance of control (SNP and Griess)

A_{bc} absorbance of blank of control (SNP)

Figure 20: Formula used for calculation of the percentage of inhibition of nitric oxide NOi.

of prostaglandins, thromboxanes, and prostacyclins [212, 223, 224]. These small molecules are involved in inflammatory conditions in certain cancers, in the brain, and in other physiologic stimuli. Thus, the inhibition of COX-2 can help to counteract an inflammatory status and may have positive effects on health conditions [225]. A peroxidase assay for measurement of COX-2 activity was used to evaluate the inhibitory potential of the extracts under study since it is a high-throughput and cheap method. In this protocol AA to generate hydroperoxide by COX-2, and TMPD an electron donor, which turns blue upon reduction, were used. TMPD is oxidated by the heme peroxidase producing a highly colored product that absorbs at 611 nm. Thus, if inhibitory compounds of COX-2 are present TMPD is less oxidized avoiding the production of the highly colored product [226].

The effects of *A. venetum* extracts on COX-2 was analyzed through the COX-2 peroxidase assay conduct according to the method described by Mogana Wiart [227]. Briefly 20 μl of extracts dissolved in DMSO were plated in triplicates at different concentrations (10, 25, 50, 125 and 250 μg dwr /ml) in a 96-well microplate followed by 20 μl of 10 U/mL of COX-2 enzyme solution. Then, 180 μl of endpoint assay mix containing 100 μM bovine hemin chloride, 10 mM of AA, 17 mM of TMPD, and 1 M of buffer Tris-HCl pH 8.1. After 5 minutes of incubation at room temperature in the dark, the absorbance was measured at 595 nm. Negative and positive controls in which the extract was replaced by 20 μl DMSO or COX-2 inhibitor (*methyl*[5-*methylsulfonyl*-1-(4-*chlorobenzyl*)-1H-2-*indolyl*]carboxylate), respectively were done. Blanks were also included where 180 μl endpoint assay mix and 20 μl of COX-2

was replaced by buffer Tris-HCl. Results were expressed as cyclogenase-2 inhibition (ICOX-2) relatively to the negative control using the formula present in figure 21. The IC_{50} (half-maximal inhibitory concentration) value was then obtained, as previously described for both the extract and the COX-2 inhibitor.

$$\% ICOX = \frac{Abs_c - (Abs_s - Abs_b)}{Abs_c} \times 100$$

Abs_c – Absorbance of negative control

Abs_b – Absorbance of blank

Abs_s – Absorbance of sample

Figure 21: Formula used for calculation of Cyclogenase-2 inhibition (ICOX-2) percentage.

4.2.9 STATISTICAL ANALYSIS

In general, experiments were performed independently three times, with at least three replicas. Data were analyzed in GraphPad Prism v. 8.0 Software (San Diego, CA, USA). EC_{50} and EC_{25} values were also calculated in GraphPad via non-linear regression analysis. Data are shown as mean $\pm$ standard deviation (SD) of three independent experiments. Results were analyzed by ANOVA analyses. Differences were considered significant at a * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

4.3 Results and discussion

4.3.1 ANTIOXIDANT ACTIVITIES

Various methods have been developed to measure the antioxidant activity of several samples such as compounds, plants, and food among others. These methods are widely used due

to their rapidity, and convenience and are based mainly on three methods: hydrogen atom transfer (HAT), single electron transfer (SET), and the ability to chelate transition metals SET [207, 228]. FRAP, ABTS are based on SET, DPPH is based on both, while ICA is based on the ability to chelate transition metals. Each method is performed in specific conditions such as pH, time, polarity, and temperature, therefore, the performance of only one assay will not reflect the total antioxidant capacity [214]. Furthermore, the fact that compounds found in plants are mostly bound to other molecules such as sugars and proteins can interfere with their antioxidant effects and may lead to lower results when compared to free forms of the compounds [46].

In this work, to study the antioxidant potential of AV and dAV several methodologies, including radical scavenging activities (DPPH and ABTS), ferric reducing antioxidant power, iron-chelating assay, superoxide anion radical (PMS-NADH system), nitric oxide (Griess method) scavenging activities and *in vitro* determination of COX-2 activity were used.

DPPH radical can be reduced by an antioxidant through the donation of hydrogen to form a more stable molecule. This method has some limitations related to its nitrogen atom location at the center of the structure that can enable larger molecules to assess the radical proportions due to steric hindrance. Furthermore, samples with high content in carotenoids or other compounds with strong absorbance at the same wavelength as the DPPH may interfere with the results [207, 229]. Despite these limitations and the fact that DPPH radical is not present in living organisms, DPPH based methods are widely used [207].

Quercetin is a well-known flavonoid and has been associated with several biological properties such as anti-inflammatory and antioxidant activities [185]. Studies show that quercetin can react stoichiometrically and rapidly with DPPH radicals, therefore was chosen as a standard compound and was used as a positive control in this assay [230]. The EC_{50} value is a statistical estimation of the concentration necessary to reach 50% of the observed effect calculated by using a hyperbolic fit. The lower the value of EC_{50} , the higher the antioxidant potential of the sample [231].

Results are present in figure 22, both extracts under study revealed antioxidant activity towards DPPH radical. Quercetin, the standard used showed an EC_{50} value of 3.1 ± 0.1

$\mu\text{g/ml}$, showing a higher antioxidant activity than both extracts under study according to the ANOVA analysis performed ($p\text{-value} < 0.0001$). The AV inhibited 50 % of the radicals at a concentration of $118.7 \pm 6.6 \mu\text{g dwr/ml}$ while the dAV had an EC_{50} value of $314.5 \pm 5.6 \mu\text{g dwr/ml}$. The EC_{50} value of the non-digested extract was significant lower than the value of the digested extract ($p\text{-value} < 0.0001$) which shows an effect of the digestion process on *Apocynum venetum* antioxidant activity towards DPPH.

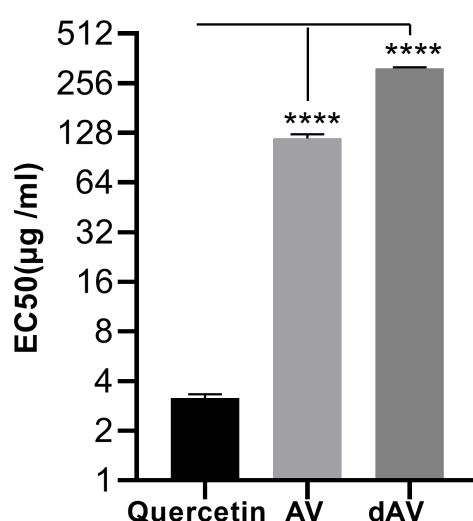


Figure 22: Antioxidant activity by DPPH. The results are presented as EC_{50} values of AV, dAV and quercetin (standard reference). Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the quercetin **** $p\text{-value} < 0.0001$ determined by ANOVA analysis.

DPPH scavenging potential of *A. venetum* have been already reported in literature, for instance Liang et al., 2010, [126] showed that *A. venetum* leaves methanolic extracts had a lower EC_{50} in the DPPH assay than butylhydroxytoluene, the standard used in the study. Another study show that *Apocynum venetum* leaves were able to scavenge 22.4% to 55.9% of DPPH in the concentration range of 0.2–1.0 mg/mL [8]. The DPPH scavenging activity of *Apocynum venetum* have been attributed to the presence of quercetin glycosides. Isolated quercetin glycosides from *Apocynum venetum* also revealed activity towards DPPH radical, showing higher activity than trolox, the standard compound used in the study [10]. Results are also in line with results found by lee and colleagues [232], that reported a

decrease of activity towards DPPH of onion quercetin and grape resveratrol after being subjected to an *in vitro* digestion.

The ABTS assay is very similar to the DPPH method since both of them use a strongly colored stable radical which is not present in living organisms [207]. However, ABTS has the advantage of being freely soluble in both organic and aqueous solvents so it can be used to screen both hydrophilic and lipophilic compounds, unlike other antioxidant methods [229]. In this assay Trolox, a water-soluble analog of vitamin E was used as a standard compound. The results are shown in the figure 23.

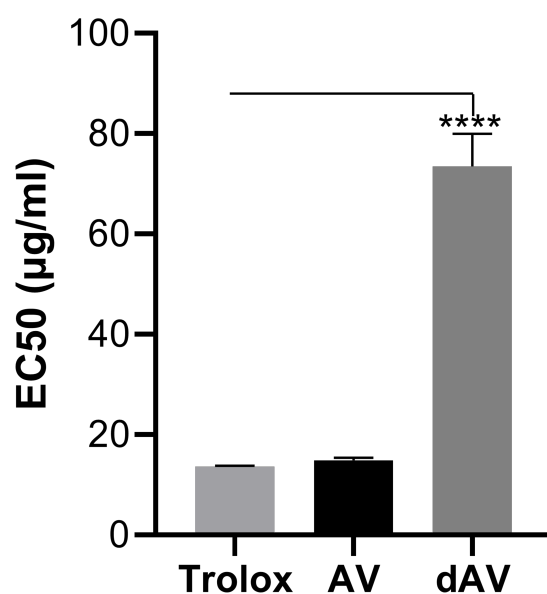


Figure 23: ABTS scavenging activity. The results are presented as EC_{50} values of AV – non-digested and dAV- digested *Apocynum venetum* methanolic leaves extract and trolox (standard reference). Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the trolox ****p-value <0.0001 determined by ANOVA analysis.

The EC_{50} value for AV was $14.9 \pm 0.5 \mu\text{g dwr/ml}$ and dAV was $73.5 \pm 6.5 \mu\text{g dwr/ml}$. The EC_{50} of dAV was significant higher than the EC_{50} of trolox ($13.9 \pm 0.1 \mu\text{g dwr/ml}$) (p-value= 0.0001) while the AV did not show significant differences in comparison with trolox according to the ANOVA analysis performed. Again, the digestion process impaired the antioxidant activity of the extract, leading to a significant increase of EC_{50} value (p-value <0.0001) according to the ANOVA performed.

Previous work has shown the capability of compounds found in *Apocynum venetum* to scavenge ABTS radical, however the analysis of *Apocynum venetum* leaves extract has not been addressed [12, 127]. Here, we prove that *Apocynum venetum* has great antioxidant activity in ABTS, and despite the decrease of activity provoked by the digestion process, *Apocynum venetum* still showed antioxidant ability in the assay.

