Universidade do Minho
Escola de Ciências

Inês Martins Laranjeira

Medicinal Plants and their relevance in Oxidative and Inflammatory processes

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Inês Martins Laranjeira

Medicinal Plants and their relevance in Oxidative and Inflammatory processes

Doctoral thesis
PhD Program in Agricultural Production Chains
– From Fork to Farm (Agrichains)

Work supervised by
Professor Doctor Alberto Carlos Pires Dias
and
Professor Doctor Filipa Santos Costa Pinto-Ribeiro

april of 2024

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Plantas medicinais e a sua relevância nos processos oxidativos e inflamatórios

Resumo

As plantas aromáticas e medicinais têm sido uma parte integrante da civilização humana desde a antiguidade, como terapias para várias doenças e contribuindo para o desenvolvimento da medicina tradicional em todo o mundo. Estas plantas contêm uma diversidade de compostos bioativos com propriedades terapêuticas, tornando-as recursos valiosos para as indústrias da saúde e farmacêutica. Com o crescente interesse em abordagens naturais e holísticas, o estudo e a utilização de plantas aromáticas e medicinais continuam a atrair a atenção de investigadores, profissionais de saúde e consumidores.

Este trabalho teve como objetivo a valorização das plantas medicinais e aromáticas, com um foco específico nas *Coreopsis tinctoria*, *Baccharis dracunculifolia* e *Genista tridentata*. Ao aumentar a consciencialização sobre o seu potencial como fontes de compostos bioativos com valor medicinal e económico significativo, procurámos identificar extratos que exibam atividades bioativas empregáveis nas indústrias farmacêutica, alimentar e cosmética.

Demonstramos que os extratos das plantas estudadas (*C. tinctoria, B. dracunculifolia* e *G. tridentata*) possuem um elevado teor de compostos fenólicos, os quais contribuem para as suas fortes atividades antioxidantes no sequestro de radicais livres, antinociceptivas em modelos experimentais de osteoartrose e diabetes tipo 2, assim como atividades anti-inflamatórias em modelos de irritação dérmica e de feridas em animais diabéticos. Assim, estes resultados apoiam a utilização destas plantas em formulações farmacêuticas ou como ingredientes alimentares funcionais. A interação sinérgica entre plantas medicinais e terapias convencionais revela-se promissor para melhorar a eficácia de tratamentos pré-existentes e reduzir efeitos colaterais.

Palavras-chave: Plantas medicinais, inflamação, *Diabetes mellitus, Coreopsis tinctoria, Baccharis dracunculifolia, Genista tridentata*

Medicinal Plants and their relevance in oxidative and inflammatory processes

Abstract

Aromatic and medicinal plants have been an integral part of human civilization since ancient times, providing therapies for various diseases and contributing to the development of traditional medicine worldwide. These plants contain a diverse array of bioactive compounds with therapeutic properties, making them valuable resources for the healthcare and pharmaceutical industries. With the growing interest in natural and holistic approaches, the study and use of aromatic and medicinal plants continues to attract the attention of researchers, healthcare professionals and consumers.

This work aimed to highlight the valorization of medicinal and aromatic plants, with a specific focus on *Coreopsis tinctor*ia, *Baccharis dracunculifolia* and *Genista tridentata*. By raising awareness of their potential as sources of bioactive compounds with significant medicinal and economic value, we sought to identify extracts that exhibit bioactive activities applicable across the pharmaceutical, food and cosmetic industries.

Our findings demonstrate the extracts of the studied plants (*C. tinctoria, B. dracunculifolia* and *G. tridentata*) have a rich phenolic compounds content, which contribute to their strong antioxidant activities in free radical scavenging, analgesic activities in experimental models of osteoarthritis and type 2 diabetes, as well as anti-inflammatory activities in models of dermal irritation and diabetic-induced wounds. Thus, these results support the use of these plants in pharmaceutical formulations or as functional food ingredients. The synergistic interaction between medicinal plants and conventional therapies shows promise for improving the efficacy of pre-existing treatments and reducing side effects.

Keywords: Medicinal plants, inflammation, *Diabetes mellitus, Coreopsis tinctoria, Baccharis dracunculifolia, Genista tridentata*

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List of Abbreviations and Acronyms

ESI-Q-TOF/MS: Electrospray Ionization Quadrupole Time-of-flight Tandem Mass Spectrometer
ETOH: ethanol
FBS: Fetal Bovine Serum
FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FeSO4: Ferrous Sulfate
FST: Forced Swimming Test
GABA: γ-Aminobutyric acid
GAE: Gallic Acid Equivalent
Gf: Grams of Force
H_2O_2: Hydrogen peroxide
HA: Hyaluronic Acid
HeLa: Human Cervical Adenocarcinoma Cell Line
HePG2: Human Hepatocarcinoma Cell Line
HMG-CoA: Methanesulfonic acid against 3-hydroxy-3-methylglutaryl-coenzyme A reductase
HNO2: Nitrous Acid
HPLC: High-Performance Liquid Chromatography
HPLC-DAD: High-Performance Liquid Chromatography with Diode Array Detection
HTAB: Hexadecyltrimethylammonium Bromide
i.d.: Isoflavone derivative
i.p.; Intraperitoneally
IBA-1: Ionized Calcium Binding Adaptor Molecule 1
ICA: Iron Chelation Activity
ICU: Intensive Care Unit
IDF: International Diabetes Federation
IL: Interleukin
IL-12: Interleukin-12
IL-1β: interleukin-1 beta
IL-33: Interleukin-33
IL-6: interleukin-6
ILC: Infralimbic Cortex
iNOS: Inducible Nitric Oxide Synthase
IZ: Inhibitory Zone
JAK-STAT: Janus kinase-signal transducer and activator of transcription
K/C: Kaolin/Carrageenan
LB: Luria Broth
LDL: Low-density Lipoprotein
LDLR: Low-density Lipoprotein Receptor
LPAG: Lateral Periaqueductal Grey Matter
LPS-NO: Lipopolysaccharide-nitric Oxid
LT: Leukotrienes
LTB4: Leukotriene B4
LWT: Limb Withdrawal Threshold
MAPK: Mitogen-Activated Protein Kinases
mAU: milli Absorbance Units
$^1O_2$ - Singlet oxygen
5-LO: Arachidonate 5-lipoxygenase
AA- Ascorbic Acid
AAE: Ascorbic Acid Equivalent
ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid
ACh: Acetylcholine
AGEs: Advanced Glycation End Products
AMY: Amygdala
ANOVA: Analysis of variance
ARTH: animals injected with kaolin/carrageenan in the right knee and treated with Phosphate Buffer Saline
ARTH_Bd100: animals injected with kaolin/carrageenan in the right knee and treated 100 mg/kg of *B. dracunculifolia* extract
ARTH_Bd50: animals injected with kaolin/carrageenan in the right knee and treated 50 mg/kg of *B. dracunculifolia* extract
Bd: *Baccharis dracunculifolia* extract
Caco-2: Human Colorectal Epithelial Adenocarcinoma Cell line
CAE: Caffeic Acid Equivalent
CC: Cingulate Cortex
CDC: Center for Disease Control and Prevention
CGC: *Caenorhabditis* Genetics Center
CK2: protein kinase kinase 2
ClAE: Chlorogenic Acid Equivalent
CNS: Central Nervous System
CO: Croton Oil
COVID-19: Coronavirus disease 2019
COX-1: Cyclooxygenase-1
COX-2: Cyclooxygenase- 2
CQAs: Caffeoylquinic Acids
DAMPs: Damage-associated molecular patterns
DEX – Dexamethasone
diCQAs: dicaffeoylquinic acids
DlPAG: Dorsolateral Periaqueductal Grey Matter
DM: *Diabetes mellitus*
DMM: Destabilization of the medial meniscus
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic Acid
DPPH: 2,2′-diphenyl-1-picrylhydrazyl radical
EDTA: Ethylenediaminetetraacetic Acid
EOs: Essential Oils
ESI: Electrospray Ionization

PKC: Protein Kinase C
PLA2: Phospholipase A2
PLC: Prelimbic Cortex
PLP2: Porcine Liver
POWO: Plants of the World Online
PPARy: Peroxisome proliferator-activated receptor-y
PPARα: Peroxisome proliferator-activated receptor-α
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PRRs: Pattern recognition receptors
PtL: *Pterospartum tridentatum* leaf extract
q.d.: Quercetin derivative.
QTOF–MS: Quadrupole Time-of-flight Mass Spectrometry
RAGE: Receptor for AGEs
RAS: Renin-angiotensin system
RCAAP: Portugal Open Access Science Repository
RNS: Reactive Nitrogen Species
ROS: Reactive Oxygen Species
RT: Retention Time
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEM: Standard Error of the Mean
SO: Superoxide
SPSS: Statistical Package for Social Science for Windows
SPT: Sucrose Preference Test
SREBP-2: Sterol regulatory element-binding protein 2
STZ: Streptozotocin
T: Trolox
T1DM: Type 1 Diabetes mellitus
T2DM: Type 2 Diabetes mellitus
TAC: Total Antioxidant Capacity
TBARS: Thiobarbituric Acid Reactive Substances
TNF: Tumor necrosis factor
TPA: 12-o-tetracanoilphorbol-13-acetate
UFJF: Faculty of Pharmacy at the Federal University of Juiz de Fora
UPLC: Ultra-performance Liquid Chromatography
UPLC-ESI-QTOF-MS: Ultra-high Performance Liquid Chromatography Coupled with Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry
UPLC-MS: Ultra Performance Liquid Chromatography Mass Spectrometry
USFDA: United States Food and Drug Administration
UV: Ultraviolet
WHO: World Health Organization
WT – Wild Type
MCF-7: Breast Adenocarcinoma
Me: Medrol
MIA: Monoiodoacetate
MIC: Minimal Inhibitory Concentration
monoCQAs: mono-caffeoylquinic acids
mPFC: Medial Prefrontal Cortex
MPO: Myeloperoxidase
MTD: Maximum Tolerance Dose
N2: Molecular Nitrogen
N2O3: dinitrogen Trioxide
NA: Nicotinamide
NaCl: Sodium Chloride
NaClO: Sodium Hypochlorite
NADH: Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NAG: N-acetyl-β-D-glucosaminidase
NaOH: Sodium Hydroxide
NBT: Nitro Blue Tetrazolium
NCBI: National Center for Biotechnology Information (USA)
NCI-H460: Non-small Cell Lung Cancer
ND: Not Detected
NF-κB: Nuclear Factor Kappa B
NGM: Nematode Growth Medium
NO: Nitric Oxide
NO_2: Nitrogen Dioxide
NPs: Natural Products
NR: Not Reported
NSAIDs: Non-steroidal Anti-inflammatory Drugs
O_2: Oxygen
O^{2-}: Superoxide
OA: Osteoarthritis
OD: Optical Density
OFT: Open Field Test
$OH^{\cdot}$: Hydroxyl Radical
OONO: Peroxynitrite
OP50: strain of *Escherichia coli* used for maintenance of *Caenorhabditis elegans* cultures.
P/O: Ratio of Adenosine Diphosphate Molecules Phosphorylated to Atoms of Oxygen Consumed
PAF: Platelet Activation Factor
PAG: periaqueductal Grey Matter
PAM: Pressure Application Measurement
Papp: Apparent Permeability Coefficient
PBS: Phosphate-buffered Saline
PCs: Phenolic Compounds
PDB: Protein Data Base
PFA: Paraformaldehyde
PFC: Prefrontal Cortex
PG: Prostaglandins

List of publications

The work performed during this PhD thesis resulted in the following publications:

SCI-indexed journals

- **Laranjeira, I. M.**, Apolinário, E., Amorim, D., Silva-Filho, A., Dias, A. C. P., & Pinto-Ribeiro, F. (2024). *Baccharis dracunculifolia* DC consumption improves nociceptive and depressive-like behaviour in rats with experimental osteoarthritis. *Foods, 13(4), 535.* https://doi.org/10.3390/foods13040535
- **Laranjeira, I. M**., Dias, A. C., & Pinto-Ribeiro, F. (2023). *Genista tridentata* phytochemical characterization and biological activities: A systematic review. *Biology*, *12 (11)*, 1387. https://doi.org/10.3390/biology12111387
- **Laranjeira, I. M**., Gonçalves, J. N., Gonçalves, C., Silva, M., Mouta, N., Dias, A. C., & Pinto-Ribeiro, F. (2023). Anti-Inflammatory effect of *Pterospartum tridentatum* leaf extract in acute and chronic inflammation. *Applied Sciences*, *13*, 4494. https://doi.org/10.3390/app13074494

Manuscripts in preparation

- **Laranjeira, I. M**., Vilasboas-Campos, D., Pinto-Ribeiro, F., Dias, A. C. P., Teixeira-Castro, A., & Maciel, P. Exploring the antioxidant properties and toxicology of *Coreopsis tinctoria* Nutt.: A potential therapeutic agent for *Diabetes mellitus*.
- **Laranjeira, I. M.**, Apolinário, E., Amorim, D., da Silva Filho, A. A., Dias, A. C. P., & Pinto-Ribeiro, F. Exploring the phytotherapeutic potential of *Baccharis dracunculifolia* extract: Anti-Inflammatory effects in experimental acute inflammation.
- **Laranjeira, I. M.**, Vilasboas-Campos, D., Silva, M., Rodrigues-Fonseca, D., Gonçalves, C., Teixeira-Castro, A., Maciel, P., Dias, A.C.P., & Pinto-Ribeiro, F. The multi-faceted benefits of *Genista tridentata* extract: From alleviating pain in diabetic rats to extending *C. elegans* lifespan in glucotoxic conditions.
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Technical Publications

- **Laranjeira, I. M**.; Dias, A.C.P.; Pinto-Ribeiro, F. L. (2023). *Genista tridentata* phytochemical characterization. Encyclopedia. Available online: https://encyclopedia.pub/entry/51257
- **Laranjeira, I. M**., Vilasboas-Campos, D., Teixeira-Castro, A., Almeida, A., Maciel, P., Pinto-Ribeiro, F., Dias, A.C.P. (April 2021). *Pterospartum tridentatum:* phytochemical profile and antioxidant activity of methanolic extracts. *International Conference on Medicinal and Food Plant Research & 3rd Sino-CPLP Symposium on Natural Products and Biodiversity Resources, 9-10th April 2021, Hanzhong, China.* DOI: 10.3389/978-2-88966-548-8

International Oral Communications:

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- **Laranjeira, I. M.**, Vilasboas-Campos, D., Silva, M., Rodrigues-Fonseca, D., Gonçalves, C., Teixeira-Castro, A., Maciel, P., Dias, A.C.P., & Pinto-Ribeiro, F. Effect of *Pterospartum tridentatum* on experimental type II *Diabetes mellitus*. Sino-PT Joint Meeting for the Research of Medicinal Plants, 2021.
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- **Laranjeira, I. M.**, Apolinário, E., Amorim, D., Silva-Filho, A., Dias, A. C. P., & Pinto-Ribeiro, F. *Baccharis dracunculifolia* ameliorated nociceptive, emotional impairments, and decrease microglia activation in rats with osteoarthritis. PSE Meeting - Natural Products in Drug Discovery and Human Health, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal, 2019.