Superoxide anions can be very harmful to cellular components due to their ability to generate hydroxyl radicals and their role in lipid peroxidation initiation [233]. Therefore, several compounds such as flavonoids have been screened for their scavenging activity of superoxide anions [234]. The scavenging activity of *A. venetum*, before and after performing *in vitro* digestion, was assessed through a non-enzymatic method. The results are present in figure 24, where AV exhibited a lower EC_{50} value ($13.33 \pm 2 \mu\text{g dwr/ml}$) than quercetin standard (EC_{50} value of $27.28 \pm 3 \mu\text{g/ml}$). The differences between these two values were significant according

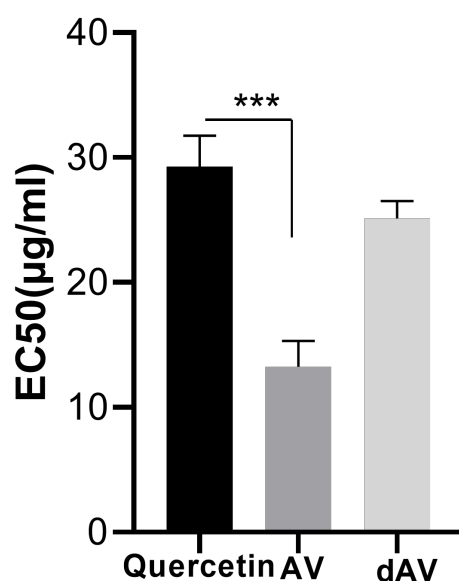


Figure 24: Superoxide scavenging activity. The results are presented as EC_{50} values of AV – non-digested and dAV- digested *Apocynum venetum* methanolic leaves extract and quercetin (standard reference). Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the ***p-value <0.001 determined by ANOVA analysis.

to ANOVA analysis (p-value = 0.0002) performed. The EC_{50} of the dAV was $24.4 \pm 1 \mu\text{g}$

dwr /ml which did not show significant differences from the quercetin EC_{50} value. Regarding the effects of the digestion, a decrease of the ability to scavenge superoxide anion (SO) was also observed. The differences between the EC_{50} of both extracts were significantly different with a p-value = 0.0009 (ANOVA analysis performed).

The ability of *Apocynum venetum* to scavenge SO, was also revealed by Li et al. [8]. Regarding the effects of digestion, the decreased pattern was similar to the one observed in DPPH and ABTS.

Transition metal ions such as Fe^{2+} are capable of perpetuating the formation of free radicals by the gain or loss of electrons. Therefore, chelating agents can prevent the formation of reactive oxygen species [235]. The ferric reducing antioxidant power of *Apocynum venetum* was determined using FRAP a convenient and reproducible method [236]. The AV was able to reduce the ferric-TPTZ complex in a concentration-dependent manner and showed chelating activity at concentrations of 25 μ g dwr/ml, the results are shown in figure 25. The *in vitro* digestion also diminished the ability of *Apocynum veentum* to reduce the ferric-TPTZ complex and dAV was not able to chelate in the minor tested concentration, 25 μ g dwr/ml, however, still reduced the complex in a concentration-dependent manner. Our results are in line with Record Lane [237] that also reported a decrease of antioxidant activity in FRAP analysis after the intestinal digestion of green and black tea.

The iron-chelating activity of *Apocynum venetum* extracts was also measured and Na_2EDTA was used as the positive control for this assay. Since EC_{50} was not achieved using the higher concentration of 2 mg dwr/ml the EC_{25} was determined. The EC_{25} value is a statistical estimation of the concentration necessary to reach 25% of the observed effect calculated by using a hyperbolic fit. The screening of *Apocynum venetum* as chelating agent showed that the extract was able to chelate iron showing an EC_{25} value of 396.1 ± 22.66 μ g dwr/ml, the results are present in figure 26. The ANOVA analysis revealed that Na_2EDTA EC_{25} value (2.5 ± 2 μ g/ml) was significantly minor than AV (p-value < 0.0001), showing a higher ICA potential. The dAV did not show ICA in the performed assay, showing once again the effects caused by the *in vitro* digestion.

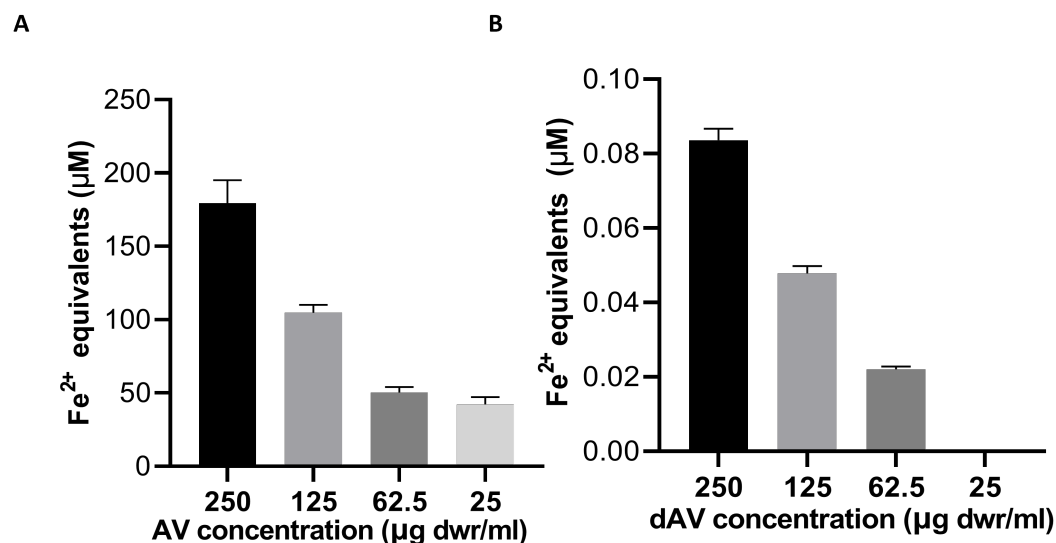


Figure 25: Ferric reducing antioxidant power of non-digested, AV present in **A** and digested extract of *Apocynum venetum* dAV in **B**, respectively. Results are expressed as Fe^{2+} equivalents and data is represent mean $\pm$ SD for three independent experiments.

As previously mentioned NO plays a relevant role in inflammatory processes and chronic expression of NO is associated with various carcinomas and inflammatory conditions [238]. In this assay, nitric oxide generated from sodium nitroprusside reacted with oxygen to form nitrite. The ability of *Apocynum venetum* extract to compete directly with oxygen in the reaction with nitric oxide translates its ability to scavenging NO generation. Quercetin was once again used as a positive control due to its role in antioxidant pathways [185]. The results are shown in figure 27.

The EC_{25} value was determined here instead of EC_{50} . The EC_{25} value of AV was $13.3 \pm 0.9 \mu g$ dwr/ml which was significantly minor than the quercetin ($28.96 \pm 1.7 \mu g/ml$) according to the ANOVA analysis performed (p -value < 0.0001). The *in vitro* digestion performed caused a decrease of the ability to inhibit NO generation, revealing an EC_{25} value of $27.70 \pm 0.3 \mu g$ dwr/ml. There were no significant differences between dAV and quercetin. Nonetheless the EC_{25} of dAV and AV were significant different (p -value < 0.0001). Despite this, the extract still revealed scavenging activity towards NO even after performing *in vitro* digestion. To sum up the performed *in vitro* digestion lead to a decrease of antioxidant activity in all tested assays performed here. So, we can conclude that the modifications occurring in simulated

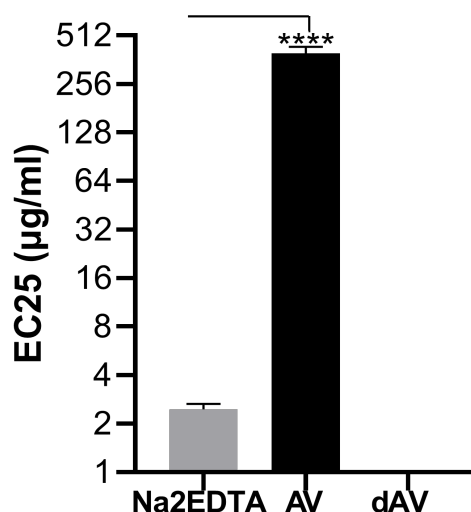


Figure 26: Iron chelating activity (ICA). The results are presented as EC_{50} values of AV – non-digested and dAV- digested *Apocynum venetum* methanolic leaves extract and Na_2EDTA (standard reference). Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the control ****p-value < 0.0001 determined by ANOVA analysis.

digestion lead to an impairment of antioxidant activity of the extract under study. Wang et al., [198] have also reported a decrease of antioxidant activity in red grape pommance after gastrointestinal *in vitro* digestion. The same article also found a decrease of quercetin and its main derivatives in the composition of red grape pommance in percentages between 25% and 46% after digestion protocol. According to Boyer 2005 [239], a 50%–75% loss of quercetin and 10% loss of quercetin-3-glucoside occurred after *in vitro* intestinal conditions.

4.3.2 PEROXIDASE ASSAY FOR MEASUREMENT OF COX-2 ACTIVITY

Following the evaluation of NO scavenging activity the extracts were screened for the inhibitory ability of COX-2 activity through the peroxidase assay for the measurement of COX-2 activity. The results are shown in figure 28. Both AV and dAV were able to inhibit COX-2 showing a half-maximal inhibitory concentration (IC_{50}) value of 16.13 ± 2.4 µg dwr /ml and 28.2 ± 3.2 µg dwr/ml, respectively. Both IC_{50} were significantly different from the inhibitor used as

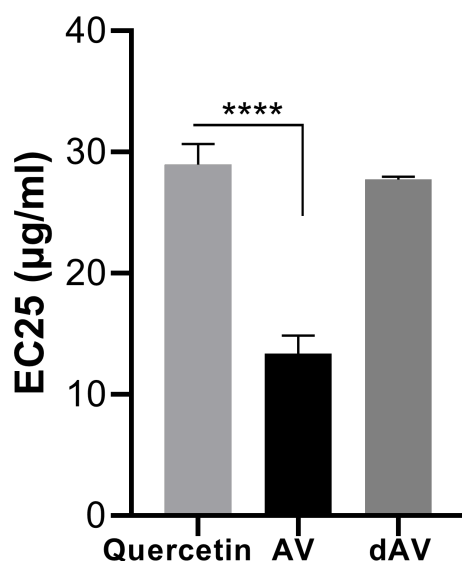


Figure 27: Nitric oxide (NO) scavenging activity. The results are presented as EC_{50} values of AV – non-digested and dAV- digested *Apocynum venetum* methanolic leaves extract and quercetin (standard reference). Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the control ****p-value < 0.0001 determined by ANOVA analysis.

control ($60.3 \pm 2 \mu\text{g dwr /ml}$), according to ANOVA analysis performed (p-value = 0.0002 for AV and p-value = 0.0005 for dAV). Regarding the effects of digestion the dAV revealed a higher IC_{50} value, nonetheless no differences were found between both values by the ANOVA analysis performed. The performed assay has been considered as a high throughput method however, other assays regarding COX-2 activity should be done such as an ELISA to confirm these results since some compounds may interfere and cause nonspecific interactions [212, 225, 226].

4.4 Conclusions

Literature describes *Apocynum venetum* as possessing antioxidant and anti-inflammatory properties. Here, several antioxidant assays were used to evaluate the antioxidant properties

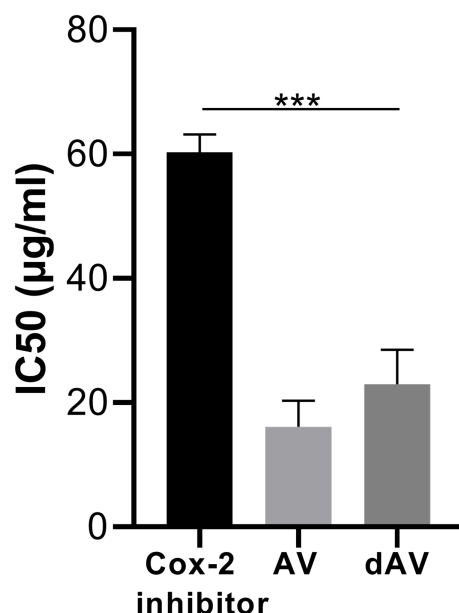


Figure 28: Inhibition of Cyclooxygenase-2 (COX-2) by the peroxidase assay. The results are presented as IC_{50} of COX-2 inhibition by AV – non-digested and dAV- digested *Apocynum venetum* methanolic leaves extract and a COX-2 inhibitor. Each bar represents the means $\pm$ SD of three independent experiments. Asterisks mean significantly differences from the control ***p-value <0.001 determined by ANOVA analysis.

of *Apocynum venetum*. Furthermore, the effects of *in vitro* gastrointestinal digestion on the antioxidant activity of the extract were also a target of study. The extract was exposed to oral, gastric, and small intestinal conditions.