National Oral Communications

- **Laranjeira, I. M.**., Gonçalves, J. N., Gonçalves, C., Silva, M., Mouta, N., Dias, A. C. P., & Pinto-Ribeiro, F. The potential effect of *Pterospartum tridentatum* leaf extract in acute and chronic inflammation. I Simpósio PhDBio, Universidade do Minho, Escola de Ciências, Braga, Portugal, 2023.
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- **Laranjeira, I.,** Vilasboas-Campos, D., Gonçalves, C., Teixeira-Castro, A., Almeida, A., Maciel, P., Dias, A.C.P., Pinto-Ribeiro, F. (July 2021). Antioxidant and antinociceptive activity of *Pterospartum tridentatum* methanolic exctracts. Vanguards of Natural Product Research 2021 on virtual chair, America Society of Pharmacognosy.
- Gonçalves, J., Fonseca-Rodrigues**, D., Laranjeira, I.,** Almeida, A., Pinto-Ribeiro, F. (June 2021). Time-dependent characterization of sex-differences on the chronic constriction injury (CCI) model of neuropathic pain. IASP 2021 Virtual World Congress on Pain.
- **Laranjeira, I.M.,** Almeida, A., Pinto-Ribeiro, F., Dias, A.C.P. (February 2021). *Pterospartum tridentatum*: phytochemical profile and antioxidant activity of methanolic extracts. 1st Online International Congress on Natural products application: Health, Cosmetic and Food.

- **Laranjeira, I.M.,** Almeida, A., Pinto-Ribeiro, F., Dias, A.C.P. (November, 2020). Evaluation of phytochemical profile and antioxidant activity of *Pterospartum tridentatum.* International IBERPHENOL Conference "Advances in the role of phenols in health effects and other uses.
- Lima, A.C., Amorim, D., **Laranjeira, I.**, Almeida, A., Reis, R. L., Ferreira, H., Pinto-Ribeiro, F., Neves, N.M. (2019) A dual targeting strategy using antibodies immobilized at the surface of biodegradable nanoparticles for local osteoarthritis treatment. Paper presented in *Achilles 2019*, 2019.
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Chapter 1: Introduction

1. Introduction

1.1. Oxidative stress

Metabolic processes of living organisms and/or environmental stresses can produce reactive species, such as free radicals, especially reactive oxygen and nitrogen species (ROS/RNS) (He *et al.*, 2017). During metabolic processes, free radicals are continuously generated and act as mediators in the transfer of electrons in several biochemical reactions (Uttara *et al.*, 2009). ROS are partially reduced forms of atmospheric oxygen (O_2), and include $O_2^{\cdot}$ (superoxide radical), $OH^{\cdot}$ (hydroxyl radical), as well as non-radical forms: H_2O_2 (hydrogen peroxide) and $^1O_2$ (singlet oxygen) (Gupta and Igamberdiev, 2015). In addition to ROS, RNS such as nitric oxide (NO), peroxynitrite ($OONO^-$), nitrogen dioxide ($NO2^-$), dinitrogen trioxide (N_2O_3), and nitrous acid (HNO_2) also promote oxidative stress (Blanco *et al.*, 2017; He *et al.*, 2017). Furthermore, within the framework of the Fenton reaction, there is an amplification in the generation of highly reactive $OH^{\cdot}$, worsening oxidative stress in biological systems (Dong *et al.*, 2022).

ROS are produced as cellular metabolic bioproducts and are involved in the synthesis of essential biological metabolites, in energy production, phagocytosis, cell growth and in cell signaling (Thannickal and Fanburg, 2000; Gülcin, 2012). However, the excess of ROS has a harmful impact, such as DNA damage, alteration the structure of proteins and cellular organelles, and, consequently, impairing their function (Newsholme *et al.*, 2016). To protect against the impacts of ROS, cells possess enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which act as antioxidants, resulting in an adequate balance between antioxidants and pro-oxidants in normal conditions (Thannickal and Fanburg, 2000; Gülcin, 2012). However, when either the production of ROS/RNS is increased considerably or the levels of antioxidants are decreased, the resulting imbalance is termed oxidative stress (Thannickal and Fanburg, 2000; Gülcin, 2012; Sena and Chandel, 2012).

Oxidative stress is often associated with several pathologies, including cancer, early aging, cardiovascular diseases, diabetes, degenerative and neurological disorders, among others (Gülcin, 2012; Sena and Chandel, 2012). In addition to natural antioxidants produced by the body, alternative sources such as food and beverages offer substances capable of sequestering free radicals (Halliwell *et al.*, 1995; Abuajah *et al.*, 2015; Shahidi *et al.*, 2015).

1.2. Natural products

The use of natural products (NPs) with therapeutic properties is as old as human civilization and for a long-time minerals, plants, marine organisms, and animal products were the principal sources of medicines (Zanin *et al.,* 2012).

During the 18th century, the development of chemistry and the advances in technology led to the synthesis of new active compounds and the purification of plant extracts used as medicines (Schmidt *et al.,* 2008; Krishnamurti and Rao, 2016). Plant extracts comprised a series of pharmacologically active components, but in undefined quantities and variable proportions (Krishnamurti and Rao, 2016). It was between 1803 and 1817 that the German pharmacist Friedrich Wilhelm Adam Seturner (1783-1841) successfully isolated and extracted morphine crystals in their pure form from *Papaver somniferum* L. seeds, promoting a new branch of science (Krishnamurti and Rao, 2016; Devereaux *et al.,* 2018). Regardless of the limited knowledge of its structure and mechanism of action, pure morphine became a common pain treatment. Nowadays, morphine is considered the "gold-standard" treatment for severe pain in conventional medicine (Devereaux *et al.,* 2018).

In the 19th century, the Industrial Revolution led to the development of organic chemistry and the preference for synthetic products increased because of their advantages. Synthetic pure compounds were easier to acquire and to change structurally to produce potentially more active drugs (Rates, 2001). Additionally, as society has progressed the use of natural products has had religious and/or exoteric implications, with distinct beliefs on the concepts of health and disease within cultures. Therefore, the use of NPs was opposed to the new lifestyle of industrialized western societies, where the application of NPs as medicines was considered an option for people with low profits and education or as religious superstition, and consequently, of no pharmacological importance (Rates, 2001, Andrade *et al.,* 2007).

in fact, the financial support for research in drug discovery based on NPs decreased considerably from 1984 to 2003 (Schmidt *et al.,* 2008). However, since the turn of century the interest in alternative therapies and the pharmacological use of NPs has grown, specifically in plant-derived products (Figure 1.2.1) (Salmerón-Manzano *et al.*, 2020). This interest in medicines of natural origin is due to the abusive and/or incorrect use of certain drugs in conventional medicine leading to the absence of results and/or adverse side effects. Moreover, the high percentage of the world population without access to conventional medicine and pharmacological treatments remains a problem (Rates, 2001).

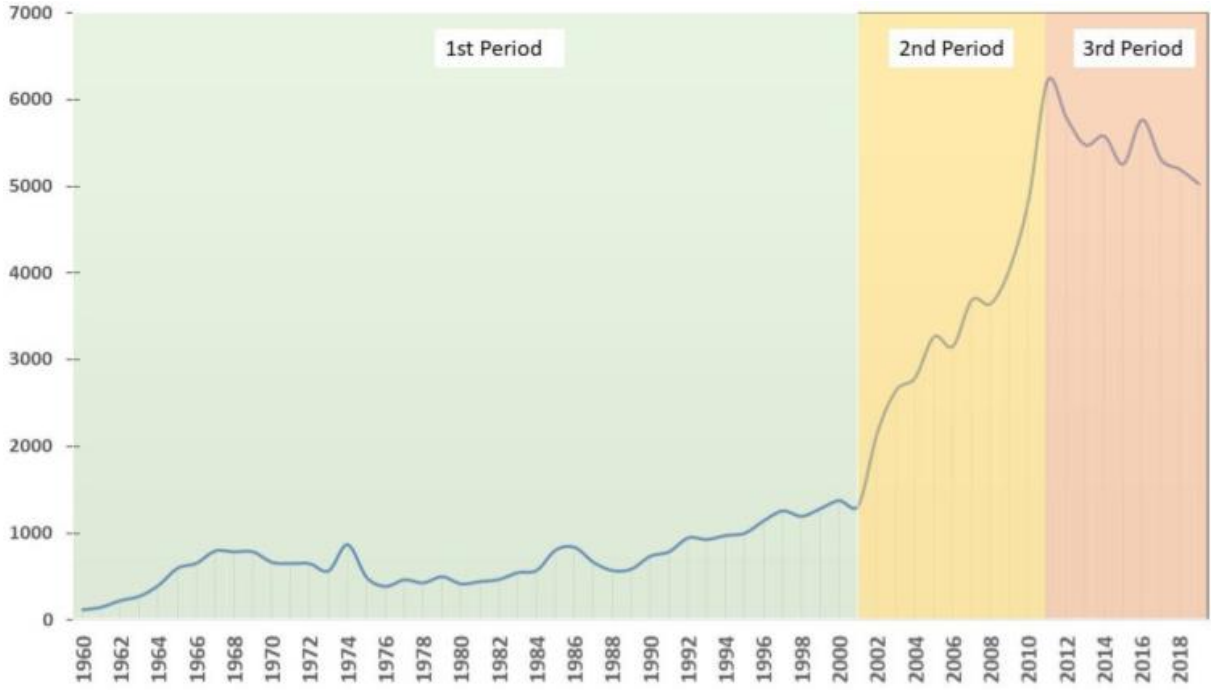


Figure 3.2.1. - Mapping the Growth of Medical Plant Research Worldwide (Salmerón-Manzano et al., 2020)

Even though there is low financial support for research in NPs drug discovery, these remain an important supply for new pharmaceutical drugs (Schmidt *et al.,* 2008). A comprehensive review of NPs drug discovery indicates that in the global medicine market, around 35 percent of medicines are derived directly or indirectly from NPs, including plant- (25%), microorganisms- (13%), and animal- (about 3%) based products (Calixto, 2019). Moreover, Newman and Cragg (2016) reported that concerning the period between 1981 and 2014 of the 1562 drugs recognized by the USFDA, 320 (21%) were resultant of natural-based products, 141 (9.1%) were botanical mixtures, 64 (4%) were pure NPs, and 61 (4%) were synthetic drugs with pharmacophores from NPs. Importantly, NPs continue to play a vital role in cancer therapy. Of the 175 anticarcinogenic drugs approved from 1940 to 2014, only 25% could not be traced to NPs, with 49% being a pure NP or a direct derivation therefrom (Newman and Cragg, 2016).

Furthermore, the reasons for studying NPs, particularly medicinal plants, include obtaining knowledge concerning the pharmaceutical potential effect of a vast diversity of native plants, creating a basis for the medicinal use of plant-based products, developing herbal medicines with low cost and relevant activity, to find new drug prototypes, to uncover novel structures and mechanisms of action, and to gain scientific evidence about traditional medicines (Zanin *et al.,* 2012; Najmi *et al.,* 2022). Thus, plant-based NPs can be considered a valuable resource for the screening of bioactive compounds, which can be a successful approach to the discovery of new therapeutical approaches.

1.2.1. Bioactive compounds

In scientific jargon, the term "bioactive" is an alternative term for "biologically active" (Cammack *et al.,* 2006). A bioactive compound is a substance with biological activity that positively or negatively affects, initiates a reaction, or triggers a response at the living tissue (Guaadaoui *et al.,* 2014). The effect is dependent on the substance, the dose, or the bioavailability (Guaadaoui *et al.,* 2014).

The term "food bioactive component" implies non-essential compounds present in foods that can modulate metabolic processes and promote better health (Guaadaoui *et al.,* 2014). So, food bioactive components are biologically active ingredients present in foods or dietary complements, other than those required to achieve basic nutritional needs, that impact the health status (Studdert *et al.,* 2020).

Plant bioactive compounds include chemicals, found in small quantities in plants and in foods (such as fruits, vegetables, oils, and whole grains) that act in the organism to promote health effects (More *et al.,* 2022). These compounds are produced as secondary metabolites and play a vital role in competition, defence, and signalling (More *et al.,* 2022). Bioactive compounds can also be found in other living organisms, such as marine organisms (Suarez-Jimeneze *et al.,* 2012**;** Jesumani *et al.,* 2019), bacteria (Chaouachi *et al.,* 2021), and mushrooms (Venturella *et al.,* 2021). Bioactive compounds are divided into several classes comprising terpenoids, alkaloids, nitrogen-containing compounds, organosulfur compounds, and phenolic compounds (Liu *et al.,* 2014; Altemimi *et al.,* 2017).

1.2.1.1. Phenolic compounds

In recent years, phenolic compounds (PCs) have been of growing interest to science and the food industry for their beneficial health effects. PCs comprise one of the major groups of metabolites produced in plants and several beneficial properties related to a high intake of phenolic-rich foods have been recognized in these compounds, which include a decreased rate of chronic diseases such as cardiovascular diseases, diabetes, Alzheimer's and Parkinson's disease, and inflammation (Shukla and Gupta, 2010; Mohamed, 2014). PCs result from two main biosynthetic pathways: via the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or via the "polyketide" acetate/malonate pathway, which can generate simple phenols or both, thus producing monomeric and polymeric phenols and polyphenols (Lattanzio, 2013; Santos-Sánchez *et al.,* 2019).

PCs are recognized to display around 8000 different skeleton structures (Cosme *et al.*, 2020). Nevertheless, they are characterized by the presence of a minimum of one phenol group (Cosme *et al.,* 2020). Based on their structure, the PCs are categorized into several classes: simple phenolics, tannins, coumarins, flavonoids, chromones and xanthones, stilbenes, and lignans (Lattanzio, 2013; Cosme *et al.,*

2020). Structurally, simple phenolics exhibit a single C6 phenyl ring with one or more hydroxyl, aldehydic, or carboxylic groups. Tannins are composed of phenyl rings with two or three hydroxyl groups. Coumarins are byproducts of benzo-α-pyrones (lactones), composed of C6-C3 carbon skeleton and occur in a free or condensed form with sugars as glycosides (Cosme *et al.,* 2020; Merecz-Sadowska *et al.,* 2021). Flavonoids, the most numerous subgroup, comprise more than 6000 distinct compounds (Panche *et al.*, 2016). Their carbon skeleton has C6-C3-C6 organized as a closed pyran ring C as the central carbon chain and two benzene rings A and B, linked in the C ring in positions 2, 3, or 4 (Figure 1.2.2) (Panche *et al.,* 2016; Vuolo *et al.,* 2019). Isoflavones and neoflavanoids are another class of flavonoids in which the B ring is linked to the C ring in positions 3 and 4, respectively. If the B ring is linked in position 2 of the C ring the flavonoids can be subdivided into several categories, including flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins, and chalcones, depending on the structural features of the C ring (Panche *et al.,* 2016).

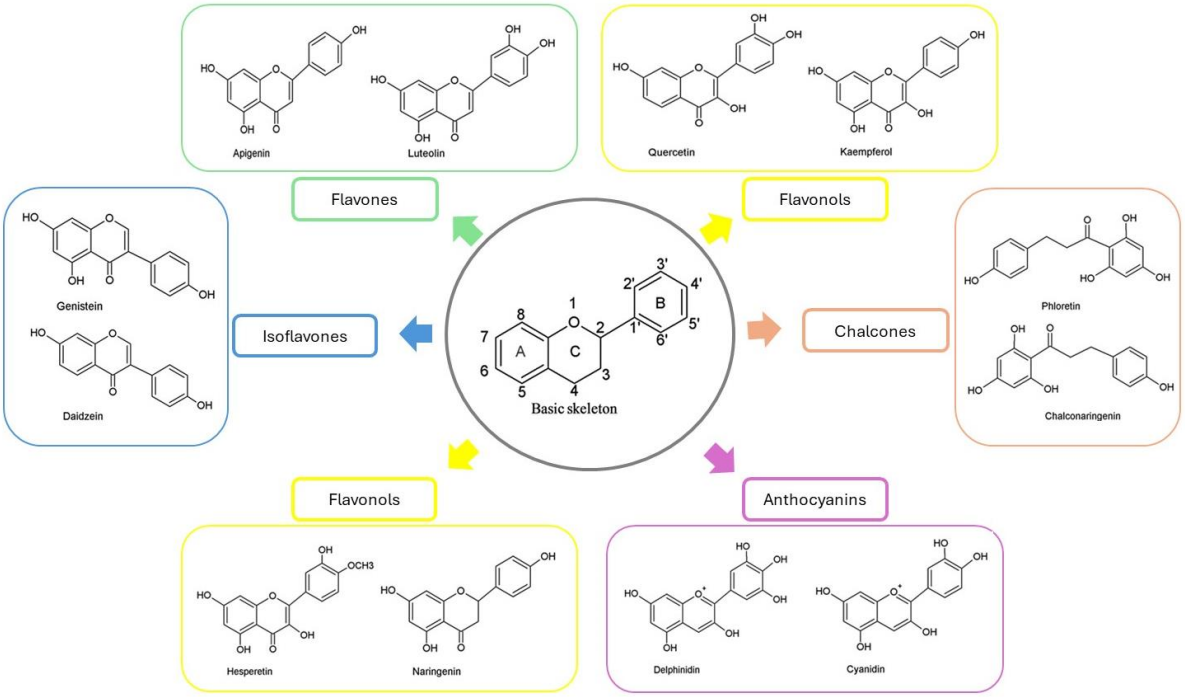


Figure 1.4.2. - Overview of the fundamental structural framework of flavonoids and their respective classes (Adapted from Panche et al., 2016).

Other common groups are the chromones and xanthones, derivatives of benzo-γ-pyrone. Chromones include a C6-C3 carbon skeleton with methyl or alkyl substituents at position 2 of the C ring and hydroxyl or alkoxyl substituents at positions 5 and 7, while xanthones including a C6-C1-C6 carbon skeleton with several substituents (Merecz-Sadowska *et al.,* 2021). As above mentioned, several scientific evidence

demonstrates the anti-inflammatory (Ambriz-Pérez *et al.,* 2016) and antioxidant (Nagarajan *et al.,* 2020) properties of phenolic compounds. Thus, it is necessary to find new sources of phenolic compounds with therapeutic properties (Shukla & Gupta, 2010). Medicinal plants used in folk medicine have been described to possess high levels of these compounds, consequently, there's a need for increasing knowledge on these plants to identify new bioactive compounds.

1.2.1.1.1. Phenolic compounds as antioxidants agents

The aromatic feature and highly conjugated system with multiple hydroxyl groups make PCs good electron or hydrogen atom donors, neutralizing free radicals and other reactive oxygen species (Zhang and Tsao, 2016).

The antioxidant activity of PCs is linked with its capability of neutralization of the reactive radical species, which occurs when the antioxidant transfers its electron and/or hydrogen atom to the radical species (Zhang and Tsao, 2016). PCs scavenging of free radicals can occur through different chemical pathways: Proton Coupled-Electron Transfer, consisting in a donation of a hydrogen atom to the radical species; Electron Transfer-Proton Transfer, involving the transference of an electron from the antioxidant to the radical (Zhang and Tsao, 2016); Sequential Proton Loss Electron Transfer, which comprises in the first stage the dissociation of antioxidant leading to the formation of aroxyl anion (ArO^-) and in the second stage the transference of the electron from anion to the radical (Olszowy, 2019; Vuolo *et al.,* 2019); and Adduct Formation, resulting in a formation of a new product (adduct) which also display antioxidant activity (Olszowy, 2019).

Nevertheless, the antioxidant potential of a specific phenolic compound depends on the number and the position of hydroxyl groups in the molecule (Soobrattee *et al.,* 2005; Zhang and Tsao, 2016), establishing a relation between structure and activity (Bendary *et al.,* 2013). Likewise, the degree of hydroxylation also influences antioxidant activity. The longer distance separating the carbonyl group and the aromatic ring of a phenolic compound appears to improve the antioxidant capability. Additionally, a greater number of hydroxyl aromatic rings, such as flavonoids, increases the antioxidant activity (Zhang and Tsao, 2016).

1.2.1.1.2. Phenolic compounds as anti-inflammatory agents

As already mentioned, PCs have also demonstrated anti-inflammatory properties. Even though the mechanisms of action of the PCs anti-inflammatory activity are not fully clarified, there is an association between the high PCs rich food-intake and the downregulation of the inflammatory response (Ambriz-Pérez *et al.,* 2016). The anti-inflammatory effects of PCs are multifaceted and involve a combination of

antioxidant, enzyme inhibitory, signaling modulation, immune-regulatory, and cytoprotective mechanisms (Sobhani *et al.*, 2021).

Previous studies demonstrated that PCs exert their anti-inflammatory through the inhibition of T lymphocyte production (Wong *et al.,* 2011; Shi *et al.,* 2011), the inhibition of pro-inflammatory cytokines (such as TNFα and interleukins) (Yang *et al.,* 2012; Wang *et al.,* 2013; Mateus *et al.,* 2022), or the control of enzymes derived from the arachidonic acid pathway (Rahman *et al.,* 2021). Moreover, recent findings suggest PCs modulate pro-inflammatory mediators by regulating eicosanoid synthesis (Rahman *et al.,* 2021), inhibiting stimulated resistant units (Xie *et al.,* 2011), and regulating NO synthase and COX-2 activity through inhibitory effects on NF-κB (Yang *et al.,* 2021; Mateus *et al.,* 2022).

As in the antioxidant activity, PCs anti-inflammatory mechanisms are interrelated to its chemical structure. The PCs structural requirements for the anti-inflammatory activity are a planar ring arrangement (Lago *et al.,* 2014; Wang *et al.,* 2021), the unsaturation in the C ring (Lattig *et al.,* 2007), the number and position of hydroxyl groups (for example, the catechol group at ring B), and the hydroxyl groups in B ring and at C5 and C7 (Kim *et al.,* 2004; Wang *et al.,* 2021). Additionally, flavones and flavanols with a hydroxyl group at position 4 of the B ring showed higher activity (Kim *et al.,* 2004; Lago *et al.,* 2014), as well as the methylation of the hydroxyl groups at positions 3, 4, or 5 (Lago *et al.,* 2014; Ambriz-Pérez *et al.,* 2016). Also, the methylation of the 3-hydroxyl group reduced the cytotoxicity (Ambriz-Pérez *et al.,* 2016). Furthermore, the role of glycosides is controversial since they reduce the anti-inflammatory activity (Kim *et al.,* 2004) although, they facilitate absorption of glycoside PC (Chuang *et al.,* 2017). Nevertheless, compounds without these structural characteristics can also exhibit anti-inflammatory activity by affecting the enzymes of the inflammatory cascade (Ambriz-Pérez *et al.,* 2016).

1.2.2. Medicinal plants

As mentioned, among NPs, plants have played a key role, with a wide range of medicinal properties, in the treatment of various diseases such as diabetes (Jacob and Narendhirakannan, 2019), inflammation (Bouyahya *et al.,* 2022), and cancer (Kuruppu *et a.,* 2019). It is estimated that approximately 70,000 plant species, including lichens and trees, have been used for medicinal purposes throughout history (Kuruppu *et al.,* 2019).

A recent review on research trends in medicinal plants (Salmerón-Manzano *et al.*, 2020) showed that from 1960 to 2019, over 110,000 studies on medicinal plants were published. Most of the studies were published in the categories of Pharmacology, Toxicology, and Pharmaceutics, making up 27.1% of the total. Other notable categories include Medicine (23.8%), Biochemistry, Genetics, and Molecular Biology

(16.7%), Agricultural and Biological Sciences (11%), and Chemistry (8.7%). Lower percentages of studies were published on Immunology and Microbiology (2.5%), Environmental Science (2.1%), and Chemical Engineering (1.5%). Categories with less than 1% include nursing, multidisciplinary sciences, and engineering (Salmerón-Manzano *et al.*, 2020).

A total of 159 countries published research on medicinal plants. With China and India contributing to most publications, likely due to the influence of traditional medicine, followed by America, especially the United States of America and Brazil (Salmerón-Manzano *et al.,* 2020).

In this research work, we focused on three medicinal plants, either native or cultivated in different continents (*Coreopsis tinctoria* from China, *Baccharis dracunculifolia* from Brazil, and *Pterospartum tridentatum* from Portugal).

The use of native/traditional plants can bring many benefits at the local, regional or country levels, including economic (Volenzo and Odiyo, 2020), environmental, and cultural heritage (Shelef *et al.,* 2017). In terms of creating economic value, these plants can be used for a variety of purposes, such as in the production of food, medicine (Volenzo and Odiyo, 2020), or in conservation projects, such as the restoration of degraded or disturbed ecosystems (McMahan, 2006), which can bring economic benefits through carbon sequestration and water conservation (Shelef *et al.,* 2017).

Promoting the use of native plants can also help to support local communities and economies, by providing jobs and income through the cultivation and harvest of native plants, and by helping to conserve traditional knowledge and practices related to the use of these plants (Volenzo and Odiyo, 2020). In terms of environmental value, native plants play an important role in maintaining the ecological balance of an area, by providing habitat and food for native wildlife, and by helping to prevent soil erosion and water loss (Shelef *et al.*, 2017). Finally, the use of native plants can also bring cultural value by preserving traditional knowledge and practices related to the use of these plants (Berhanemeskel, 2009; Kachmar *et al.*, 2021), and by promoting the conservation of cultural landscapes and heritage (Berkes and Davidson-Hunt, 2006). However, it is important to note that the sustainable and legal harvesting, cultivation, and trade of these plants are important to ensure the conservation of biodiversity and the rights of indigenous communities (Chen *et al.*, 2016b).

1.2.2.1. *Coreopsis tinctoria*

Coreopsis tinctoria Nutt. is an annual herbaceous plant that belongs to the Asteraceae family (Zhang *et al.*, 2016). It is also known as *garden tickseed*, *plain coreopsis*, *plains tickseed*, *golden coreopsis*, and *golden tickseed* (Tripathy *et al.,* 2021). This plant typically grows to be about 1-2 feet tall and 1-1.5 feet

wide. The flowers are yellow with a maroon dark centre disk, bloom in the summer, and can continue to bloom into the fall if deadheaded regularly. *C. tinctoria* is a hardy plant, tolerant of a wide range of soil conditions and able to withstand heat and drought (Tripathy *et al.,* 2021).

It is a native plant of North America, Canada, and Eastern Mexico (Tripathy *et al.,* 2021). However, it is also widely cultivated in China (Zhang *et al.,* 2016), particularly in southern Xinjiang, where it can be found growing in the Karakorum and Kunlun Mountains at elevations of up to 3,000 meters above sea level (Zhang *et al.,* 2016; Abdurehman *et al.,* 2022). Due to the lower pollution level and higher elevation, the Kunlun Mountains in Xinjiang provide a unique environment for *C. tinctoria*. The plant found in this area, due to its specific environmental conditions, displays unique biological activities (Yao *et al.*, 2016).

C. tinctoria has been traditionally used for medicinal purposes by several Native American tribes (Begmatov *et al.*, 2020; Shen *et al.*, 2021). The Navajo people in the Southwest U.S. used the whole herb for ceremonial chant lotion, cold infusion of dried plant taken with salt for "lightning infection" and as a fumigant for sexual infections. The Zuni Tribe in New Mexico and Arizona used the aerial parts of the plant for reproductive aid drugs and to influence the gender of their babies. The Cherokee, who lived in Eastern Tennessee and western North and South Carolina, used the root of the plant as an antidiarrheal and a "life medicine" (Shen *et al.*, 2021).

C. tinctoria, was introduced to China around 1900 (Chen *et al.*, 2017) and is widely cultivated as an ornamental plant in 34 provinces and cities (Wu *et al.*, 2010). According to Shen and colleagues (2021) it was first used as a medicinal plant in China in 1986 and is used for treating acute and chronic dysentery, red-eye swelling, and pain. *C. tinctoria*, known as Gulqai or Snow Chrysanthemum by the local Uygur people in Hotan region, Xinjiang, is consumed as a tea for high blood pressure, diarrhea (Yao *et al.*, 2019), decrease hypertension and hyperlipidemia (Li *et al.*, 2015), detoxifying, promoting blood circulation, and removing blood stasis, strengthening the stomach and spleen, as well as for treating heat and thirst, palpitation, gastrointestinal discomfort, loss of appetite, dysentery and sore swelling poison (Shen *et al.*, 2021). In Portugal, the flowering tops infusion of *C. tinctoria*, also known as "Estrelas do Egipto", has been used to treat hyperglycemia in diabetics by drinking two cups of this infusion a day (Dias *et al.*, 2010).

C. tintoria has been the subject of extensive research in recent decades for its pharmacological properties. Extracts and individual compounds from the plant have been shown to possess antidiabetic (Dias *et al.*, 2010; Begmatov *et al.*, 2020), cardioprotective (Zhang *et al.*, 2016), antioxidant (Chen *et al.*, 2016a; Begmatov *et al.*, 2020), and anti-inflammatory (Yao *et al.*, 2019; Begmatov *et al.*, 2020) properties, as

well as exhibit neuroprotective (Li *et al.,* 2015), antimicrobial (Guo *et al.,* 2017), and antitumor (Wufuer *et al.,* 2020) activity. Despite these findings, the activity of most of the plant's constituents remains uncertain (Shen *et al.*, 2021).

1.2.2.2. *Baccharis dracunculifolia*

The genus *Baccharis* comprises more than 500 species distributed mainly in the tropical areas of South America, predominantly in southeastern Brazil, Argentina, Paraguay, Uruguay, Colombia, Chile, Mexico, and Bolivia (Abad and Bermejo, 2007). Several plants from the genus *Baccharis* have been traditionally used by indigenous people, for the treatment and prevention of various diseases, particularly for their anti-inflammatory, analgesic, and anti-diabetic effects (Abad and Bermejo, 2007; Massignani *et al.,* 2009; Rodrigues *et al.,* 2009; Guimarães *et al.,* 2012).

The present research work focuses on the species *Baccharis dracunculifolia* DC (Asteraceae). *B. dracunculifolia* is plant species, native to Central and South America, popularly known as "Alecrim do campo" (Guimarães *et al.,* 2012). This medicinal plant is the major Brazilian green propolis botanical supply of resin and organic components (Guimarães *et al.,* 2012). In traditional medicine, it is used as a gastroprotective and anti-inflammatory (Guimarães *et al.,* 2012; Ramos Campos *et al.,* 2016).

Currently, the interest in studying the phytochemical profile and biological activity of Brazilian green propolis is increasing, as well as the studying of its major botanical source*, B. dracunculifolia* (Cestari *et al.,* 2011; Guimarães *et al.,* 2012). Even though the nature of Brazilian green propolis is more complex and unpredictable than *B. dracunculifolia*, its pharmacological activities can be related to several phytochemicals compounds known as constituents of *B. dracunculifolia* (Cestari *et al.,* 2011).