In vitro biochemical antioxidant assays are the target of much criticism due to the lack of a unified terminological approach and accepted units of measurement among other criticism regarding the variety of methods performed. Despite this, these assays are low-cost, high-throughput tools used to screen the antioxidant potential of samples as well as to conduct quality control protocols.

The AV exhibited activity in all the antioxidant assays performed which is a good indicator for future research. Its activity was significantly higher than the control in superoxide anion radical (PMS-NADH system) and nitric oxide (Griess method) scavenging activities and COX-2

inhibitory activity which also shed light on the possible good effects of the *Apocynum venetum* on inflammatory conditions.

Results obtained showed a decrease in the antioxidant capacity after digestion, which was more prominent in the ICA, where EC_{50} value was not achieved, and in FRAP where the lower concentration did not show activity. For the other assays, the dAV still showed good antioxidant activity. The results prove that *Apocynum venetum* has maintained its antioxidant properties to some extent throughout the digestive system. This also suggests that *Apocynum venetum* may be able to act as a potent antioxidant and may intervene in oxidative and inflammatory processes since they are related processes, as previously explained.

In this way, *Apocynum venetum* may be useful as a therapeutic for diseases such as neurodegenerative diseases where oxidative stress and inflammatory responses play a major role.

CHAPTER 5

Antioxidant and anti-inflammatory effects of
the extracts: animal cell lines experiments

5.1 Introduction

Neurodegenerative diseases (NDDs) are an increasing cause of mortality and morbidity worldwide, especially among the elderly population. These are multi-factorial and complex diseases where oxidative effects and inflammation seems to play a major role [81, 240].

In the brain, inflammation is mainly regulated by microglia, the resident macrophages of the central nervous system. These cells are essential to promote the well-being of the brain by avoiding damages provoked by pathology or injuries among other functions (see section 1.4). To perform these functions microglia produces pro-inflammatory mediators such as ROS, cytokines, prostaglandins and NO through the increment of COX-2 and iNOS activities, respectively, after the activation of inflammatory pathways. Furthermore, ROS itself can enhance neuroinflammation through the activation of those pathways, which may cause an overload of those mediators. This may provoke the disruption of neurotrophin signaling, damage to cells, promotion of protein accumulation and cell death. Consequently, the loss of neuronal cells damages synapses and the production of neurotransmitters, leading to the cognition and movement impairments typical of NDDs [241–243]. Therefore, the blockage of over-activation of microglia may be a promising approach to prevent or delay the onset of neurodegenerative conditions.

The lack of effective therapeutic agents for NDDs has led to the search for preventive ways and therapeutic agents to combat inflammatory and neurological disorders in natural sources [242]. Plants have been re-discovered as a valuable source of many phytochemicals capable of exercising positive effects in the treatment of neurodegenerative diseases. Rather than providing just one mechanism, like drugs currently used, treatment with a single plant can promote many beneficial effects through the intervention in antioxidant, pro-survival, or pro-apoptotic inflammatory pathways [56, 105, 110, 244, 245]. Having this in mind the protective effects of digested and non-digested *Apocynum venetum* leaves methanolic extract in an immortalized murine microglial cell line (BV-2) were evaluated. First, the possible cytotoxicity and cytoprotective effects against t-BHP (an oxidative insult) were analyzed. The protective effects against inflammation induced by LPS (inflammatory insult) in the same

cell line were also evaluated. For that purpose, the expression of genes was evaluated by Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and NO levels of cells supernatant were measured.

Finally, before proceeding with the study of possible positive effects, plant extracts were screened for possible toxicities to avoid negative effects [244]. The liver plays a pivotal role in the metabolism and biotransformation of compounds, an activity that can cause hepatotoxicity which can lead to systemic toxicity [246]. Thus, the toxicities of extracts under study were evaluated using a HepG2 cell line (human hepatocyte carcinoma). This is a widely used model to measure cytotoxicity since this cell line retained all the enzymes responsible for the metabolism of xenobiotics [228, 247]. So the cytotoxicity of dAV and AV were first evaluated in this cell line. Then the cytoprotective effects of both extracts were also evaluated in the HepG2 cell line through the measurement of cell metabolic activity (MTT assay) and ROS intracellular levels, through the use of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a probe for redox state.

5.2 Material and methods

5.2.1 CHEMICALS AND REAGENTS

For the cell culture assays, high glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution, antibiotic-antimycotic solution, t-BHP, LPS (lipopolysaccharides from *Escherichia coli*, O111:B4), DCFH-DA, sodium bicarbonate, and MTT (97.8% purity), were purchased from Sigma, (Darmstadt, Germany). GRS Total RNA Kit – Blood Cultured Cells, Xpert cDNA Synthesis Mastermix and Xpert Fast SYBR Green Master Mix were purchased from GrisP, (Porto, Portugal). Primers for mouse interleukin-1BETA (IL-1BETA), IL-6, iNOS, COX-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and NF-KB were purchased from StabVida, (Oeiras, Portugal).

5.2.2 CELL CULTURE

Two cell lines were used in this work: HepG2 (human hepatoma) and BV-2 (mouse microglia). BV-2 cell line was kindly donated by Dr. Annika Höhn (German Institute of Human Nutrition, Germany) and HepG2 was obtained from the American Type Culture Collection (ATCC).

Both cells were kept in DMEM (13.4 g/l, 3.7 g/l sodium bicarbonate, pH 7.1-7.3) supplemented with 10% (v/v) heat-inactivated FBS, and 1% (v/v) antibiotic–antimycotic solution. After filtering in a laminar flow chamber into an autoclaved flask using a 0.1 Whatman nitrocellulose membrane filters Sigma, (Darmstadt, Germany) the medium was kept at 4 °C.

Cultures were maintained in 25 cm² flasks in a humidified incubator containing 95% air and 5% CO₂ at 37°C (HERACELL 150i, Thermo scientific). Cell morphology was monitored regularly by observation under a microscope (Inverted microscope Olympus CK2). BV-2 cells were sub-cultured three times a week with trypsin 0.05% and HepG2 cells two times a week with trypsin 0.25%.

To perform assays a cell suspension with a cell density of 2×10^5 cell/ml for both cell lines was used to plate on a 96 (200 µl / well). For the 12 and 48 plates both cells were plated at 2.5×10^5 500 µl and 1 ml/ well, respectively. After plating, cells were left for adhesion for 24 hours.

5.2.3 MTT ASSAY

Cell metabolic activity (viability) was determined using the MTT assay as described before [187]. This assay is based on the ability of viable cells to convert the water-soluble yellow dye MTT into an insoluble purple formazan through the action of mitochondrial reductase present in the cell. Following this, formazan can be solubilized and can be quantified by optical density at 570 nm. The amount of formazan formed is proportional to the number

of functional mitochondria and therefore proportional to the number of metabolic active cells which is an indicator for the number of viable cells [248, 249].

The protocol was performed after cell incubation with the extracts and compounds under study. The medium was aspirated and 100 µl of a solution with a 1:10 proportion of MTT (5 mg/ml in PBS) and DMEM (without FBS) was added to each well. Immediately after, cells were kept protected from light and incubated for 1.5 hours at 37 °C in a 5% CO₂ incubator. After incubation time, the supernatant was aspirated and 100 µl of DMSO: ethanol solution (1:1) was added to re-suspend formazan crystals. Finally, absorbance was read at 570 nm in the SpectraMaxPlus microplate reader. The results were analyzed and expressed as % of cell viability according to the formula presented in figure 29.

$$\% \text{ cell viability} = \frac{A_s}{A_c} \times 100\%$$

A_s Absorbance of the sample tested

A_c Absorbance of control (untreated cells)

Figure 29: Formula used to calculate % of cell viability.

5.2.4 CYTOTOXICITY ASSAY

To assess the potential cytotoxicity of *Apocynum venetum* BV-2 and HepG2 cells were seeded in 96-well culture plates at density of 2×10^5 cells /ml (200 µl/well) in complete DMEM (with FBS). After the incubation period (24 hours), the medium was discarded, and cells were incubated with different concentrations of the extracts (concentrations tested were 500, 100, 50 µg dwr/ml) for 4h or 24h. In each experiment cells in DMEM (without the extracts) or with 10% DMSO in DMEM were used as normal growth and death controls, respectively. Cells with 0.05% DMSO were used as solvent control (to make sure that DMSO present in the extracts was not harmful to cells).

Cell viability was determined using the MTT assay previously described (see section 5.2.3). The cell viability was expressed as a percentage of the control (cells just with DMEM).

5.2.5 CYTOPROTECTIVE ASSAY AGAINST AN OXIDATIVE INSULT

To evaluate the antioxidant cytoprotective effects of the extracts t-BHP was used as an oxidative insult [247]. This is a compound commonly used to induce oxidative stress in cells to evaluate the cytoprotective effects of the extracts under study the following protocol was carried out [247, 250, 251].

Cells were seeded in the same conditions as for the cytotoxicity assay and were first pre-incubated with extracts for 20h at 500, 100, 50 $\mu\text{g dwr/ml}$ concentrations, and then co-incubated with t-BHP (0.5 mM) for additional 4 hours. Additionally, cells were co-incubated with the extract and t-BHP for 4 hours. Cellular viability was assessed by the MTT assay (see section 5.2.3). In each experiment cells in DMEM (not incubated with the extracts or oxidant insult; normal growth), 0.05% DMSO in DMEM (solvent control) and in DMEM with t-BHP (0.5 mM), were used as controls. The cell viability was expressed as percentage of the control (cells just with DMEM).

Results were plotted as the means $\pm$ SD of three independent experiments performed in triplicates for each experimental condition. All parameters were graphically represented using the software GraphPad 8 (Prism, USA).

5.2.6 EVALUATION OF INTRACELLULAR ROS THROUGH DCFH-DA IN HEPG2 CELLS

The t-BHP is easily diffused through the cell membrane and generates intracellular alkoxyl and peroxy radicals causing several damages to the cell and consequently cell death as previously discussed [252]. Intracellular levels of ROS were measured through the DCFH-DA, a commonly used method. DCFH-DA is a non-fluorescent compound capable of entering the cell to the cytoplasm where it is hydrolyzed by intracellular esterases to the non-fluorescent compound

(DCFH). Then, DCFH can be oxidized by free radicals and converted into a fluorescent compound 2',7'-dichlorodihydrofluorescein (DCF). Thus, the measurement of the fluorescent directly measures the redox state of a cell [253] (see figure 30).

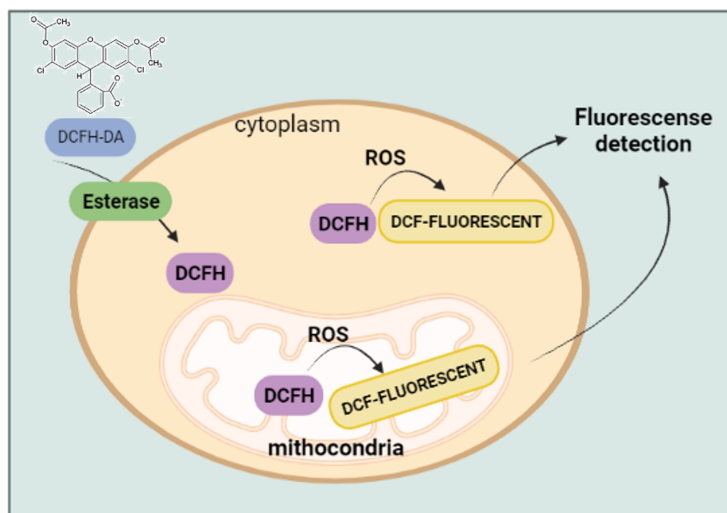


Figure 30: Mechanism of action of DCFH-DA probe inside the cell. DCFH-DA (2',7' – *dichlorodihydrofluorescein diacetate*), a cell permeable non-fluorescent dye enters the cell where is hydrolyzed into DCFH (2',7' – *dichlorodihydrofluorescein*) through the action of esterases. DCFH is then oxidized by peroxidases and intracellular reduced oxygen species (ROS) into its fluorescent form DCF(2',7' – *dichlorofluorescein*) which can then be quantified.

HepG2 cells were seeded in 48-well plates at a density of 2.5×10^5 cell/ml (500 μ l/well). After the adhesion time (24 hours) cells were incubated with 500 μ l of DCFH-DA (25 μ M in PBS) and incubated for 30 min. at 37 $^{\circ}$ C and 5% CO₂, in the dark. DCFH-DA solution was then removed, and cells were washed with warm PBS. Next, cells were pre-incubated at previous conditions for 4 hours with the extracts at 50, 250, and 500 μ g dwr/ml of both AV and dAV. Then, t-BHP was added at 0.5 mM and left for incubation for 1 hour. After this, cells were washed with warm PBS and lysate with a DMSO/PBS solution (9:1 solution), at room temperature, for 10 min. in a shaker. In each experiment cells in DMEM (not incubated with the extracts or oxidant insult; normal growth), 0.05% DMSO in DMEM (solvent control) and in DMEM with t-BHP (0.5 mM), were used as controls. Changes in intracellular levels of ROS were monitored using a fluorescent microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, ThermoScientific) at excitation and emission wavelengths of 485 nm and 538 nm, respectively. The results were expressed as a percentage and normalized

relative to the control (cells just with DMEM) according to the equation present in figure 31.

$$\% \text{fluorescence intensity} = \frac{A_s}{A_c} \times 100$$

A_s – Sample absorbance

A_c – Control (Cells in DMEM) absorbance

Figure 31: Equation used to determine the percentage of fluorescence intensity.

5.2.7 QUANTIFICATION OF NO PRODUCED BY LPS-INDUCED BV-2 CELLS

In this procedure, cells were plated in a 12-well plate, 2.5×10^5 cells/ml (1 ml per well) and after the adhesion time (24 hours), cells were pre-incubated with the extracts under study for 30 min.. After that, cells were supplemented with LPS (2 $\mu\text{g}/\text{ml}$ final concentration) and incubated for 20 hours. Then, the supernatants of the culture medium were collected and 50 μl were transferred to a 96-well plate. Next, 50 μl of 1% (w/v) sulfanilamide in phosphoric acid buffer 2 % (w/v) was added to each well, and cells were incubated at room temperature in the dark for 10 minutes. Next, 50 μL of 0.1% (w/v) NED in phosphoric acid buffer 2 % (w/v) was added and incubated under the same conditions. The absorbance was then, read at 543 nm in the SpectraMaxPlus microplate reader. Cells in the medium, without any addition of LPS were used as negative control. As a positive control, cells were only incubated with LPS, without a pre-incubation with extracts. The cells incubated with the extracts and LPS were recovered for ribonucleic acid (RNA) extraction to evaluate gene expression.

5.2.8 QUANTIFICATION OF GENE EXPRESSION OF PRO-INFLAMMATORY GENES (RT-PCR)

To perform RNA extraction, BV-2 cells were recovered. Plated BV-2 cells after performing an pre-incubation period with AV and dAV at a concentration of 125 µg/ml for 30 min. and co-incubation with LPS for 20 hours were washed with warm sterile PBS (37°C) and trypsinized (0.05 %). Cells were then recovered in DMEM medium with 10 % FBS to marked eppendorfs (for each condition) and kept on ice. Then, eppendorfs were centrifuged (centrifuge 5418R Eppendorf Fischer Scientific) at 1000 rpm for 5 minutes, and the yielded pellet was washed with PBS ice-cold. Once again, eppendorfs were centrifuged in the same conditions and the resulting pellet was kept at -80°C for further assays.

Total RNA was isolated from recovered BV-2 cells using the GRS Total RNA Kit – Blood Cultured Cells (grisPin), following standard protocol. RNA purity and concentration were evaluated using a Nanodrop spectrophotometer (ND-100, Thermo Fischer) through the analysis of 260/280 and 260/230 ratios. The integrity of RNA was confirmed through the performance of electrophoresis in a 1% agarose gel (agarose in tris-acetate-EDTA buffer (TAE) 0.5x buffer). The addition of cybersafe dye (grisPin) in the samples allowed the visualization of RNA bands and a specific marker Bioron Ladder 1 kb was used to quantify RNA bands. The electrophoresis was performed at 100 V voltage and the gel was visualized in ChemiDoc (ChemiDoc Imaging System VWR. Geno-Smart).

Isolated RNA was reverse-transcribed into complementary DNA (cDNA) (0.5 µg of each sample) using the Xpert cDNA synthesis master mix following the standard protocol. The samples were run in a thermocycler (C1000, Bio-Rad) under the following conditions: 5 minutes at 65 °C, 2 minutes on ice, 10 minutes at 25 °C after the addition of RNA transcriptase, 15 minutes at 50 °C and lastly 5 minutes at °C, to inactive reverse transcriptase enzyme. To each cDNA sample 55 µl of RNase free water was added reaching a final concentration of 6.7 ng/ µl and samples were kept on ice to the posterior step.

Quantitative real-time polymerase chain reaction (PCR) was carried out with Xpert Fast SYBR Green Master Mix (GrisP) and performed in 96-well plates using a CFX96 Real-Time

Detection System (Bio-Rad). First, a primer solution containing both forward and reverse primers at a concentration of 10 μM was made for each primer under study. Then a reaction mix was done by mixing 5 μl of blue master mix (Xpert Fast SYBR Green Master Mix), 0.6 μl of each primer solution at 10 μM and 3.4 μl of nuclease-free water to each well. Finally, 1 μl of cDNA was added to each well.

The conditions used in the cycler were 15 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 55°C and 30 s at 72°C. Experiments were done in triplicates and analyzed with the software Bio-Rad CFX Manager (Bio-Rad), using GAPDH as an internal control. For each well, melting curves were analyzed and the results were optimized IL-1BETA, IL-6, iNOS and COX-2 were the genes under evaluation and the specific primers were designed with Quantprime Software (see table:3).

Table 3: Sequence of primers used in this study.

Gene	Primer sequence	
	Forward	Reverse
GAPDH	5'-GTGGACCTCATGGCCTACAT-3'	5'-TGTGAGGGAGATGCTCAGTG -3'
IL-1β	5'TGTGTAATGAAAGACGGCACACC-3'	5'-GTATTGCTTGGGATCCACACTCTC-3'
IL-6	5'-TCCTCTCTGCAAGAGACTTCCATC-3'	5'-TGGTTGTCACCAGCATCAGTCC-3'
iNOS	5'-TCCTCACGCTTGGGTCTTGTTTC-3'	5'-TCCAACGTTCTCCGTTCTCTTGC-3'
COX-2	5'-TCAGCCAGGCAGCAAATCCTTG-3'	5'-AATCCAGTCCGGGTACAGTCAC-3'

5.2.9 STATISTICAL ANALYSIS

All experiments were performed at least in triplicate, with data presented as the means $\pm$ SD of three independent experiments and analyzed using GraphPad 8 (Prism, USA). Comparisons between control and treatment groups were made using one-way ANOVA, the p-value was considered significant when $t * p\text{-value} < 0.05$; $** p\text{-value} < 0.01$; $*** p\text{-value} < 0.001$; $**** p\text{-value} < 0.0001$. All analysis were performed using the software GraphPad 8 (Prism, USA).

5.3 Results and discussion

5.3.1 CYTOTOXICITY AND CYTOPROTECTIVE ASSAY AGAINST AN OXIDATIVE INSULT OF EXTRACTS IN HEPG2 CELLS

The liver is the main detoxifying organ in the human body being responsible for drug metabolism. Hence hepatic cells are important to understanding possible side effects of drugs [254]. The HepG2 cell line is a human liver cancer cell line well adapted to different environments and a well-known model to study the action of many chemicals and medications [255]. Therefore, this cell line was used, to evaluate the possible toxicity of the extracts, under study, through the analysis of the cells metabolically active.

Cytotoxicity of AV and dAV were evaluated through the MTT assay, at three different concentration (50, 100 and 500 µg dwt/ml) and during 4 and 24 hours of incubation, mimicking acute and chronic cell exposure (figure 32A and figure 32B, respectively). Results showed that both AV and dAV did not induce toxicity for any tested concentrations after 4 h of incubation as shown in figure 32A. At 24 hours of incubation, there were also no significant differences in cellular viability when comparing the cell viability percentage of the different extract concentrations with the control (cells with medium) (figure 32B). According to the results present in figure 32, the *in vitro* digestion did not induce changes on % cell viability.

Previous work had already elucidated on cytotoxic effects of *Apocynum venetum* in the HepG2 cell line. Our results are in line with Zhang and colleagues [124] that revealed that an ethanolic extract of *Apocynum venetum* leaves showed no cytotoxicity effects on HepG2 cells even up-value 400 µg dwt/ml during 24 hours of incubation. The cytotoxicity effects were evaluated both through MTT assay and lactate dehydrogenase (LDH) release assay. The same study also revealed non-symptoms of toxicity or mortality during the experiments on mice with the same extract after 14 consecutive days of treatment.