Several findings recognized *B. dracunculifolia* as a valuable source of antimicrobial (Filho *et al.,* 2008) and gastroprotective active compounds, with protective potential against gastric ulcers, in reducing gastric juice and increasing stomach pH (Lemos *et al.,* 2007; Massignani *et al.,* 2009). Moreover, *B. dracunculifolia* is also described by its hepatoprotective (Rezende *et al.,* 2014), antigenotoxic and antimutagenic capacity, when used in low doses (Andrade *et al.,* 2008; Rodrigues *et al.,* 2009), as well as antioxidative (Nakanishi *et al.,* 2003; Guimarães *et al.,* 2012). immunomodulator (Missima *et al.,* 2007), anti-stress (Missima and Sforcin, 2008), and anti-inflammatory potential (Paulino *et al.,* 2008; Cestari *et al.,* 2011).

1.2.2.3. *Genista tridentata*

Genista tridentata L. Willk (previously known as *Chamaespartum tridentatum* P. Gibbs. and *Pterospartum tridentatum* (L.) Willk), commonly known as "prickled broom", is a European endemic Leguminosae (Fabaceae) species belonging to the subfamily *Papilionoideae* (Coelho *et al., 2011*; Ferreira *et al., 2012*), autochthonous in the Iberian Peninsula and Morocco. This shrub can be found in the understory of *Arbutus unedo, Pinus,* and *Eucalyptus* forests, as well as in abandoned lands. It grows spontaneously up to 100 cm in acidic soils (Vitor *et al., 2004*), and presents yellow flowers with a typical odour in alternate branches and coriaceous winged stems (Novais *et al., 2004*). Traditionally it is harvested in the spring between March and June.

G. tridentata is an aromatic plant, important in Portuguese gastronomy, the leaves are conventionally used as a condiment/food spice for seasoning traditional dishes of rice and meat (Grosso *et al., 2007*). Moreover, fresh, or shade-dried flowers of *G. tridentata* are also used in folk medicine, in a form of infusions, decoctions, and tonics (Pinela *et al.,* 2011) as an anti-inflammatory (Gião *et al., 2007*; Bremner *et al., 2009*), diuretic and depurative of the liver (Grosso *et al.,* 2007; Coelho *et al., 2011*; Gonçalves *et al., 2013*; Aires *et al., 2016*). It is commonly used to ameliorate colds (Coelho *et al.,* 2011; Balanč *et al.,* 2016), in digestive disorders (Gião *et al.,* 2008; Coelho *et al.,* 2011; Serralheiro *et al.,* 2013; Balanč *et al.,* 2016), intestinal (Coelho *et al.,* 2007; Falé *et al.,* 2014) and urologic problems (Grosso *et al.,* 2007; Bremner *et al.,* 2009; Coelho *et al.,* 2011; Gonçalves *et al.,* 2013; Balanč *et al.,* 2016), and rheumatism (Grosso *et al.,* 2007; Coelho *et al.,* 2011; Gonçalves *et al.,* 2013). Additionally, it's is also used for respiratory disorders (Gião *et al.,* 2007; Coelho *et al.,* 2011; Luis *et al.,* 2011; Ferreira *et al.,* 2012; Balanč *et al.,* 2016), headaches (Coelho *et al.,* 2011), to lower blood pressure (Coelho *et al.,* 2011; Ferreira *et al.,* 2012; Balanč *et al.,* 2016), against hypercholesterolemia (Coelho *et al.,* 2011; Ferreira *et al.,* 2012; Serralheiro *et al.,* 2013; Falé *et al.,* 2014; Balanč *et al.,* 2016), and hyperglycemia (Grosso *et al.,* 2007; Coelho *et al.,* 2011; Luis *et al.,* 2011; Ferreira *et al.,* 2012; Gonçalves *et al.,* 2013; Aires *et al.,* 2016; Balanč *et al.,* 2016; Gonçalves *et al.,* 2018), and in weight loss programs (Coelho *et al.,* 2011).

1.3. Inflammation and pain

Inflammation is an immune system protective mechanism against infection or injury to repair tissue structure and its physiological function, restoring homeostasis (Freire and Van Dyke, 2013). Inflammation can be divided into acute and chronic inflammation. The acute inflammatory response occurs immediately after injury and results in the elimination of the infectious agents, followed by a repair phase, that allows the return to homeostasis (Freire and Van Dyke, 2013; Furman *et al.*, 2019). Non-resolving acute

inflammation could lead to an increased and persistent inflammatory response, becoming chronic inflammation (Furman *et al.*, 2019). Clinically, inflammation is characterized by five cardinal signs: redness, increased heat, swelling, pain, and loss of function (Ji *et al.*, 2016).

The inflammatory response is a coordinated process that involves activating signalling pathways to increase levels of inflammatory mediators in both resident tissue cells and inflammatory cells that are recruited from the bloodstream (Ji *et al.*, 2016). The specific mechanisms of this response depend on the type of stimulus and its location in the body, but all involve recognizing harmful stimuli through pattern receptors on cell surfaces (Gong *et al.*, 2020; Marchi *et al.,* 2022), activating inflammatory pathways (Furman *et al.*, 2019; Marchi *et al.,* 2022), releasing inflammatory markers (Furman *et al.*, 2019), and recruiting inflammatory cells (Freire and Van Dyke, 2013).

Inflammation is typically triggered by the activation of pattern recognition receptors (PRRs) found on both immune and non-immune cells (Seong and Matzinger, 2004; Gong *et al.*, 2020). These receptors can be activated by a variety of molecules, including those associated with viral and bacterial infections (microorganism-associated molecular patterns or pathogen-associated molecular patterns) as well as endogenous molecules known as damage-associated molecular patterns (DAMPs) (Furman *et al.*, 2019; Gong *et al.*, 2020), such as nucleic acids, small metabolites, and proteins (Vanpouille-Box *et al.,* 2019). Under normal conditions, DAMPs cannot activate PRRs because they cannot access the subcellular compartments where PRRs are located (Vanpouille-Box *et al.,* 2019). However, when cells are under stress or dying, the permeability of these compartments can change, allowing DAMPs to activate PRRs and start an inflammatory response (Kroemer *et al.*, 2022).

Inflammatory stimuli activate several intracellular signalling pathways that mediate the inflammatory response. These include the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell proliferation and differentiation (Zhang *et al.*, 2019), the nuclear factor kappa-B (NF-κB) pathway, which controls the expression of genes involved in inflammation and immunity (Furman *et al.*, 2019; Zhang *et al.*, 2019), and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which regulates the activity of various signalling molecules (Purohit *et al.,* 2023). The activation of these pathways leads to the production and release of inflammatory mediators such as cytokines, chemokines, and other inflammatory molecules including interleukins (IL)-1β, IL-6, IL-8, tumour necrosis factor (TNF)-α, reactive oxygen species (ROS), nitric oxide (NO), and prostaglandins (PGs) (Furman *et al.*, 2019). These inflammatory mediators contribute to the recruitment and activation of immune cells and promote tissue repair (Freire and Van Dyke, 2013).

The overproduction of inflammatory mediators in chronic inflammation has been linked to the development of chronic degenerative diseases such as arthritis (Buch *et al.*, 2021), atherosclerosis (Galkina and Ley, 2009; Moriya, 2019), asthma (Broide, 2008), Alzheimer's disease (Baulch *et al.*, 2020), and cancer (Ferrari *et al.*, 2019).

Current clinical anti-inflammatory therapies are nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. These drugs work by inhibiting the activity of enzymes that produce pro-inflammatory mediators, reducing inflammation (Tabas and Glass, 2013). However, pharmacological options available comprise considerable potential for gastric, cardiovascular, renal, and hepatic adverse effects, especially in cases of prolonged usage (Ambriz-Pérez *et al.*, 2016; Ghlichloo and Gerriets, 2019). Therefore, in recent years there has been a growing interest in alternative therapies with anti-inflammatory effects and proven safety.

Inflammation and pain are closely related and often occur together (Pinto-Ribeiro *et al.*, 2017). When the body experiences injury, infection, or tissue damage, it triggers an inflammatory response that is intended to help repair the damage and protect the body from further harm. However, this response can also cause pain (Ji *et al.*, 2014; Matsuda *et al.*, 2019). Pain is a result of the complex interplay between various biological systems in response to injury, pathogens, and irritants (Basbaum *et al.*, 2009).

During inflammation, various chemical mediators are released, such as prostaglandins, histamine, bradykinin, and substance P (Amaya *et al.*, 2013), which sensitize nerve endings and make them more responsive to pain signals (Ji *et al.*, 2014; Matsuda *et al.*, 2019). Prostaglandins, for example, are produced by damaged cells and are responsible for the sensation of pain, fever, and swelling (Azizaram *et al.*, 2021). Histamine, a chemical mediator released by immune cells, causes blood vessels to dilate and increase blood flow to the affected area (Saheera *et al.,* 2022), which further contributes to the sensation of pain (Amaya *et al.*, 2013; Matsuda *et al.,* 2019). Bradykinin and substance P are other pain-inducing molecules that are released during inflammation, they are neurotransmitters that sensitize nociceptors (Matsuda *et al.,* 2019), which are specialized sensory neurons that respond to various forms of tissue damage and injury (Basbaum *et al.*, 2009).

Additionally, inflammation can cause structural changes in the nervous system, such as the hyperexcitability of pain receptors, leading to chronic pain (Ji *et al.*, 2016; Matsuda *et al.,* 2019). When inflammation persists for prolonged periods, it can lead to chronic pain conditions (Matsuda *et al.,* 2019) such as fibromyalgia (Uçeyler *et al.*, 2013) and osteoarthritis (Scanzello, 2017). These chronic conditions

are characterized by pain that lasts for more than three months (Basbaum *et al.*, 2009) and can be the result of changes in the nervous system caused by chronic inflammation (Matsuda *et al.,* 2019).

Moreover, chronic pain can lead to low-grade systemic inflammation, which in turn can contribute to the development of chronic pain conditions (Matsuda *et al.,* 2019). This creates a feed-forward cycle of pain and inflammation that can be difficult to break (Ji *et al.*, 2016).

1.3.1. Aging

Human population aging is a worldwide phenomenon. Currently, the median age of the population has been increasing because of increasing life expectancy (Officer *et al.,* 2020) associated to a declining fertility rate (Lee *et al.,* 2014). In 2020, According to the Federal Interagency Forum on Aged-Related Statistics, the proportion of the U.S. population over 65 years old was about 15%, and it's projected to reach about 20% by 2050 (Carr, 2023). In China and Japan, as of 2020, the proportion of the population over 65 years old was approximately 13.5 (Liu *et al.,* 2023) and 28% (Yamada and Arai, 2020), respectively. However, it is projected to reach about 27.6% (Feng *et al.,* 2018) and 40% (Yamada and Arai, 2020), respectively, by 2050. In Europe, the population is also aging. According to Eurostat (2020), the proportion of the population over 65 years old in the European Union (EU) was 17.3% in 2020, and it's projected to reach about 34% by 2070. So, the proportion of the world's population over 60 years is expected to double from 12% in 2015 to 22% in 2050 (Officer *et al.,* 2020), with significant social, economic, and political implications (Lopreite and Zhu, 2020; Officer *et al.,* 2020).

The scientific process of aging is a multidisciplinary field that studies the biological, genetic (Booth and Brunet, 2016; López-Otín *et al.,* 2013), environmental, and social factors (George, 2011) that contribute to the aging process. One of the key biological theories of aging is the "free radical theory" (Gerschman *et al.,* 1954) which proposes that aging is caused by the accumulation of damage to cells and molecules caused by ROS (Warraich *et al.,* 2020). These highly reactive molecules can damage DNA, proteins, and lipids, and over time, this damage can accumulate and contribute to the aging process (Capel *et al.,* 2005; López-Otín *et al.,* 2013; Warraich *et al.,* 2020).

Another theory of aging is the "telomere theory," which proposes that aging is caused by the shortening of telomeres, the protective caps on the ends of chromosomes (Blasco *et al.,* 1997; Lee *et al.,* 1998; López-Otín *et al.,* 2013). Telomeres shorten as cells divide, and eventually, cells can no longer divide and die, which can contribute to aging and age-related diseases (Herbig *et al.,* 2004; Fumagalli *et al.,* 2012; López-Otín *et al.,* 2013).

In addition to biological theories, there are also several genetic theories of aging (Hannum *et al.,* 2013; Chen *et al.,* 2016). For example, research has identified genetic variations that are associated with an increased risk of age-related diseases and may influence the aging process (Perna *et al.,* 2016). Moreover, lifestyle factors such as diet, exercise, stress, and sleep can also influence the aging process (Melancon *et al.,* 2014; George, 2021). A diet high in fruits, vegetables, and whole grains, and low in saturated fat and processed foods as well as caloric restriction (Ma *et al.,* 2020; Green *et al.,* 2017; Green *et al.,* 2022), can help to reduce the risk of age-related diseases and promote healthy aging. Regular physical activity can also help to improve cardiovascular health, build muscle mass, and maintain a healthy weight (Taylor, 2021). Overall, aging is a complex process that involves multiple factors. While the process of aging is largely determined by genetics (Chen *et al.,* 2016; Warraich *et al.,* 2020), lifestyle choices can also play a role in how quickly someone ages (George, 2021).

Researchers in this field use a variety of techniques to understand the underlying mechanisms of aging and to develop interventions that can delay or even reverse the aging process. Some of the key areas of research in the scientific process of aging include studying the role of inflammation (Davizon-Castillo *et al.,* 2019), oxidative stress (Capel *et al.,* 2004; López-Otín *et al.,* 2013), and cellular senescence (Herbig *et al.,* 2006) in the aging process, as well as identifying genetic (Fahy *et al.,* 2019 Warraich *et al.,* 2020); and environmental (Soltani *et al.,* 2019; George, 2021) factors that influence aging.

Aging is associated with an increased risk of several diseases, including cardiovascular disease, cancer, diabetes, and neurodegenerative diseases such as Alzheimer's and Parkinson's (Burkle *et al.,* 2007; Atella *et al.,* 2018). These diseases, also known as age-related diseases or geriatric diseases, can have a significant impact on an individual's quality of life and represent burden on healthcare systems (Atella *et al.,* 2018). As the global population ages, the number of individuals affected by these aging-related diseases is expected to increase (Atella *et al.,* 2018) with significant implications for healthcare systems and economies, as the demand for services and treatments for age-related diseases is expected to increase (Atella *et al.,* 2018).

1.3.1.1. Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and typically affects the joints in the hands, hips, knees, and spine (Cho *et al.,* 2015; Van der Kraan *et al.,* 2016). OA is the most prevalent disability among the elderly population (Allen *et al.,* 2022), affecting about one-third of people over the age of 65 (Van der Kraan *et al.,* 2016). Osteoarthritis is also a major cause of work-related disability (Hunter *et al.,* 2020) and an economic burden, with healthcare costs and lost productivity due to osteoarthritis estimated to be

in the billions of dollars globally (Hunter *et al.,* 2020; Shi *et al.,* 2022). It affects over 7% of adults worldwide (Hunter *et al.,* 2020), with the condition being the 4th disease most common in women and the 8th in men, (Lawrence *et al.,* 2008; Rutjes *et al.,* 2012; Van der Kraan *et al.,* 2016). The prevalence of OA varies across regions and countries, with the highest rates being observed in developed countries (Allen *et al.,* 2022). For example, the prevalence of osteoarthritis in the adult population is estimated to be around 13.4% in Europe, 19.2% in Asia, 15.8% in North America, and 4,1% in South America (Cui *et al.,* 2020). It's important to note that these numbers are just estimates since it is difficult to provide exact numbers on the incidence and prevalence of OA as there is no direct correlation between the observed radiographic abnormalities and the degree of joint pain described by the patients and vice-versa (Van der Kraan *et al.,* 2016). Moreover, OA is a slow-progressing pathology, rare in people under 40 years old, but increasing with aging (Deshpande *et al.,* 2016; Allen *et al.,* 2022). Curiously, below 45 years old, men are more affected than women, while this effect is reversed afterwards (Deshpande *et al.,* 2016; Van der Kraan *et al.,* 2016).