On the other hand, an article published by Chong Li [131] revealed an inhibitory effect in the growth of HepG2 cells within a concentration range between 50 and 200 µg dwt/mL, after

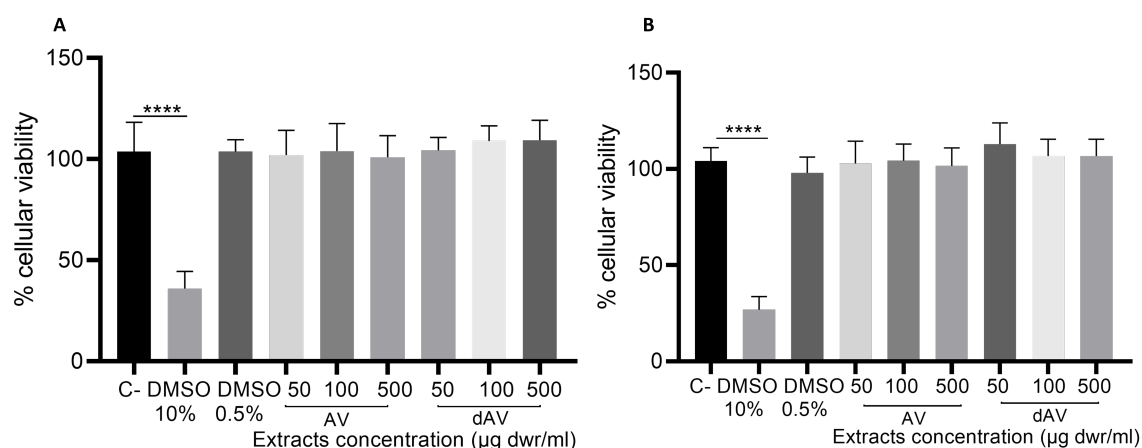


Figure 32: Cytotoxicity of AV and dAV on HepG2 cells. HepG2 cells were incubated with AV and dAV at 50 100, 500 $\mu\text{g dwr/ml}$ for 4h of incubation (**A**) or for 24 h (**B**). Cellular viability was assessed with MTT assay. AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract. Each bar represents the mean $\pm$ SD of the results obtained in three independent experiments. Asterisks mean significant differences from the control (cells in medium), as indicated: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

48 h of incubation. However, there are no more scientific reports regarding this incubation period on HepG2 cells.

After the study of cytotoxicity of the extracts, the cytoprotective effects against t-BHP were analyzed. The t-BHP is a commonly used compound for evaluating the cytoprotection of natural antioxidants. This oxidant insult is able to induce ROS, accelerate lipid peroxidation and cause DNA damages leading to cell death [256]. Cytoprotective results of AV in HepG2 for both 4 hours and 24 hours are represented in figure 33A and 33B, respectively. First, the results confirmed that t-BHP causes cell death, inducing more than 50 % of cell death in the concentration used (0.5 mM). Furthermore, results show that incubation with AV had a positive effect on cell viability when compared with the control (cells incubated with t-BHP 0.5 mM), revealing protective effects against the damage induced by the insult. For the 4 hours of incubation, the increase in cell viability caused by the extract occurred in a dose-dependent manner revealing significant differences from all tested concentrations. The minor concentration showed a p-value of 0.0418, the concentration of 100 $\mu\text{g dwr/ml}$ revealed a

p-value of 0.0153 while the highest concentration (500 $\mu\text{g dwr/ml}$) revealed a p-value = 0.0023 in the ANOVA analysis performed.

Results from 24 hours of incubation also displayed an increase in cell viability, when compared with the control (cells incubated with t-BHP) in a dose-dependent manner (figure 33B). The increase of cell viability was statistically significant in all tested concentration displaying a p-value = 0.0033 for the 50 $\mu\text{g dwr/ml}$ and a p-value <0.0001 for the two highest concentration according to the ANOVA analysis performed. The positive effects of the extract were increased after the increment of incubation time proving that the extract had a more prominent effect over time, unveiling a possible indirect effect of *Apocynum venetum* extracts through the increment of antioxidant enzymes activities. Previous work studying the cytoprotective effects of *Apocynum venetum* tea extract against hydrogen peroxide revealed an up-regulation of glutathione peroxidase, SOD and catalase in 293T cells [8]. In the literature, the cytoprotective effects of *Apocynum venetum* against t-BHP in HepG2 have not yet been a target of study. However, a cytoprotective study against CCl_4 of flavonoids of *Apocynum venetum* in HepG2 revealed protective effects in both MTT and LDH [124].

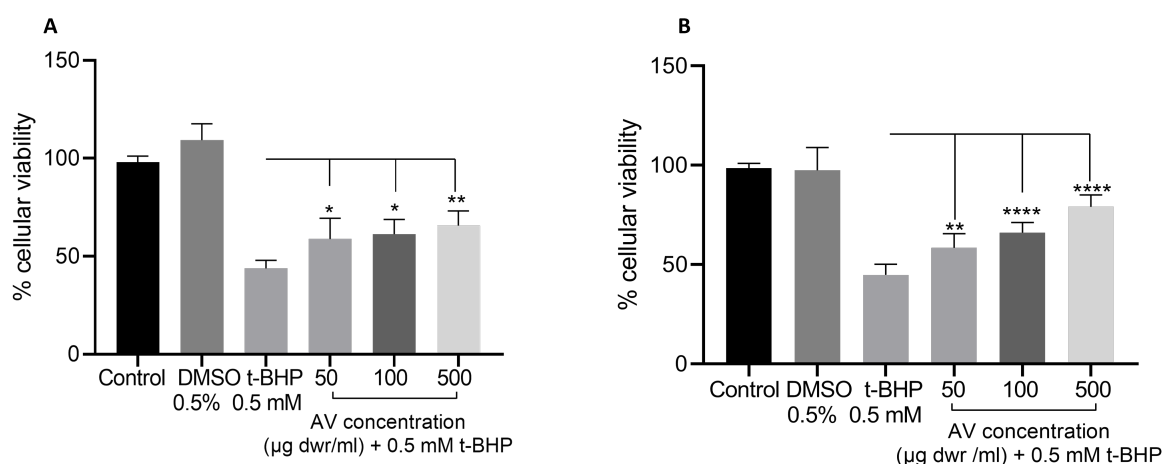


Figure 33: Cytoprotective potential against tert-butyl hydroperoxide (t-BHP) -induced damages. HepG2 cells were co-incubated for 4 hours with both dAV (50 100, 500 $\mu\text{g dwr/ml}$) and t-BHP(A); or pre-incubated with the extracts (50 100, 500 $\mu\text{g dwr/ml}$) for 20 h and co-incubated with t-BHP for 4 hours (B). AV-non-digested *Apocynum venetum* methanolic leaves extract; Cellular viability was assessed by the MTT assay. Each bar represents the mean $\pm$ S.D. of the results obtained in three independent experiments. Asterisks mean significant differences from the cells only incubated with t-BHP 0.5 mM, as indicated: **** p-value < 0.0001.

The cytoprotective results for the dAV are represented in figure 34. Results of the cytoprotective effects of the incubation with digested extract for 4 and 24 hours are presented in figure 34A and figure 34B, respectively. The incubation with the dAV showed protective effects against damages induced by t-BHP in all tested concentrations in both incubation times, once again in a dose-response manner. For the 4 hours of incubation extracts revealed a significant increment of % cell viability with p-values of 0.0059, 0.0023 and <0.0001 for each of concentration 50 $\mu\text{g dwr/ml}$, 100 $\mu\text{g dwr/ml}$, 500 μg , respectively. The protection was more significant after 24 hours of incubation were all tested concentrations (50, 100, 500 $\mu\text{g dwr/ml}$) revealed a p-value <0.0001 according to the ANOVA analysis performed, showing once again the increment of the protective effects over time.

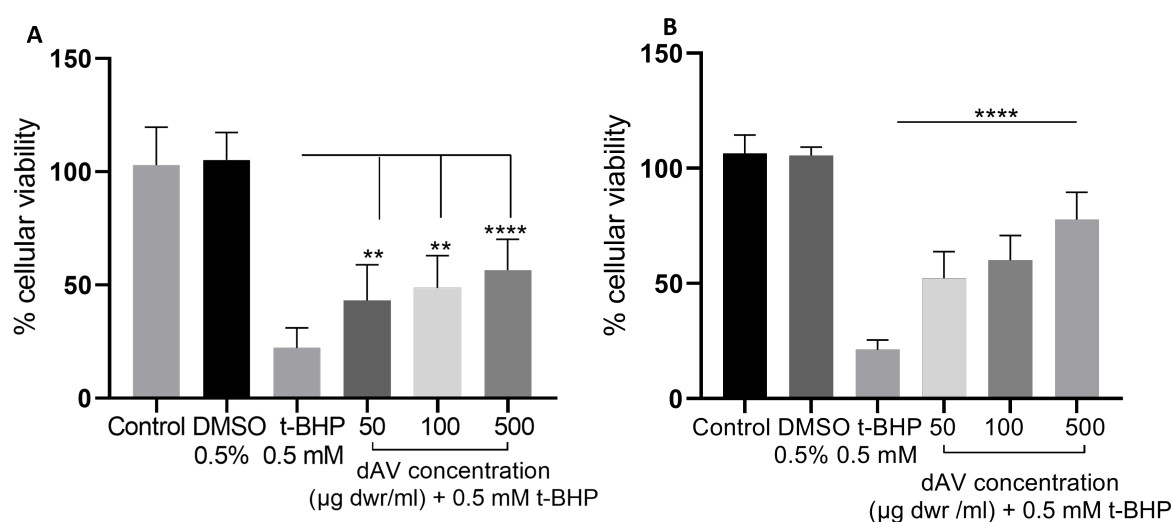


Figure 34: Cytoprotective potential against tert-butyl hydroperoxide (t-BHP)-induced damages. HepG2 cells were co-incubated for 4 hours with dAV (50 100, 500 $\mu\text{g dwr/ml}$) and t-BHP (**A**); or pre-incubated with the extracts (50 100, 500 $\mu\text{g dwr/ml}$) for 20 h and co-incubated with t-BHP for 4 hours (**B**). dAV-digested *Apocynum venetum* methanolic leaves extract; Cellular viability was assessed by the MTT assay. Each bar represents the mean $\pm$ S.D. of the results obtained in three independent experiments. Asterisks mean significant differences from the cells only incubated with t-BHP 0.5 mM for 4 h), as indicated: * p-value < 0.05 ; ** p-value < 0.01 ; *** p-value < 0.001 ; **** p-value < 0.0001 .

To compare the differences in cytoprotective effects of digested and non-digested extracts the percentage of protection was determined through the difference between the cell viability % of cells incubated with t-BHP and the obtained cell viability % for each tested concentration,

results are present in table 4. No significant differences were found between the digested and non-digested extract, revealing that the *in vitro* digestion did not impair or increased the protective effects towards t-BHP-induced damages.

In the literature, several effects of *in vitro* digestion on antioxidant effects are described, sometimes there is an increment and other times a decrease of activities. In fact, the different *in vitro* digestion protocols, as well as food matrix can be crucial, leading to different results. Furthermore, since plants' compositions are different, and digestion has different effects on different compounds, several outcomes may occur [61, 62, 64–66]. Nevertheless, digestion aims to facilitate the uptake of compounds through the improvement of solubility, which can also increase properties of compounds [239]. The results proved that antioxidant cytoprotective activity was maintained despite the modifications that may occur in *in vitro* digestion.

Table 4: Percentage of protection against tert-butyl hydroperoxide (t-BHP) effects after the pre-incubation of HepG2 cells with both AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract (50 100, 500 $\mu\text{g dwr/ml}$) for 4h or 20 hours. The results are represent as mean $\pm$ S.D. of the results obtained in three independent experiments.