OA is a chronic, degenerative joint condition impacting the cartilage between bones, resulting in alterations to surrounding and internal tissues of affected joints, with its causative factors not fully elucidated (Van der Kraan *et al.,* 2016; Tong *et al.,* 2022). The structural changes of OA comprise loss of articular cartilage, fibrillation, development of osteophytes, changes in subchondral bone plaque, fibrosis, and synovitis (Maldonado and Nam, 2013; Van der Kraan *et al.,* 2016). Clinically, OA is characterized by joint pain, stiffness, limited range of motion, sensitivity, bone crepitation, and various degrees of inflammation (Houard *et al.,* 2013; Van der Kraan *et al.,* 2016).

Currently, OA clinical management relies on pain control and joint replacement (Van der Kraan *et al.,* 2016; Ghomrawi., *et al.,* 2020). NSAIDs are the most frequently prescribed agents for OA, although they present adverse effects, such as gastrointestinal and cardiovascular events (Rutjes *et al.,* 2012; Van der Kraan *et al.,* 2016). An alternative treatment is hyaluronic acid viscosupplementation however the advantages of this treatment are minimal while the technique might cause damage to the knee (Rutjes *et al.,* 2012; Pereira *et al.,* 2022).

Even though aging contributes significantly to cartilage degeneration (Allen *et al.,* 2022), additional important risk factors include obesity, metabolic syndrome (Courties *et al.,* 2019), stress, injury (Poulsen *et al.,* 2019), and genetic predisposition (Tachmazidou *et al.,* 2019). As the population is aging and obesity rates are increasing, it is expected that the number of people affected by osteoarthritis will continue to rise in the future (Houard *et al.,* 2013).

Several animal models are commonly used to study OA as they mimic several characteristics of human disease (Bendele, 2001) and comprise various species, such as zebrafish (Mitchell *et al.*, 2013), rodents (mice, rats, guinea pigs, Syrian hamsters), rabbits, primates, and dogs, (Bendele, 2001; Teeple *et al.*, 2013), sheep, goats, and horses (Gregory *et al.*, 2012; Teeple *et al.*, 2013). These models can be broadly categorized into three main types: induction models, genetic models, and spontaneous models (Serra and Soler, 2019), each presenting advantages and limitations (Malfait and Little, 2015).

Induction models involve surgically or chemically induced OA (Serra and Soler, 2019). For example, researchers can use a surgical technique designated as destabilization of the medial meniscus (DMM) (Sophocleous and Huesa, 2019; Shao *et al.*, 2020), or they can use a chemical agent such as monoiodoacetate (MIA) to damage the knee joint in rodents (Sahin *et al.*, 2021). These models closely mimic the degenerative changes seen in human OA (Serra and Soler, 2019) but they may not fully replicate the complex interactions between genetic, environmental, and lifestyle factors that contribute to the development of OA in humans (Malfait and Little, 2015).

Genetic models consist of creating genetically modified animals that are predisposed to developing OA (Cope *et al.*, 2019), like mice that lack certain genes involved in cartilage development or maintenance, such as the aggrecan or collagen type II genes (Ala-Kokko *et al.*, 1990, Jalbă *et al.*, 2011). Although important to understand the mechanisms underlying joint degeneration, results may not be directly applicable to human OA (Malfait *et al.*, 2015; Cope *et al.*, 2019; Serra and Soler, 2019).

Spontaneous models include the studying of naturally occurring OA in animals (Serra and Soler, 2019). For example, horses that have developed the disease due to aging or injury (Boyce *et al.*, 2013). These models are useful for studying the natural progression of OA (Cope *et al.*, 2019; Serra and Soler, 2019), but the results may not be directly comparable to human OA (Cope *et al.*, 2019).

In the present work, experimental OA was induced using the Kaolin/Carrageenan (K/C) model. In this model, a mixture of kaolin (a clay mineral) and carrageenan (a sulfated polysaccharide that causes inflammation) is injected into the synovial cavity of the knee joint (Pinto-Ribeiro *et al.*, 2008; Amorim *et al.*, 2022). In the K/C model, the disorder develops within a few hours and persists for several weeks, triggering cartilage damage, inflammation of the synovium, and exudate of synovial fluid (Neugebauer *et al.*, 2007). This model leads to the gradual degeneration of the articular structures, such as the medial femoral plateaux, inducing the increase of subchondral bone volume and the decrease of bone marrow area as well as the formation of a subchondral cyst (Amorim *et al.*, 2014).

1.3.1.2. *Diabetes mellitus*

Diabetes mellitus (DM) is a group of metabolic diseases characterized by abnormally high levels of glucose in the blood, hyperglycemia, resulting from a deficiency in insulin secretion, insulin action, or both (Chaudhury *et al.,* 2017), that often leads to a disturbance in glucose homeostasis and fat and protein metabolism (Kalasz *et al.,* 2017). The reasons for the escalating epidemic of *Diabetes mellitus* are multiple, including population aging, economic development, urbanization, unhealthy eating habits, and sedentary lifestyles (Zheng *et al.,* 2018).

The prevalence of DM has reached epidemic proportions worldwide. The International Diabetes Federation (IDF) (IDF, 2017) estimated that 463 million adults aged 20–79 years had DM in 2017 and that this value is expected to increase to 700 million by 2045, proving that DM is one of the fastest increasing global health emergencies of the 21st century. According to IDF, the economic impact of DM has been growing considerably from USD 232 billion spent worldwide in 2007, to USD 727 billion in 2017 (for adults aged 20–79 years) and it is projected that will reach USD 845 billion by 2045. Importantly, in these predictions, they assume that the mean expense per person and DM prevalence remain constant while considering only demographic changes (IDF, 2017).

Several processes are implicated in the development of DM as the autoimmune destruction of the pancreatic β-cells with consequential insulin deficiency and abnormalities that causes resistance to insulin action (Zheng *et al.,* 2018). Deficient insulin action results from ineffective insulin production and/or deficient action of insulin on target tissues which leads to an alteration in carbohydrate, protein, and fat metabolism (American Diabetes Association, 2014; Ozougwu *et al.,* 2013). Diminishing insulin secretion and deficiencies in insulin action commonly coexist in the same patient, and it is often unclear which is the primary cause of hyperglycemia (American Diabetes Association, 2014).

Symptomatology of DM includes polyuria, polydipsia, weight loss despite polyphagia, blurred vision, hyperglycemia, glucosuria, acidosis, ketosis, and in more severe cases, coma (American Diabetes Association, 2014; Rodrigues and Mota, 2012). Long-term DM complications comprise Central and peripheral nervous systems alterations, namely stroke, autonomic and peripheral neuropathy (motor and sensory dysfunctions) (Bax *et al.,* 1995; Brown *et al.,* 1982; Hewston and Deshpande, 2016). Several studies demonstrated that DM increased Cardiovascular System diseases (Cardiomyopathy, Myocardial infarction; Atherosclerosis, and Hypertension) (Kannel & McGee, 1979; De Visser *et al.,* 2002; Rawshani *et al.,* 2017; Madmoli *et al.,* 2018). Other symptoms of long-term DM are disorders in the renal system, such as nephropathy, proteinuria, glucosuria, kidney failure, and in the gastrointestinal system, as delayed

gastric emptying, diarrhea constipation, dyspepsia, exocrine gland insufficiency (Feldman *et al.,* 1983; Fujishiro *et al.,* 2017; Vilarrasa *et al.,* 2017). Diabetic patients also exhibit a higher prevalence of oral illnesses comprising xerostomia, taste impairment, oral candidosis, and sialosis, furthermore, diabetic patients are 2 to 3 times more likely to develop periodontal disease and caries (Allen *et al.,* 2008). Moreover, DM is associated with retinopathy and presents a 5-fold higher prevalence of cataracts, the major cause of blindness in the world (Obrosova *et al.,* 2010). Genital system disorders are also associated with DM in terms of impotence, sexual dysfunction, urogenital dysfunction, and infections (Kolodny, 1971; Malavige and Levy, 2009; Nitzan *et al.,* 2015). Additionally, DM has been associated with an increased risk of fractures (Vestergaard *et al.,* 2009) and risk of foot ulceration and amputation (Hunt, 2011).

Most cases of diabetes fall into two etiopathogenetic categories, type1 (T1DM) and type2 (T2DM) (IDF, 2017). T1DM, which has immune-mediated or idiopathic etiology, is caused by the destruction of pancreatic beta cells, leading to a complete deficiency of insulin production (Asmat *et al.,* 2016; IDF, 2017). T1DM accounts for 5-10% of cases and patients with this type of DM requires exogenous insulin (IDF, 2017). This type of DM can be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers (American Diabetes Association, 2014).

On the other hand, for T2DM, the cause comprises both, insulin resistance and an inadequate compensatory insulin secretory response (American Diabetes Association, 2014; Asmat *et al.,* 2016). In T2DM, a low degree of hyperglycemia is enough to cause pathological and functional alterations in different target tissues (American Diabetes Association, 2014) and may be present for a long period of time without clinical symptoms before the diagnosis of DM (IDF, 2017). However, during the asymptomatic period, it is possible to detect abnormalities in carbohydrate metabolism by the evaluation of plasma glucose (Asmat *et al.,* 2016). In some T2DM patients, adequate glycemic control can be accomplished with lifestyle modifications, such as oral glucose-lowering agents, weight reduction, and exercise (Asmat *et al.,* 2016). However, DM patients with β-cell destruction, T1DM, require insulin for survival (IDF, 2017).

Several studies suggest that hyperglycemia (glucotoxicity) and hyperlipidemia (lipotoxicity) are involved in β-cell dysfunction by increasing ROS production (De Souza-Ramalho *et al.*, 2009; Pitocco *et al.*, 2013; Mikrut *et al.*, 2016). Curiously, the negative effects of lipotoxicity only appear in the presence of glucotoxicity, establishing the term glucolipotoxicity to describe the harmful relationship between lipids and β-cell function (Harmon *et al.*, 2001; El-Assaad *et al.*, 2003).

Glucotoxicity leads to an increase in the production and accumulation of advanced glycation end products (AGEs) and increased expression of their receptors RAGE (Receptor for AGEs), an increase of protein kinase C (PKC) isoforms and an increase in the activation of the polyol and hexosamine pathways (Luo *et al.*, 2016; Zheng *et al.*, 2016). Additionally, glucolipotoxicity induces disturbances in the lipid profile, making the cells more susceptible to lipid peroxidation (Asmat *et al.*, 2016).

The activation of these pathways results in glucotoxicity, and glucolipotoxicity leads to an increase in mitochondrial ROS, further aggravating cellular redox imbalance and oxidative stress (Del Prato, 2009; Zheng *et al.*, 2016). Furthermore, hyperglycemia and hyperlipidemia, combined with insulin resistance, are involved in diabetic vascular complications through metabolic and structural imbalances (Arman *et al.*, 2019).

The use of animals in experimentation has played a crucial role in advancing our understanding of diabetes and its complications. Inducing diabetes in animals through experimental models is crucial for studying the various aspects of the disease's development and ultimately finding new treatments and cures (Kleinert *et al.,* 2018; Rais *et al.,* 2022). There are several types of animal models of *Diabetes mellitus*, which can be broadly classified into two categories: genetic and chemically induced models (Al-awar *et al.*, 2016; Maqbool *et al.,* 2019).

Genetic models of diabetes in animals involve the genetic manipulation of animals to induce the disease (Allen *et al.*, 2004). These models can include creating mutations in genes known to be associated with diabetes (Allen *et al.*, 2004), as well as introducing genetic variations found in humans with the disease (Richards *et al.,* 2014). These models allow researchers to study the genetic factors that contribute to the development of diabetes and its complications and can also be used to test potential therapies (King, 2012; Rais *et al.,* 2022). Some examples of genetic models of diabetes in animals include the spontaneous mutation in the non-obese diabetic (NOD) mouse, and the use of transgenic mice that express human diabetes-associated genes (King *et al.*, 2012; Al-awar *et al.*, 2016).

Chemical models of diabetes are induced by administering specific chemicals or drugs to animals that disrupt normal insulin production or function (Al-awar *et al.,* 2016; Rais *et al.*, 2022). Some examples of chemical models of diabetes include the use of Streptozotocin (STZ) and Alloxan, the most potent diabetogenic chemicals, that specifically target and kill the beta cells of the pancreas, leading to a deficiency in insulin production and hyperglycemia (Al-awar *et al.,* 2016; Rais *et al.*, 2022).

Moreover, both type 1 and type 2 diabetes are complex diseases that involve different bodily systems. In T1DM, animals used to study the disease are often highly inbred and have a limited number of pathways

to the disease, which may not comprehensively reflect the diverse mechanisms and triggers implicated in human T1DM development (Al-awar *et al.,* 2016). This can include chemical ablation of the beta cells or breeding rodents that spontaneously develop autoimmune diabetes (Al-awar *et al.,* 2016; Maqbool *et al*., 2019).

In T2DM, several animal models have been developed to understand the pathophysiology of the disease and its complications. These models include both models of insulin resistance and models of beta cell failure (Al-awar *et al.,* 2016). Many of these models are also obese, as obesity is closely linked to the development of T2DM in humans (Kleinert *et al.*, 2018). These models often display abnormalities in one or multiple genes related to obesity, glucose intolerance, and/or insulin resistance leading to high blood glucose levels. The progression and development of diabetic complications are also affected by various factors including obesity, insulin resistance, hyperglycemia, and hyperlipidemia (Al-awar *et al.,* 2016; Kleinert *et al*., 2018).

Consequently, it is important to carefully select the appropriate animal model for diabetes research based on the specific aspect of the disease being studied (Al-awar *et al.,* 2016; Kleinert *et al.*, 2018). For example, if the focus of the research is on the autoimmune mechanism of T1DM, an animal model with a spontaneous autoimmune disorder would be more appropriate than one induced chemically (Al-awar *et al.,* 2016; Maqbool *et al.,* 2019). Similarly, if the research is focused on the role of obesity in the development of T2DM, an obese animal model would be more suitable than a lean model (Kleinert *et al*., 2018; Maqbool *et al*., 2019). By selecting the appropriate animal model, researchers can more accurately study the specific aspect of the disease and increase the prospect of obtaining relevant and useful results (Kleinert *et al.*, 2018).

In this work, we focused on the study of T2DM. Therefore, the chemical-induced model of streptozotocin and nicotinamide (STZ-NA) was used. The STZ-NA model of diabetes was first described by Masiello and colleagues (1998) which treated rats with a combination of STZ and nicotinamide (NA) to induce diabetes. The use of this combination to induce T2DM resulted in a more gradual onset of diabetes and a lower mortality rate compared to the traditional STZ model. The STZ-NA model has since been widely used in diabetes research to study the early stages of T2DM and the underlying disease mechanisms (Rais *et al*., 2022). STZ selectively destroys the insulin-producing beta cells in the pancreas, while NA protects the remaining beta cells from the toxic effects of STZ (Kaikini *et al*., 2020).