HEPG2	Extract	AV			dAV		
	Concentration ($\mu\text{g dwr}$)	50	100	500	50	100	500
% of Protection	4h	16.2 $\pm$ 8.0	17.5 $\pm$ 6.2	24.1 $\pm$ 7.2	21.4 $\pm$ 6.5	23.3 $\pm$ 5.9	37.8 $\pm$ 8
	24h	11.5 $\pm$ 7.3	23.3 $\pm$ 3.8	35.2 $\pm$ 6.4	22.8 $\pm$ 7.2	30.4 $\pm$ 7.3	44.5 $\pm$ 8.6

5.3.2 EVALUATION OF INTRACELLULAR ROS THROUGH DCFH-DA IN HEPG2 CELLS

The t-BHP is a widely used pro-oxidant compound that can produce free radicals leading to oxidative damage and cell death [257]. In the cytoprotective assay performed, the extracts under study were able to protect cells against damages induced by t-BHP. We analyzed the effects of the extract on the redox state of the cell through the use of DCFH-DA. The t-BHP can directly oxidize DCF and it can also decompose to peroxy radicals generating lipid peroxides and ROS thus, increasing fluorescence [256].

Results are represented in figure 35 and as expected the incubation with t-BHP led to significant increase of intracellular ROS levels after one hour of incubation. Pre-incubation

with AV and dAV in the different tested concentrations (50, 250, 500 $\mu\text{g dwr/ml}$) resulted in a significant reduction of ROS (figure 35).

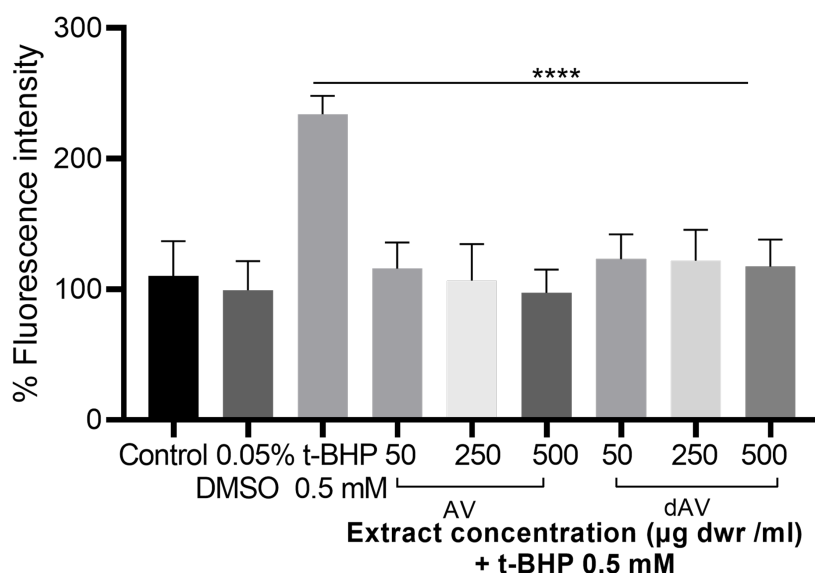


Figure 35: 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) microplate assay. HepG2 cells were labeled with DCFH-DA (25 μM) for 30 min. and then incubated with the AV and dAV at 50, 250, 500 $\mu\text{g dwr/ml}$ during 4 hours and co-incubated with tert-butyl hydroperoxide (t-BHP) 0.5 mM (for 1h). Cells were then analyzed on a fluorescent plate reader with excitation and emission wavelengths of 485/535 nm. Values are presented as % fluorescent intensity. AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract. Each bar represents the mean $\pm$ S.D. of the results obtained in three independent experiments. Asterisks mean significant differences from the cells only incubated with t-BHP 0.5 mM for 4 h), as indicated: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

Notably, all the significant reductions lead to values similar to the values of the control (cells in DMEM) revealing significant differences from cells only incubated with t-BHP (p-value < 0.0001) in the ANOVA analysis performed. The statistical comparison between the non-digested and the digested extracts, at the same concentration, did not show significant differences indicating that the digestion had no effects on the protective effects of the extracts towards ROS induced by t-BHP.

The results show that extracts under study strongly inhibited the generation of ROS induced by t-BHP in HepG2 cell lines, thus preventing and delaying oxidative damages in the cell. These effects may be related to the presence of flavonoids in the extracts that have been linked to direct (scavenging activities) and indirect antioxidant (triggering defense

systems) properties. Literature has already reported the decrease of ROS induced by t-BHP by flavonoids found in *Apocynum venetum* using DCFH-DA [256, 258, 259].

5.3.3 CYTOTOXICITY AND CYTOPROTECTIVE ASSAY AGAINST AN OXIDATIVE INSULT OF EXTRACTS IN BV-2 CELLS

Bv-2 mouse microglial is a commonly used cell line, a reliable substitute for primary cells. The inflammatory environment in the brain can be mimicked through the exposure of Bv-2 cells to LPS. LPS are a critical glycolipid component of gram-negative bacteria, which can trigger an inflammatory response (see section 1.4). After stimulation with LPS microglia initiates an inflammatory response which consequently leads to the release of pro-inflammatory cytokines, prostaglandins, ROS and RNS [68, 260].

For many years now, BV-2 cells have been used to study neuroinflammation and neurodegenerative disorders [261]. Firstly cytotoxicity and cytoprotective activities of *Apocynum venetum* leaves extract on this cell line were evaluated. The results of cytotoxicity of AV and dAV for the 4 and 24 hours of incubation are present in figure 36A and 36B, respectively. The incubation of BV-2 cells with *Apocynum venetum* extracts (both non-digested or digested) did not induce any toxicity up to 100 µg dwr/ml, regardless of incubation time (figure 36A and 36 A, B). However in the highest tested concentration (500 µg dwr/ml) a slight non-significant decrease in cell viability of < 2% for the 4 hours and < 5% in the 24 hours of incubation was observed with the incubation with AV. After the digestion process, however, the dAV did not reveal any decrease of % of cell viability, showing that digestion had abolished the observed level of toxicity. The effects of cytotoxicity of *Apocynum venetum* in BV-2 are not yet described in the literature.

After analyzing the possible toxicity of the extract toward BV-2 cells, we further analyze the cytoprotective effects of the extracts under study against an oxidative insult. The results of AV for 4 and 24 hours of incubation are represented in figure 37A and figure 37B respectively. The AV revealed some protective effects of the extract against the effects provoked by t-BHP after 4 hours of incubation. A significant increase of % cell viability was found in the

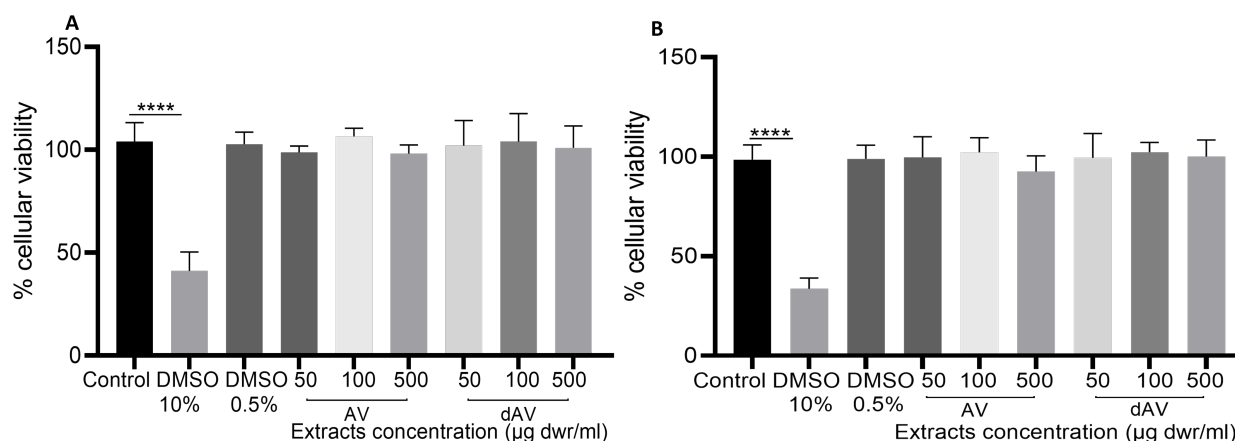


Figure 36: Cytotoxicity of AV and dAV on BV-2 cells. BV-2 cells were incubated with AV and dAV at 50 100, 500 $\mu\text{g dwr/ml}$ for 4h of incubation (A) or for 24 h (B). Cellular viability was assessed with MTT assay. AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract. Each bar represents the mean $\pm$ SD of the results obtained in three independent experiments. Asterisks mean significant differences from the control (Cells in medium), as indicated: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

two highest tested concentrations. The ANOVA analysis revealed a p-value of 0.0418 and < 0.0001 for the tested concentration of 100 and 500 $\mu\text{g dwr/ml}$ respectively when compared to the control (cells incubated with t-BHP). The pre-incubation of 20 hours further increased the protection levels of AV showing a p-value <0.0001 for the higher concentrations and a p-value of 0.0088 and of 0.0069 for the concentrations 50, 100 $\mu\text{g dwr/ml}$ respectively when compared to the control (cells incubated with t-BHP) (figure 37B). These results prove that the effects of the extract are improved over time, as happens with the incubation of the HepG2 cell line.

Following the 4 hours of co-incubation with dAV and t-BHP (see figure 38A) BV-2 cells revealed a significant viability increase in the two higher tested concentrations when compared to the control (cells incubated with t-BHP) with a p-value of 0.0125 and p-value <0.0001 for the 500 and 100 $\mu\text{g dwr/ml}$ respectively.

After 20 h pre-incubation with dAV a higher and significant cytoprotective effect was achieved for all tested concentrations (figure 38B). According to the ANOVA analysis performed the p-value was <0.0001 for 100 and 500 $\mu\text{g dwr/ml}$ and the p-value for the lowest concentration was 0.0096.

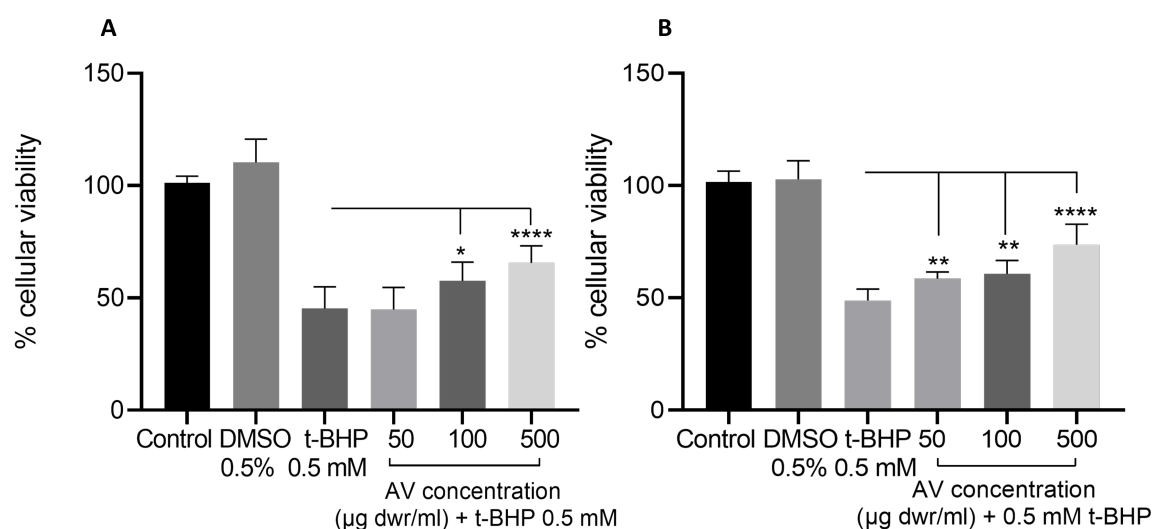


Figure 37: Cytoprotective potential against t-BHP-induced damages. BV-2 cells were co-incubated with 4 hours with dAV (50 100, 500 µg dwr/ml) and t-BHP(**A**); or pre-incubated with the extracts (50 100, 500 µg dwr/ml) for 20 h and co-incubated with t-BHP for 4 hours (**B**); dAV-non-digested *Apocynum venetum* methanolic leaves extract; Cellular viability was assessed by the MTT assay. Each bar represents the mean $\pm$ S.D. of the results obtained in three independent experiments. Asterisks mean significant differences from the cells only incubated with t-BHP 0.5 mM for 4 h , as indicated: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

To compare the differences in cytoprotective effects of digested and non-digested extracts the percentage of protection was calculated through the difference between the cell viability % of cells incubated with t-BHP and the obtained % for each study concentration the results are present in table 5 . As it happened in HepG2 cells no significant differences were found between dAV and AV protective activities against t-BHP, proving that the protective activities against t-BHP-induced damages remains after the digestion process. This is a good indicator for the use of *Apocynum venetum* as a medicinal tea.