This model is used to mimic human T2DM, which is characterized by a progressive loss of beta cell function rather than destruction as seen in T1DM (Eizirik *et al*., 2020; Kaikini *et al*., 2020). It also allows

the study of the early stages of the disease and the interplay between beta cell dysfunction, hyperglycemia, insulin resistance, and glucose intolerance (Kaikini *et al.*, 2020). STZ-NA model is also suitable for the study of other features of diabetes including long-term diabetes complications such as diabetic cardiomyopathy (Rais *et al.,* 2022), neuropathy (Kaikini *et al.*, 2020) and nephropathy (Noshahr *et al.,* 2020). Additionally, The STZ-NA induced diabetes model, as a model for non-obese T2DM, can be used to evaluate the efficacy of various treatments, such as drugs or dietary interventions, for managing diabetes (Jangale *et al.*, 2016; Rais *et al.*, 2022). Furthermore, the STZ-NA model is considered a more suitable tool for evaluating the effectiveness of natural compounds in treating diabetes and testing the potential impact of these compounds on diabetes progression (Rais *et al.,* 2022).

1.4. Research objectives

These thesis objectives collectively aim to deeply understand the antioxidant and anti-inflammatory bioactive properties of *Coreopsis tinctoria*, *Baccharis dracunculifolia* and *Genista tridentata*, highlight their potential applications in healthcare and in the food industry.

The present thesis comprises five chapters (1 to 5), each specifically designed to achieve specific objectives.

Chapter 1 embarks on an extensive literature review, centring on the key themes of the thesis. It commences with an exploration of oxidative stress and inflammatory responses. This introduction extends to chronic inflammatory diseases, experimental models, and the pharmacological potential of plant-derived products. Additionally, the chapter provides detailed insights into the traditional uses of the studied plant extracts and to the animal's models herein used.

In Chapter 2, our research delved into an extensive examination of the pharmacological properties inherent within the methanolic extract of *C. tinctoria* flowers. Our focus was on elucidating its antioxidant potential, toxicological profile, and its capacity to mitigate glucotoxicity in *C. elegans*, particularly pertinent to conditions such as *Diabetes mellitus*.

Moving forward to Chapter 3, our objective was to conduct a thorough evaluation of the anti-inflammatory attributes associated with the *B. dracunculifolia* extract. Additionally, we aimed to highlight the intricate interconnection between pain, emotional comorbidities, and microgliosis in osteoarthritis, while exploring the therapeutic promise held by *B. dracunculifolia* in managing these interconnected symptoms.

Lastly, in Chapter 4, we conducted a systematic review to attain a profound understanding of the phytochemical composition and biological activities inherent in *G. tridentata* extracts, with far-reaching

implications across various industries and potential health domains. After this review, we undertook three distinct research studies to investigate the potential therapeutic effects of *G. tridentata* methanolic extracts. Our exploration encompassed their efficacy in mitigating inflammation and alleviating nociceptive behavior in osteoarthritis, as well as in diabetic neuropathy in rats. Parallel, we evaluated their impact on diabetes-induced histopathological alterations within internal organs. In the final study, we delved into the potential of *G. tridentata* extract to improve wound healing in diabetic conditions through a combination of *in vitro* and *in vivo* experiments.

In addition to exploring the multifaceted realm of medicinal plants, this thesis seeks to valorize the studied botanical resources. Our objectives extend to highlighting the cultural, ecological, and economic significance of these plants. Through a holistic approach, we aim to shed light on the intrinsic value of medicinal plants in traditional healing practices, biodiversity conservation efforts, and sustainability. By recognizing and promoting the inherent worth of these plants, this research endeavors to foster conservation, and utilization of medicinal plants for the benefit of human health and the preservation of natural ecosystems.

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Chapter 2: *Coreopsis tinctoria* Nutt.

This chapter encloses the following manuscript in preparation:

Laranjeira, I. M., Vilasboas-Campos, D., Pinto-Ribeiro, F., Dias, A. C. P., Teixeira-Castro, A., & Maciel, P. Exploring the antioxidant properties and toxicology of *Coreopsis Tinctoria* Nutt.: A potential therapeutic agent for *Diabetes Mellitus*

2.1. Exploring the antioxidant properties and toxicology of *Coreopsis tinctoria* Nutt.: A potential therapeutic agent for *Diabetes Mellitus*

2.1.1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by abnormally high levels of glucose in the blood, hyperglycemia, resulting from a deficiency in insulin secretion, insulin action, or both (Chaudhury *et al.,* 2017) that often leads to a disturbance in glucose homeostasis and fat and protein metabolism (Kalasz *et al.,* 2017). The prevalence of DM has increased worldwide, the International Diabetes Federation (IDF) (IDF, 2017) estimated that 463 million adults aged 20–79 years had DM in 2017 and is expected to increase to 700 million by 2045, proving that DM is one of the fastest increasing global health emergencies of the 21st century.

According to IDF, the economic impact of DM has been considerably growing from USD 232 billion spent worldwide in 2007, to USD 727 billion in 2017 (for adults aged 20–79 years) and it is projected that will reach USD 845 billion by 2045. However, in these predictions, they assume that the mean expense per person and DM prevalence remain constant while considering only demographic changes (IDF, 2017).

Over the last few decades, the medical community has increasingly acknowledged and accepted the potential of traditional Chinese herbal medicine for the treatment of DM (Khan & Ahmad, 2019; Matos *et al.*, 2021). In modern pharmacological research, a growing number of plants are being investigated and utilized for their medicinal properties (Salmerón-Manzano *et al.*, 2020).

Coreopsis tinctoria Nutt. a member of the Asteraceae family (Zhang *et al.*, 2016), is an annual herbaceous plant known by various names such as *Garden tickseed, Plain coreopsis, Plains tickseed, Calliopsis, goldenwave, Golden coreopsis,* and *Golden tickseed* (Tripathy *et al.,* 2021).

Although *C. tinctoria* is a native plant of North America, Canada, and Eastern Mexico (Tripathy *et al.,* 2021), it is widely grown in China (Zhang *et al.,* 2016), particularly in southern Xinjiang. In this area, it can be found growing in the Karakorum and Kunlun Mountains at elevations of up to 3,000 meters above sea level, due to the lower pollution levels and higher altitude (Zhang *et al.,* 2016; Abdurehman *et al.,* 2022). The unique environmental conditions of the Kunlun Mountains in Xinjiang (China) create a distinctive habitat for *C. tinctoria*, which may contribute to the plant's unique biological activities (Yao *et al.*, 2019).

C. tinctoria was first employed in China as a medicinal plant in 1986 (Shen *et al.*, 2021) to treat acute and chronic dysentery, red-eye swelling, and pain. The local Uygur people in the Hotan region, Xinjiang

refer to *C. tinctoria* as Gulqai or Snow Chrysanthemum and consume it as a tea to reduce high blood pressure, treat diarrhea (Yao *et al.*, 2019), lower hypertension and hyperlipidemia (Li *et al.*, 2015), detoxify, improve blood circulation and thirst, palpitations, gastrointestinal discomfort, loss of appetite and dysentery (Shen *et al.*, 2021). In Portugal, the flowering tops infusion of *C. tinctoria*, also known as "Estrelas do Egipto," have been used to treat hyperglycemia in diabetics by drinking two cups of this infusion daily (Dias *et al.*, 2010).

In recent decades, extensive research has been conducted on *C. tinctoria* for its pharmacological properties. Both, extracts and individual compounds from the plant have exhibited a range of beneficial effects, including antidiabetic (Dias *et al.*, 2010; Begmatov *et al.*, 2020), cardioprotective (Zhang *et al.*, 2016), antioxidant (Chen *et al.*, 2016; Begmatov *et al.*, 2020), and anti-inflammatory (Yao *et al.*, 2019; Begmatov *et al.*, 2020) properties, as well as neuroprotective (Li *et al.*, 2015), antimicrobial (Guo *et al.*, 2017), and antitumor (Wufuer *et al.*, 2020) activity. However, despite these findings, the activity of most of the plant's constituents remains uncertain (Shen *et al.*, 2021).

Thus, this study aimed to investigate the phytochemical profile and antioxidant *in vitro* potential of *C. tinctoria* methanolic extract as well as its *in vivo* toxicity and the effect upon glucotoxicity in *C. elegans*.

2.1.2. Materials and methods

2.1.2.1. Preparation of *C. tinctoria* extracts

Flowers of *C.tinctoria* plants were collected in 2019 (June to September), in the region of Kunlun Mountains (41° 45' 20.24 N; 86° 8' 51.16 W; Korla city, Xinjiang, China). Biomass was triturated and macerated with methanol 70% (Sigma-Aldrich, Barcelona, Spain), in the dark, for 7 days. Afterward, the mixture was filtered and concentrated with a rotary evaporator (Buchi® RII, Flawil, Switzerland), under reduced pressure (Buchi® V-700 pump, Flawil, Switzerland), in the dark, at 50 °C. The extract was then lyophilized (Christ® Alpha 2–4, B. Braun, Tuttligen, Germany) for two days to obtain a powder and stored in a closed container (in the dark at room temperature) until further use (Dias *et al.*, 2007).

For the biochemical analyses and *in vivo* tests, the extract powder was dissolved in ethanol (Sigma-Aldrich, Barcelona, Spain) and DMSO (Sigma-Aldrich, Barcelona, Spain), respectively (stored stock concentration 100 mg/mL, 4 °C).

2.1.2.2. HPLC phenolic analysis

The phenolic content of *C. tinctoria* extracts was analyzed by HPLC-DAD as described by Dias *et al.* (2007). Briefly, *C. tinctoria* extract (3 mg/mL) extracts were solubilized in pure methanol (Sigma-Aldrich,

Barcelona, Spain) and filtered by a 0.2 µm nylon syringe filter (Sigma – Aldrich, Barcelona, Spain) with an injection volume of 20 µL. Compound separation was achieved by a chromatographic system (Merck-Hitachi Elite LaChrom, New York, NY, USA) using a gradient elution of methanol (HPLC grade, Merck, Darmstadt, Germany) with 0.1% of formic acid (P.A. grade, Merck, Darmstadt, Germany) (solvent A) and ultrapure water with 0.1% of formic acid (solvent B; Merck, Darmstadt, Germany). The optimal elution profile comprised a six-step gradient: starting with 15% of solvent A and installing a gradient to obtain 15% (solvent A) at 3 min, 90% (solvent A) at 35 min, 90% (solvent A) at 45 min, 15% (solvent A) at 50 min, and 15% (solvent A) at 60 min. Data was recorded in the range of 245–530 nm, and chromatograms were taken at 260, 280, and 350 nm.

2.1.2.3. *In vitro* antioxidant activity

2.1.2.3.1. DPPH scavenging assay.

The evaluation of radical scavenging activity was performed following a modified method based on the stable DPPH free radical (Oliveira *et al.*, 2018). In a 96-well plate (Sigma-Aldrich, Barcelona, Spain), 140 µl of a 400 µM DPPH solution (in 100% ethanol, (Sigma-Aldrich, Barcelona, Spain)) was combined with 10 µl of *C. tinctoria* extracts at varying concentrations (46.875 µg/ml; 93.75 µg/ml; 187.5 µg/ml; 375 µg/ml; 750 µg/ml and 1000 µg/ml) up to a final concentration of 1000 µg/ml. Following a 1-hour incubation in darkness at room temperature, absorbance was measured at 517 nm in a microplate reader (SpectraMax Plus 384, Molecular Devices, USA). The Graph Pad Prism software (GraphPad Software Inc., version 5, LaJolla, CA, USA) was employed to calculate the extract concentration necessary to scavenge 50% of the initial DPPH (EC50), with Quercetin (Sigma-Aldrich, Barcelona, Spain) serving as the reference standard. Measurements were conducted in three independent sets, each consisting of three replicates, and the results are presented as the mean ± standard error of the mean (SEM).

2.1.2.3.2. Nitric Oxide (NO) scavenging assay.

The assessment of NO scavenging activity followed a protocol with minor adjustments based on Silva *et al.*, (2008). In summary, a reaction mixture (200 µl) comprising Sodium Nitroprusside (SNP Sigma-Aldrich, Barcelona, Spain, 10 mM, in PBS, pH 7.4) and *C. tinctoria* extract, up to a final concentration of 1000 µg/ml, was incubated under light at room temperature for 2 hours. A control group, replacing the extract with PBS, pH 7.4, was included. Subsequently, 50 µl of Griess reagent (1% (w/v) sulfanilamide (Sigma-Aldrich, Barcelona, Spain), 0.1% (w/v) NED (N-(1-naftil) etilenodiamida) in 2% phosphoric acid) (Sigma-Aldrich, Barcelona, Spain) was added and incubated for 10 min in the dark. Two blanks (the sample blank and the control blank) with a composition like their respective counterparts but with 2%

phosphoric acid (Sigma-Aldrich, Barcelona, Spain) instead of the Griess reagent, were incorporated. The absorbance of the resulting chromophore (purple azo dye) was measured at 543 nm. Galic acid (Sigma-Aldrich, Barcelona, Spain) served as the reference standard, and EC50 values were determined as described previously. The measurements were carried out in three independent sets, with each set comprising three replicates. The results are expressed as the mean ± SEM.

2.1.2.3.3. Superoxide (SO) scavenging assay

Superoxide (SO) scavenging by the extracts was evaluated according to the protocol reported by Valentão and collaborators (2002). A reaction mixture contained 50µl NADH stock (1.97mM in phosphate buffer, pH 7.4) (Sigma-Aldrich, Barcelona, Spain), 150 µl NBT stock (Nitro blue tetrazolium, 81.5 µM in phosphate buffer, pH 7.4) (Roche, Basel, Switzerland), 50 µl PMS solution (3.26 mM in phosphate buffer, pH 7.4) (Acros Organics; Thermo Scientific Chemicals; Antwerpen, Belgium), and 50µl of *C. tinctoria* extract, up to a concentration of 1000 µg/ml, incubated for 2 minutes at room temperature. Two blanks (sample and control) mimicked their counterparts but differed in the sample blank including 50 µl of respective solutions and 50 µl of NADH stock and in the negative control contained phosphate buffer (pH 7.4), NADH stock, NBT stock, and PMS solution. Absorbance at 560 nm was measured using a SpectraMax Plus 384 microplate reader (SpectraMax Plus 384, Molecular Devices, USA), indicating SO production by a color shift from blue to yellow. Graphs were created with GraphPad Prism (GraphPad Software Inc., version 5, LaJolla, CA, USA) to determine the extract concentration yielding a 50% SO scavenging (EC50), using Quercetin (Sigma-Aldrich, Barcelona, Spain) as a reference standard. Three sets of three replicates yielded results presented as mean ± SEM.

2.1.2.3.4. Iron Chelating Activity (ICA) assay

The chelation of ferrous ions by *C. tinctoria* extracts was determined using a method previously described by Oliveira *et al.* (2018), with small adjustments. In a 96-well plate (Sigma-Aldrich, Barcelona, Spain), 50 µl of 0.12 mM FeSO4 (Sigma-Aldrich, Barcelona, Spain) was combined with 50 µl of various concentrations of the extract up to a final concentration of 1000 µg/ml. The reaction was initiated by adding 50 µl of 0.6 mM ferrozine solution (Sigma-Aldrich, Barcelona, Spain), followed by a 15-minute incubation at room temperature in the dark. Subsequently, the absorbance of the solution was measured at 562 nm SpectraMax Plus 384 microplate reader (SpectraMax Plus 384, Molecular Devices, USA). EDTA (Sigma-Aldrich, Barcelona, Spain) served as the reference standard, and the extract concentration required to chelate 50% of the ferrous ions (EC50) was calculated. Measurements were conducted in three sets, each with three replicates, and the results are presented as the mean ± SEM.

2.1.2.4. *In vivo* study

2.1.2.4.1. *C. elegans* strains, maintenance, and synchronization

C. elegans strains used in this study were provided by the Caenorhabditis Genetics Center (CGC). Standard procedures (Brenner, 1974) were used to grow animals on nematode growth medium (NGM) agar plates (LabChem, LaborSpirit Lda, Lisboa, Portugal) at a temperature of 20°C. To maintain *C. elegans*, the OP50 strain of *Escherichia coli* was seeded on plates and grown in Luria Broth (LB) media (Gibco, Life Thecnologies Corporation, Miami, FI, USA) overnight at 37°C and 180 rpm. Hypochlorite treatment, involving approximately 2.6% NaClO (Sigma-Aldrich, Barcelona, Spain) and 0.5 M NaOH (Sigma-Aldrich, Barcelona, Spain), was used for 5 minutes to synchronize *C. elegans*.

2.1.2.4.2. Toxicity evaluation of *C. tinctoria* extracts in *C. elegans* - Food clearance assay

To assess the toxicity of extracts, the *C. elegans* Bristol strain N2 was used in a 96-well plate (Sigma-Aldrich, Barcelona, Spain) assay conducted in liquid culture, following the protocol described By Teixeira-Castro *et al.*, (2015). Each well consisted of a total volume of 60 µL, comprising 15 µL of egg suspension (20-25 eggs), 25 µL of each plant extract at a range of concentrations (0.125 to 2 mg/mL), and 20 µL of OP50 bacteria (Sigma-Aldrich, Barcelona, Spain) with an OD of 0.6-0.7 at 595 nm. The OP50 bacteria were grown overnight at 37°C and 180 rpm in Luria Broth (LB) media (Gibco, Life Thecnologies Corporation, Miami, FI, USA), pelleted by centrifugation, inactivated by three cycles of freeze/thawing, frozen at -80°C, and then resuspended in complete S-medium (Sigma-Aldrich, Barcelona, Spain). Worms were cultured with continuous shaking at 180 rpm and 20°C (Shell Lab incubator shaker,Sheldon Manufacturing.Inc, Cornelius, OR, USA) for seven days. The consumption of OP50 was measured daily at 595 nm (NanoQuant Plate™-Tecan, Tecan Trading AG, Zurich, Switzerland).

2.1.2.4.3. Glucotoxicity induction

To induce glucotoxicity (Schlotterer *et al.*, 2009) gravid adult nematodes were transferred for 2 hours to NGM plates (LabChem, LaborSpirit Lda, Lisboa, Portugal) supplemented with glucose (100 mmol/L) to yield eggs and obtain a synchronized population born in hyperglycemic condition. The control groups (i: uninduced worms seeded with OP50 strain of *Escherichia coli* (Sigma-Aldrich, Barcelona, Spain) grown in LB; ii: uninduced worms seeded with OP50 strain of *Escherichia coli* grown in LB supplemented with *C. tinctoria* extract; iii: glucotoxicity-induced worms seeded with OP50 strain of *Escherichia coli* grown in LB; iv: glucotoxicity induced worms seeded with OP50 strain of *Escherichia coli* grown in LB supplemented with *C. tinctoria* extract) and experimental groups were maintained in triplicates.

2.1.2.4.4. Evaluation of *C. elegans* size

After treatment with *C. tinctoria* extracts the size (area captured) of the *C. elegans* was evaluated. Animals were grown in NGM solid plates (with and without glucose incorporation) (Sigma-Aldrich, Barcelona, Spain) and seeded with extracts (0.1,1 and 2 mg/mL resuspended in inactivated OP50). On days 4,6 and 8 after hatching, the animals were removed from the plates to unseeded NGM plates (LabChem, LaborSpirit Lda, Lisboa, Portugal). A population of animals in the NGM plate was photographed using an Olympus SZX7 stereomicroscope (Olympus Co. Ltd., Tokyo, Japan) with an integrated camera (Olympus SC30; Olympus Co. Ltd., Tokyo, Japan).

A total of 50–75 animals were scored in three independent assays. The photographs were processed using the Image J® software (National Institutes of Health, Bethesda, MD, USA; Schneider *et al.*, 2012). The area of worms after treatment with *c. tinctoria* extracts was measured as described by Hirose *et al.*, 2003. Briefly, the perimeter of the animal was traced, and the measured area was used as an estimate of its size.

2.1.2.4.5. Evaluation of *C. elegans* activity

On the day of the experiment, worms aged 4 and 6 days were synchronized by egg posture of gravid adults and, then transferred from the plates to micro-centrifuge tubes (Sarstedt AG & Co, Numbrecht, Germany) using M9 buffer (Sigma-Aldrich, Barcelona, Spain). The worms in the tubes underwent at least two washes with M9 buffer to remove any remaining OP50. Following this, approximately 150 worms in 300 μL were dispensed into each well of a 96-well plate (LabChem, LaborSpirit Lda, Lisboa, Portugal), with 3 wells allocated for each age group. Subsequently, the plate was inserted into the WMicrotracker-One™ apparatus (Phylumtech S.A., Santa Fe, Argentina) and monitored for 2 hours at room temperature, following the protocol stablished by Soh *et al.* (2020).

2.1.2.4.6 *C. elegans* lifespan assays

To assess the lifespan of *C.elegans* (Vilasboas-Campos *et al.*, 2021), animals were cultured on NGM solid plates (LabChem, LaborSpirit Lda, Lisboa, Portugal) or NGM solid plates supplemented with glucose (100mM) that were seeded with *C. tinctoria* extracts (1 mg/mL) resuspended in inactivated OP50. The L4 stage was designated as day 0. During the first two weeks, the animals were transferred daily to freshly seeded plates containing inactivated OP50 and *C.tinctoria* extracts (1 mg/mL), and subsequently every 2-3 days for the remainder of the experiment. Mortality was recorded daily, with an animal being considered dead if there was no mechanical response after ten consecutive touches with a platinum wire.

In cases where internal hatching, extrusion, or desiccation in the border of the plate had occurred, censoring was applied. Positive and negative controls consisted of daf-2 and daf-16 mutants, respectively.

2.1.2.5. Statistical analyses

Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software Inc., version 5, LaJolla, CA, USA). In the antioxidant biochemical assays, a T-test was employed to compare experimental groups (control and *C. tinctoria* extract). The Food Clearance assay was analyzed via a two-way ANOVA. Size and activity analyses were performed using a T-test to compare groups with and without glucotoxicity, followed by a one-way ANOVA to compare groups with and without *C. tinctoria* administration. The Life Span assay was analyzed using SPSS software (IBM SPSS Statistics for Windows, Version 23.0., IBM Corp, Armonk, NY, USA). Kaplan-Meier method was applied to calculate survival fractions and the log-rank (Mantel-Cox) test was used to compare survival curves. All experiments were run at least in triplicate ($n \geq 3$) and data presented correspond to mean ± standard deviation, unless otherwise stated, with significance levels of $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***), respectively.

2.1.3. Results

2.1.3.1. HPLC-DAD

Chromatographic data and identification of *C. tinctoria* compounds were obtained by HPLC-DAD, data was used for both qualitative and quantitative analysis. When possible, pure standard compounds were used for quantification (i.e. cafeic acid, cumaric acid, chlorogenic acid, kaempferol). Otherwise, caffeoylquinic acids and flavonoids were quantified as equivalents of chlorogenic acid and kaempferol, respectively.

A typical *C. tinctoria* HPLC-DAD chromatogram is shown in Figure 2.1.1. Major compounds present in *C. tinctoria* extract were identified by typical UV-Vis spectra and retention times. In Table 2.1.1., the maximum absorption of each peak is listed, as well the compound identification and concentration.

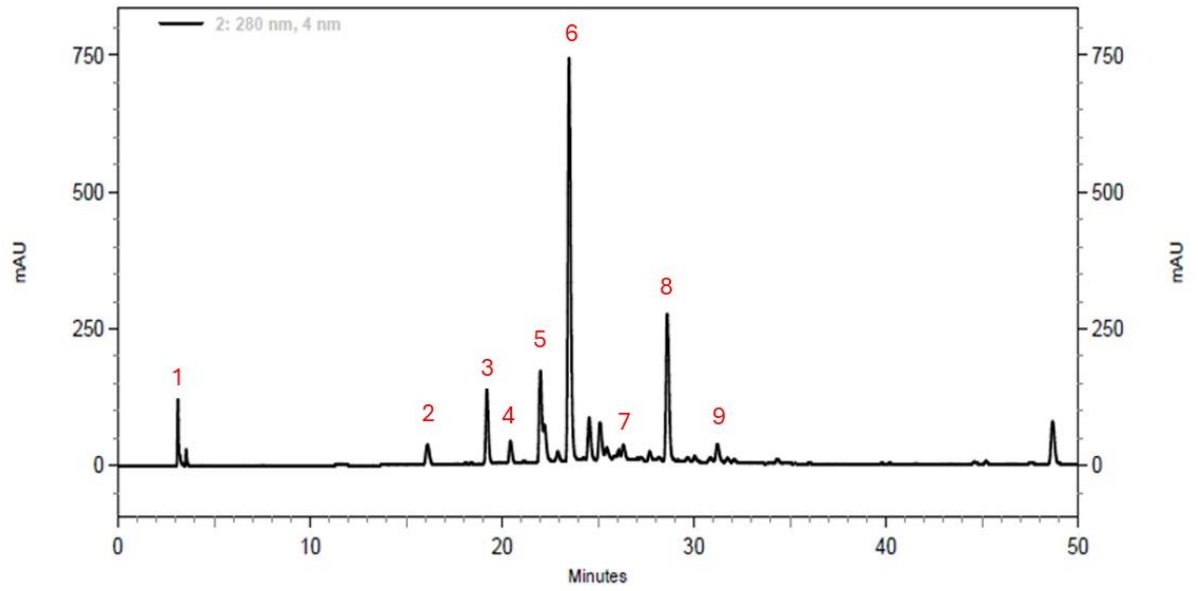


Figure 2.1.1. - HPLC-DAD-UV (280 nm) phenolic profile of the methanolic extract from flowers of C. tinctoria. Peaks 19–41 correspond to the compounds listed in Table 3.1.1. (mAU - milli Absorbance Units)

Table 2.2.1. - Phenolic composition of the C. tinctoria extract.

Peak	Retention time	Compound	mg/g Dwb
2	17,53	Caffeoylquinic acid derivate	10
3	19,16	Cathechin	35
4	20,27	Kaempherol derivate	15
5	21,85	Quercetin derivate	58
6	22,24	Quercetin derivate	144
7	23,67	Caffeoylquinic acid derivative	20
8	27,98	Quercetin	58
9	30,41	Kaempherol	6

Note: the peak numbers correspond to the ones indicated in figure 2.1.1.

2.1.3.2. Biochemical screening of antioxidant activity

The initial evaluation of antioxidant activity of methanolic extracts from *C. tinctoria* flowers was performed employing various *in vitro* methods, measuring several activities for the overall antioxidant performance

of the extracts. The summarized results are presented in Figure 2.1.2. Overall, *C. tinctoria* extracts demonstrated higher activity in all antioxidant assays and exhibited strong iron-chelating activity. The activities of the extract were significantly different from the positive controls in all assays (DPPH: $t_{(16)}$ =9.784, P<0.0001; SO: $t_{(14)}$ =15.31, P<0.0001; ICA: $t_{(15)}$ =0.07122, P =0.9442; and NO: $t_{(16)}$ =19.34, P<0.0001).

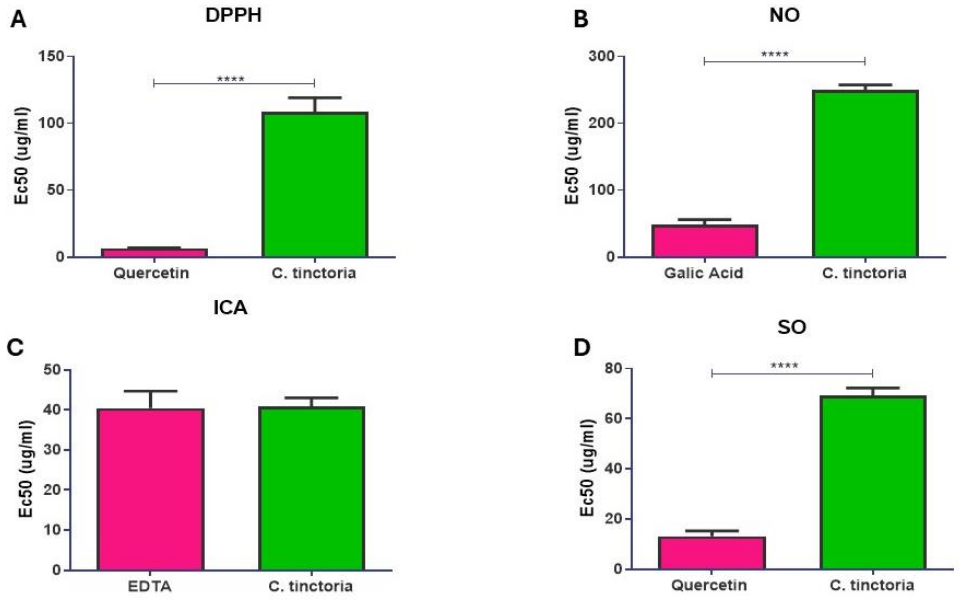


Figure 2.1.2. - Evaluation of the antioxidant potential of C. tinctoria through DPPH (A), NO (B), ICA (C), and SO (D) assays. Results are expressed as mean ± SEM, **** p < 0.0001.

2.1.3.3. Toxicity evaluation of *C. tinctoria* extracts in *C. elegans* - Food clearance assay

Initially, we evaluated the safety of various concentrations of *C. tinctoria* in wild-type *C. elegans* (N2) using a food clearance assay. This assay measured the rate of food consumption as an indirect indicator of the worms' normal growth, survival, and fecundity by monitoring the OD595nm of *E. coli* bacteria (OP50) over time. We considered a particular concentration of *C. tinctoria* as safe if the treated animals exhibited a food consumption rate like that of control animals treated with 1% DMSO (MARCA) (considered a safe condition). If the OD decrease, in animals treated with a specific *C. tinctoria* extract concentration, diverged from the 1% DMSO control condition (non-toxic vehicle) or approached the 5% DMSO control condition (considered toxic), we classified it as toxic. The obtained results are summarized in Figure 2.1.3., the statistical analysis of the toxicity assay revealed that all tested concentrations were considered safe for the animals (Treatment: $ANOVA_{Two\text{-}away}$: $F_{(10,176)}$ =8.496, P<0.0001; Time: $ANOVA_{Two\text{-}away}$: $F_{(7,176)}$ =145.3, P<0.0001; Interaction: $ANOVA_{Two\text{-}away}$: $F_{(70,176)}$ =8.496, P=0.0019).