5.3.4 QUANTIFICATION OF NO PRODUCED BY LPS-INDUCED BV-2 CELLS

As previously mentioned (see section 5.1), LPS has the ability to simulate inflammatory conditions on BV-2 cells and can trigger an inflammatory response. After stimulation with LPS microglia initiates an inflammatory response which triggers the release of pro-inflammatory mediators, such as ROS, NO, IL-1BETA, IL-6 among others (See section

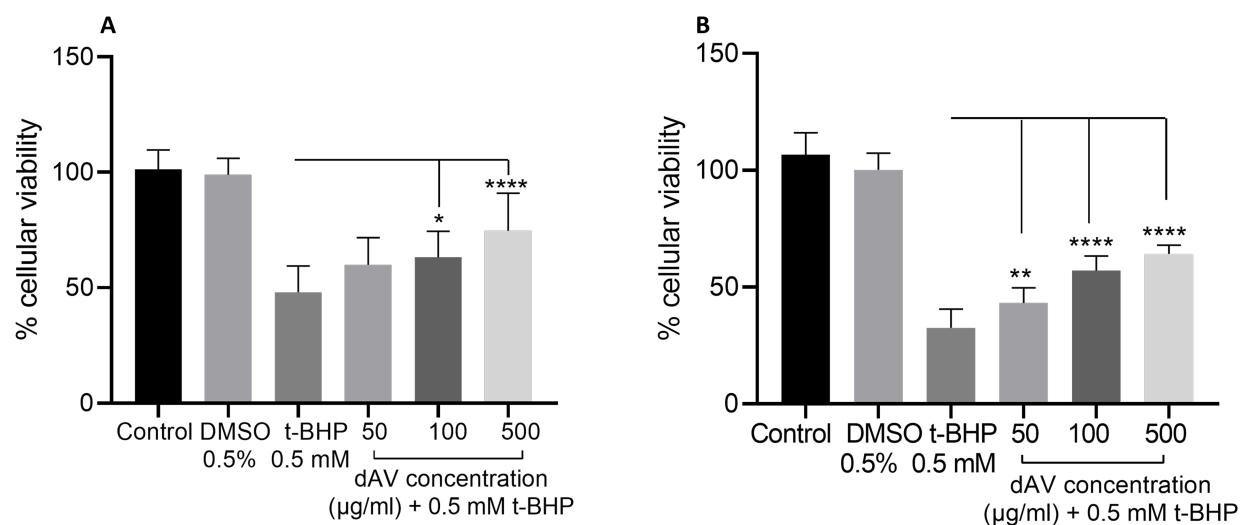


Figure 38: Cytoprotective potential against tert-butyl hydroperoxide (t-BHP) 0.5 mM. BV-2 cells were co-incubated with 4 hours with both dAV (50 100, 500 $\mu\text{g dwr/ml}$) and t-BHP (**A**) or pre-incubated with the extracts (50 100, 500 $\mu\text{g dwr/ml}$) for 20 h and co-incubated with t-BHP for 4 h (**B**). dAV-digested *Apocynum venetum* methanolic leaves extract; Cellular viability was assessed by the MTT assay. Each bar represents the mean $\pm$ S.D. of the results obtained in three independent experiments. Asterisks mean significant differences from the cells only incubated with t-BHP 0.5 mM for 4 h, as indicated: ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.

Table 5: Percentage of protection against tert-butyl hydroperoxide (t-BHP) effects after the pre-incubation of BV-2 cells with both AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract (50 100, 500 $\mu\text{g dwr/ml}$) for 4h or 20 hours. The results are represent as mean $\pm$ S.D. of the results obtained in three independent experiments.

BV2	Extract	AV			dAV		
	Concentration ($\mu\text{g dwr}$)	50	100	500	50	100	500
% of Protection 4h		16.2 $\pm$ 7.2	17.5 $\pm$ 6.4	24.1 $\pm$ 7.8	21.4 $\pm$ 6.5	23.3 $\pm$ 7.9	32.2 $\pm$ 7.6
% of Protectio 24h		22.8 $\pm$ 7.5	30.4 $\pm$ 8.1	35.2 $\pm$ 8.4	11.5 $\pm$ 6.4	23.3 $\pm$ 4.1	35.2 $\pm$ 6.3

1.4) [245, 260]. The amount of NO produced by LPS-induced BV-2 cells can be determined by using a method based on Griess reaction [262], which measures the amount of nitrite present in the culture supernatant a metabolite of NO [249, 263].

The anti-inflammatory ability of the extracts under study was evaluated by its ability to inhibit the NO production induced by LPS. For that, the NO levels in the supernatant were

measured by Griess reaction after the incubation of BV-2 cells with extracts and LPS (20h). The treatment with LPS caused a significant increase in NO levels when comparing to the cells in DMEM (see fig:39). Remarkably, the tested concentration of both AV and dAV (75 and 125 µg dwr/ml) significantly inhibited NO production induced by LPS in comparison with BV-2 cells only incubated with LPS (2 µg/ml final concentration) (p-value <0.0001). In general, the digestion process caused a significant reduction of anti-inflammatory activity of the extract revealing a p-value of 0.0175 and the p-value of 0.0133 for 75 µg dwr/ml and 125µg dwr/ml, respectively according to the ANOVA analysis.

The decrease of the ability of the extract to scavenge NO after the performed digestion was reported in section 4.3. So, the decrease of anti-inflammatory properties of dAV revealed in these results may be related to the decrease in the ability to scavenge NO. Nonetheless, the inhibition of NO can also be related to the ability of the extract to intervene in inflammatory pathways, which will be discussed in the following section.

Our results are in line with the ones described by Magalhaes et al., [187], that also reported a decrease of anti-inflammatory activities of *Lycium ruthenicum* and *Lycium barbarum* after performing *in vitro* digestion.

5.3.5 QUANTIFICATION OF GENE EXPRESSION OF PRO-INFLAMMATORY GENES (RT-PCR)

RT-PCR is an efficient method capable of carrying out a sensitive and reproducible quantification of Messenger ribonucleic acid (mRNA) levels. First, the RNA is transverse-transcribed into cDNA by RNA transcriptase, which is amplified in the next phase. A fluorescent probe is embedded in the new formed PCR product, and through the measurement of fluorescence emitted by those probes the levels of mRNA transcription is achieved. The SYBR green molecule, which possesses a high affinity for double-strand DNA was used to estimate the levels of gene expression. For this method is crucial to use a reference gene to enable the normalization of gene expression, due to the differences in the

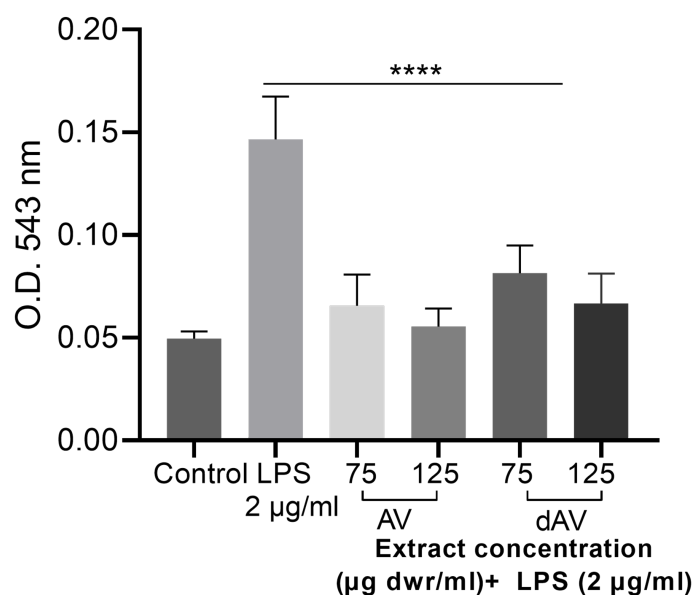


Figure 39: Nitric oxide (NO) measurement in lipopolysaccharides (LPS)-induced BV-2 cells supernatant. BV-2 cells in 12-well plates were pre-incubated (30 min.) with extracts (75 and 125 µg dwr/ml), and co-incubated with LPS (2 µg/ml) for 20h. Supernatants were collected for the measurement of NO by Griess reaction (Absorbance at 543 nm). dAV-digested and AV-non-digested *Apocynum venetum* methanolic leaves extract. Each bar represents the means $\pm$ SD of the results obtained in three independent ones experiments. Asterisks mean significant differences from the positive control (cells incubated exclusively with LPS 2 µg/ml final concentration); *p-value < 0.01 *** p-value < 0.001; **** p-value < 0.0001.

amount and quality of each sample and the ones occurring in the previous phases (BV-2 recovery, RNA preparation and cDNA synthesis) [264–266].

The expression levels of genes encoding for IL-6, IL-1BETA, COX-2 and iNOS were evaluated after the pre-incubation of BV-2 cells with AV and dAV (125 µg dwr/ml 30 min.) and co-incubation with LPS (2 µg /ml final concentration for 20 hours) through RT-PCR and are present in figure 40. As expected the treatment with LPS significantly increased the expression of IL-1BETA (figure 40A), IL-6 (figure 40B), COX-2 (figure 40C) and iNOS (figure 40D) genes, in comparison with control cells (cells not incubated with LPS). The pre-incubation with the AV resulted in a significant decrease of all genes expression in comparison to the negative control (cells in DMEM). This significant reduction was to a less extent for the iNOS gene (figure 40D) revealing a p-value of 0.0034. For the IL-6 the AV showed a significant decrease with a p-value < 0.0001, for the IL-1BETA a p-value = 0.0002 was found

and for the COX-2 a p-value= 0.0034. Notably, for these two genes, the reduction of the gene expression levels led to similar values to the control, proving the great anti-inflammatory activities of AV. Previous work have already unveil the ability of *Apocynum venetum* or some compounds found in this plant to decrease the mRNA levels of genes related to inflammatory pathways in mice [8], in rats [116] and in LPS-stimulated RAW264.7 cells [267]. However, work regarding BV-2 cell line was not yet published.

However, the pre-incubation with dAV, did not show a significant decrease for any of the genes under study (figure 40). Revealing that the anti-inflammatory activity of *Apocynum venetum* may be more susceptible to the modifications occurring in the digestion process than its antioxidant activities. The decrease of the inflammatory activities after digestion, through the decrease of ability to counteract the expression of genes, involved in inflammatory pathways, have been also described in the literature [187, 268].

These results are in line with the results obtained for the quantification of NO produced by LPS-induced BV-2 cells. Where the dAV also showed less activity towards NO inhibition. Nonetheless, the decrease of mRNA levels of iNOS by AV also shows that the extract is capable of modulating the activity of enzymes. Proving that both scavenging activity and the modulation of iNOS expression may contribute to the decrease of NO reported here. Despite the decrease in NO scavenging activity and the ability to modulate iNOS after the digestion, the dAV was still capable to counteract the NO levels induced by LPS.

To sum up, the results indicate that AV can modulate the expression of the genes encoding pro-inflammatory mediators in LPS-stimulated BV-2 cells. Although, these effects were diminished after the digestion process. This first confirms the need for more studies on the effects of digestion on bioactivities of plants and second to search for ways to avoid its negative effects.

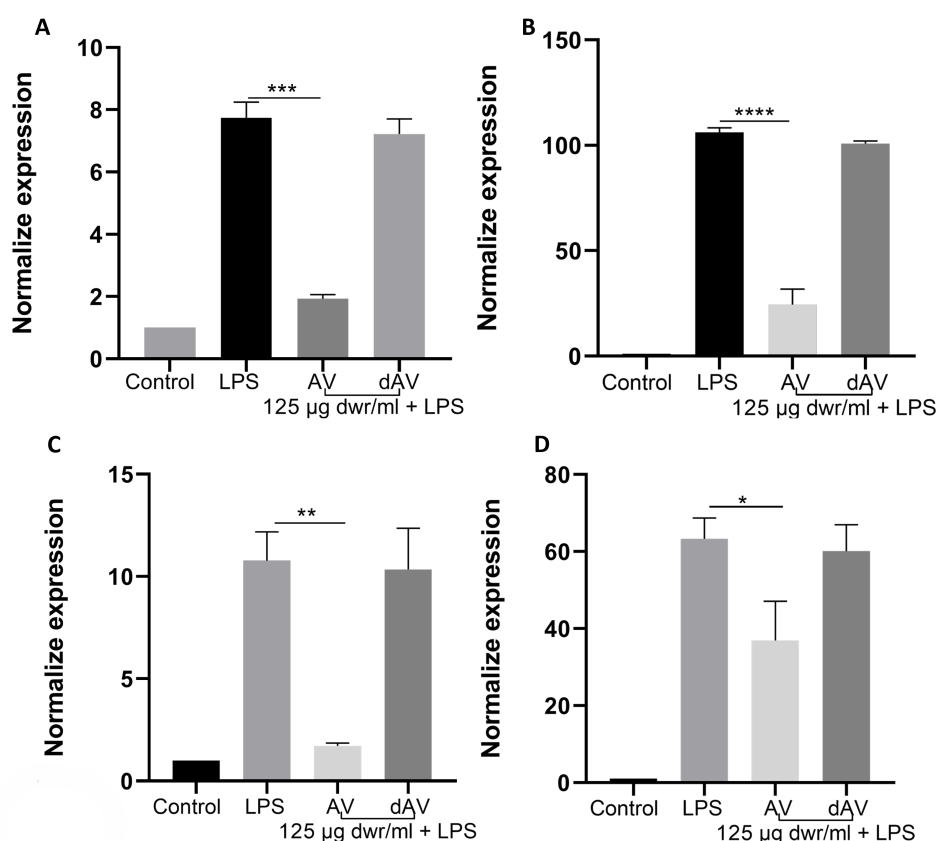


Figure 40: Real time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of genes in lipopolysaccharides (LPS)-induced BV-2 cells. BV-2 cells in 12-well plates were pre-incubated (30 min.) with extracts (125 µg dwr/ml), and co-incubated with LPS (2 µg/ml) for 20h. The cells were collected for RNA extraction and RT-PCR was performed. Gene expression of: **(A)** interleukin-1BETA (IL-1BETA); **(B)** interleukin 6 (IL-6); **(C)** cyclooxygenase-2 (COX-2); **(D)** inducible nitric oxide synthase (iNOS); were calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as an internal control. Cells incubated with medium and cells incubated with LPS were used as a negative and positive control, respectively. AV-non-digested and dAV-digested of *Apocynum venetum* methanolic leaves extract. Experiments were analyzed with the software Bio-Rad CFX Manager. Statistical differences are presented vs. positive control (cells with LPS): *p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001.

5.4 Conclusion

Following the premise that the blockage of over-activation of microglia may be a key step to intervene on NDDs and consequently cause a delay in the onset of disease symptoms, the *Apocynum venetum* was studied in this work. Both antioxidant and inflammatory capacities of

AV and dAV were evaluated to also understand the effects of digestion on *Apocynum venetum* properties.

Apocynum venetum cytotoxic and cytoprotective effects were screened in both HepG2 and BV-2. The results proved that both extracts did not induce toxicity and even protected cells from damages induced by t-BHP. The protection was more prominent in HepG2 cells and the analysis of cellular ROS levels in HepG2 corroborated the activity of the extracts to eliminate ROS-induced by t-BHP. Furthermore, studies regarding antioxidant enzymes should be done to understand if the extracts can modulate the antioxidant activity. This is a hypothesis to justify the increment of the cytoprotective effects over time observed here, which is supported by results in the literature describing the ability of *A. venetum* to modulate antioxidant enzymes' activity [8, 124, 132].

When comparing the results of the AV and dAV, it is possible to conclude that the performed *in vitro* digestion did not impair the protective activities against t-BHP-induced damages observed in both cell lines. These results validate the use of *Apocynum venetum* as an antioxidant beverage and further illuminates the antioxidant properties of *Apocynum venetum* on HepG2 cells and for the first time on Bv-2 cells.

Regarding the anti-inflammatory effects, *Apocynum venetum* methanolic leaves extracts proved to be able to reduce NO levels induced by LPS and to modulate the levels of gene expression related to inflammatory status such as IL-1BETA, IL-6, COX-2 and iNOS through RT-PCR analysis. The AV was able to significantly reduce the gene expression of all evaluated genes. However, the digested extract in the tested concentration was not able to significantly reduce the mRNA levels of genes related to inflammation. Despite this, the digested extract still proved to be able to reduce NO levels, proving that some anti-inflammatory effects remained after the performed digestion. Perhaps because a dose-response was found in the quantification of NO, if a higher concentration of the extract was chosen, a more effective activity on the modulation of gene expression could be found for the dAV. Nonetheless, the results presented in this work demonstrated the potential of both AV and dAV to revert at some levels to neuroinflammatory/oxidative stress situations. Further research aiming to enlighten how the

extracts are able to modulate gene expression should be done, to understand the involved inflammatory pathways.

CHAPTER 6

Final remarks

Currently, a renewal of interest in traditional medicines has been occurring. This led to a growth of interest by the scientific community in phytopharmaceuticals resulting in several efforts toward the understanding of natural products' composition and properties. In this work we evaluate the ability of *A. venetum* towards the reduction of neuroinflammation/oxidative stress. Following the hypothesis that attenuating these inflammatory/oxidative processes, mainly in microglia may bring positive outcomes for NDDs. We further study the effects of digestion on those activities through the performance of a *in vitro* digestion. The cytotoxicity and cytoprotective effects of *Apocynum venetum* were evaluated on HepG2 since it is a commonly used cell line to evaluate toxicities.

The first step of this work regarded the biochemical analysis of *Apocynum venetum* antioxidant/ inflammatory activities. In these analyzes both extracts, the non-digested and digested extract revealed antioxidant activities in almost all performed assays except for the dAV in ICA. AV antioxidant activity was significantly higher than the control in SO,NO scavenging activities and COX-2 inhibitory activity. Here, all reported activities were decreased after the *in vitro* digestion. Nevertheless, antioxidant activities remained as well as the ability to inhibit COX-2 activity. This is a relevant result due to the interest in finding new COX-2 inhibitors. Nonetheless, this is only a preliminary result, other analyzes such as polarographic, or prostaglandin detection should be done.

In the fourth chapter, both AV and dAV were capable of protecting against t-BHP oxidative stress-induced toxicity in both HepG2 and BV-2. These cytoprotective effects can be related to the decrease of ROS levels. Since a reduction of intracellular ROS, up to basal levels was found in HepG2 after pre-incubation with the extracts and measurement of redox state by DCFH-DA. Moreover, except for the higher concentration of AV in the BV-2 cell that revealed a non-significant decrease of % cell viability in both incubation times, no toxicities were found here. Furthermore, this decrease in viability was abolished after the *in vitro* digestion, which is a good indicator for the use of *Apocynum venetum* as a beverage. Results revealed that the digestion processes had no effects on the protection properties against t-BHP induced damages proving that the extract still maintained the ability to protect the cells. Changes in the activity to decrease intracellular levels of ROS-induced by t-BHP were also not found after the performance of *in vitro* digestion. To elucidate more

about the cytoprotective effects of the extracts under study, studies regarding antioxidant enzymes should also be performed.

In this study, the anti-inflammatory properties of the extract were the most impaired after performing the *in vitro* digestion. Both AV and dAV were able to significantly reduce NO levels induced by LPS in comparison with positive control (cells with LPS). However, the effects of dAV were lower than AV. Furthermore, the analysis of RT-PCR revealed that AV has a high capacity to modulate the gene expression levels of the genes under study (IL-6, IL-1BETA, COX-2 and iNOS). However, dAV was not able to significantly reduce the expression of any of those genes.

These results prove that *Apocynum venetum* may be able to blockage the overload of inflammation. Moreover, it is necessary to understand which pathways are involved in these activities through the evaluation of gene expression of transcription factors such as NF-KB and AP-1 as well as evaluate protein levels by western blot to screen other possible effects of the extract. Also, the evaluation of the use of nanoparticles should be assessed as an option to avoid the decreased effects provoked by the digestion.

The presence of phenolic compounds in the composition of the extracts such as quercetin glycosides and caffeoylquinic acid derivatives described in the literature as good antioxidant sources may be responsible for the discussed properties. In this study, the digested extracts displayed a lower antioxidant for the biochemical analysis and anti-inflammatory activities than their counterparts, probably due to the decreased content of phenols and flavonoids, according to reports present in the literature. Nevertheless, a phytochemical analysis should be done to analyze the compounds present in the extract after the performance of the *in vitro* digestion. Further studies to analyze which compounds are responsible for the reported activities are also necessary. Nonetheless, the digested extracts still maintain effective levels of antioxidant and anti-inflammatory activities, which might be relevant and validates their human consumption and use.

To sum up the present study suggest that digestion might modify phytochemical composition of the AV leading to a reduction in anti-inflammatory activities and antioxidant activities in the biochemistry analysis, however the protective properties against t-BHP were

not impaired. These results encourage the study of plants as a natural source for the treatment of pathological conditions and further enlightens on the properties of *Apocynum venetum*.

CHAPTER 7

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