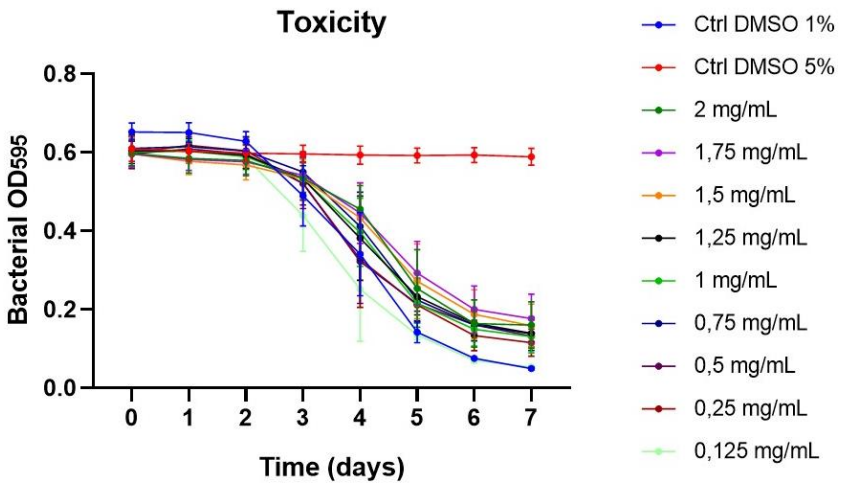


Figure 2.1.3. - Evaluation of the toxicity of C. tinctoria methanolic extracts via the food clearance assay in C. elegans. Optical density readings of the OP50 suspension treated with C. tinctoria extract in N2 animals were monitored daily at various concentrations.

2.1.3.4. *C. elegans* size evaluation

To assess the impact of *C. tinctoria* extract treatment on the size of the animals, we quantified the area of *C. elegans* treated with various concentrations of the extract on days 4, 6, and 8. Under conditions of glucotoxicity, we observed an increase in the size of worms cultivated in a glucose concentration of 100 mM compared to control worms (Figure 2.1.4. A: $T_{(138)}$=9.056; P<0.0001; B: $T_{(134)}$=12.75; P<0.0001; C: $T_{(128)}$=11.90; P<0.0001) .

However, no detectable influence of *C. tinctoria* extract treatment was noted in WT animals (Figure 2.1.4. D: $ANOVA_{one\text{-}way}$: $F_{(3,299)}$=2.221, P=0.0862; E: $F_{(3,241)}$=0.6272, P=0.5981; F: $F_{(3,230)}$=1.085, P=0.3564), confirming the safety of the extract, the application of *C. tinctoria* extracts in *C. elegans* under glucotoxicity resulted in a reduction in the body area (Figure 2.1.4. G: $ANOVA_{one\text{-}way}$: $F_{(3,256)}$=7.151, P=0.0001; H: $F_{(3,250)}$=16.81, P<0.0001; I: $F_{(3,248)}$=6.502, P=0.0003).

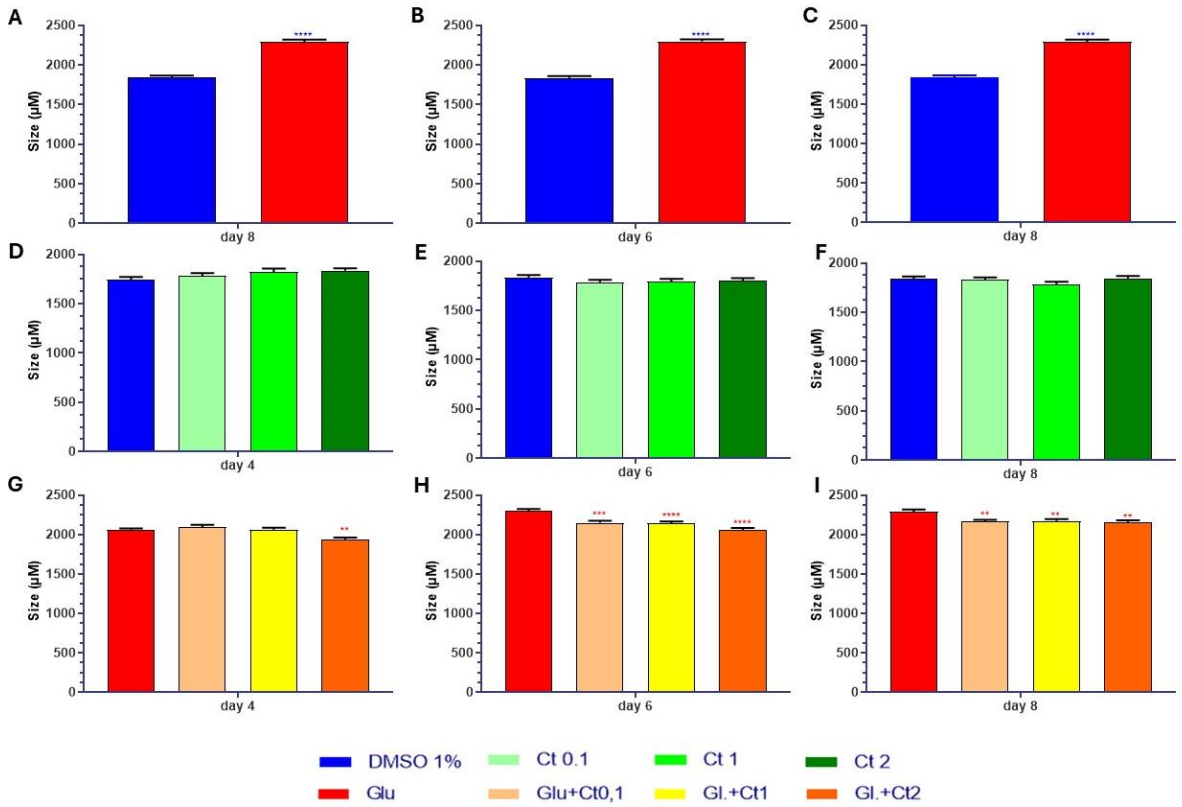


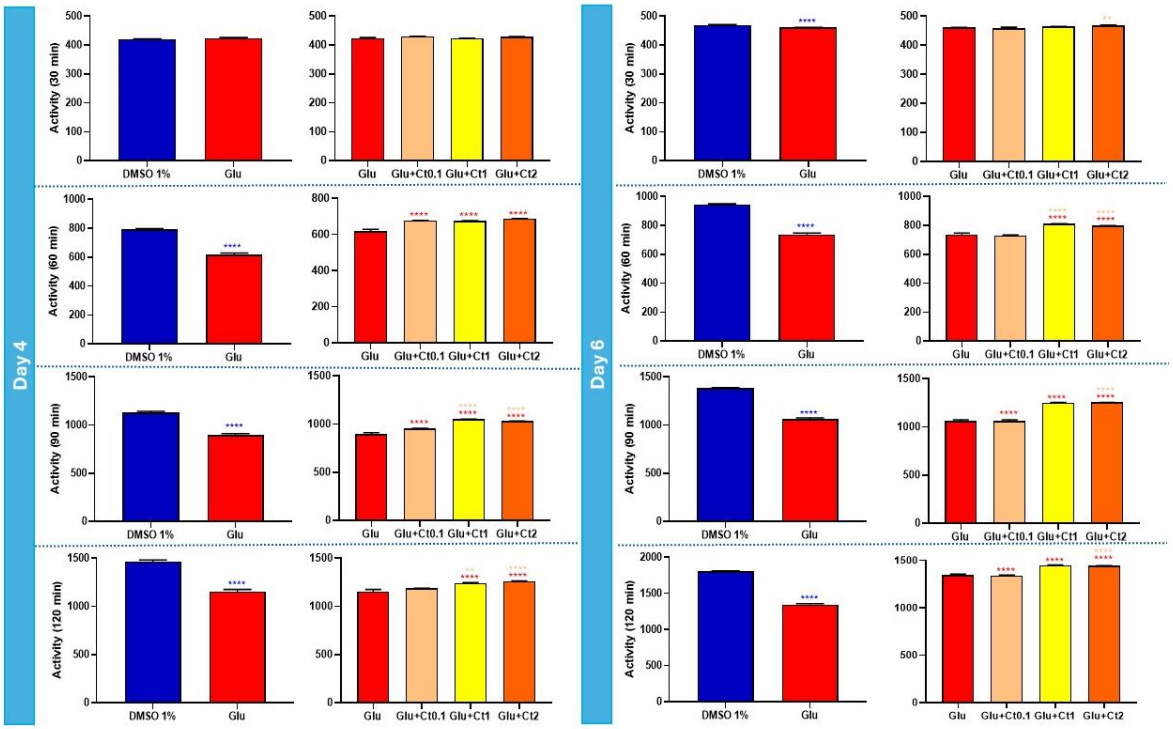
Figure 2.1.4. Assessment of *C. elegans* body size across varied culture conditions on days 4, 6 and 8. A-C: Comparison of control animals treated with 1% DMSO and 100mM Glucose on days 4, 6, and 8, respectively; D-F: Comparison of animals treated with 1% DMSO and animals treated with extract at different concentrations on days 4, 6, and 8, respectively; G-I: Comparison of animals under glucose conditions treated with extract at different concentrations on days 4, 6, and 8, respectively. (Ct 0.1: animals treated with 0.1 mg/kg of *C. tinctoria* extract; Ct 1: animals treated with 1 mg/kg of *C. tinctoria* extract; Ct 2: animals treated with 2 mg/kg of *C. tinctoria* extract; Glu: animals under glucotoxicity, treated with glucose 100mM; Glu+Ct 0.1: animals under glucotoxicity , treated with 0.1 mg/kg of *C. tinctoria* extract; Glu+Ct 1: animals under glucotoxicity, treated with 1 mg/kg of *C. tinctoria* extract; Glu+Ct 2: animals under glucotoxicity, treated with 2 mg/kg of *C. tinctoria* extract). The control DMSO (1%) was utilized as the drug vehicle. Data obtained from n = 3 independent experiments. Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ and **** $p < 0.0001$.

2.1.3.5. Spontaneous activity assay

The spontaneous activity assay indicated a decreased in the spontaneous activity of animals under glucotoxicity at day 4 and 6 (Figure 2.1.5.: Day 4: 30 min. – $T_{(298)}$= 1.406; P= 0.1608 ; 60 min. - $T_{(298)}$= 13.78; P< 0.0001; 90 min. - $T_{(298)}$= 11.25; P< 0.0001.; 120 min. - $T_{(298)}$= 11.44; P< 0.0001; Day 6: 30 min. - $T_{(298)}$= 4.233; P< 0.0001; 60 min. - $T_{(298)}$= 19.58; P< 0.0001; 90 min. - $T_{(298)}$= 23.89; P< 0.0001.; 120 min - $T_{(298)}$= 30.35; P< 0.0001).

Post-hoc tests highlighting that the treatment with *C. tinctoria* in the context of glucotoxicity, led to an increase in the spontaneous activity of *C. elegans* (Figure 2.1.5.: Day 4: 30 min. - $ANOVA_{one\text{-}way}$: $F_{(3,596)}$=5.479, *P*= 0.0010; 60 min. - $ANOVA_{one\text{-}way}$: $F_{(3,596)}$= 34.41, *P*< 0.0001; 90 min. - $ANOVA_{one\text{-}way}$: $F_{(3,596)}$= 66.78, *P*<

0.0001; 120 min. - ANOVA$_{one\text{-}way}$: $F_{(3,596)}$= 19.83, P< 0.0001; Day 6: 30 min. - ANOVA$_{one\text{-}way}$: $F_{(3,596)}$= 3.841, P= 0.0096; 60 min. - ANOVA$_{one\text{-}way}$: $F_{(3,596)}$= 74.79, P< 0.0001; 90 min. - ANOVA$_{one\text{-}way}$: $F_{(3,596)}$= 181, P< 0.0001;



120 min - ANOVA$_{one\text{-}way}$: $F_{(3,596)}$= 74.47, P< 0.0001).

Figure 2.1.5. Assessment of *C. elegans* spontaneous activity across varied culture conditions on days 4 and 6. (Glu: animals under glucotoxicity, treated with glucose 100mM; Glu+Ct 0.1: animals under glucotoxicity, treated with 0.1 mg/kg of *C. tinctoria* extract; Glu+Ct 1: animals under glucotoxicity, treated with 1 mg/kg of *C. tinctoria* extract; Glu+Ct2: animals under glucotoxicity, treated with 2 mg/kg of *C. tinctoria* extract). DMSO (1%) was utilized as the vehicle. Results are expressed as mean ± SEM. ** $p < 0.005$, and **** $p < 0.0001$.

2.1.3.6. Lifespan assay

Since glucose has been identified as a factor influencing lifespan, we investigated the impact of cultivating worms in media enriched with glucose and the effect of *C. tinctoria* extracts on the lifespan of the WT strain with and without glucotoxicity. Despite *C. elegans* exposed to glucotoxicity conditions exhibiting a decrease in lifespan, contrary to expectations, there was no noticeable effect on the lifespan of *C. elegans* treated with *C. tinctoria* extracts compared to the control group (Figure 2.1.6, Table 2.1.1.).

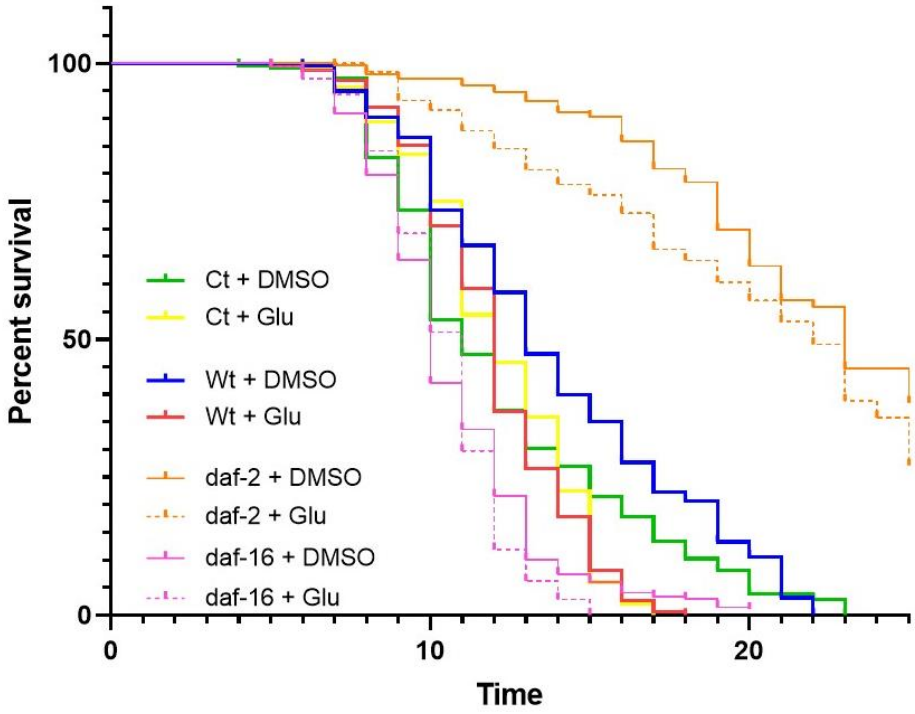


Figure 2.1.6. Kaplan-Meier survival curve depicting the response of *C. elegans* under glucotoxicity to treatment with 1 mg/ml of *C. tinctoria* extract. We observe increased survival rates of animal exposed to glucotoxicity after treatment with *C. tinctoria* methanolic extracts without affecting WT animals. Positive and negative controls consisted of daf-2 and daf-16 mutants, respectively (n = 300 per condition, across three independent assays). The assay spanned 25 days, until the mortality of all animals. (Ct + DMSO: Wild type animals and treated with *C. tinctoria* extract; Ct + Glu: Wild type animals under glucotoxicity and treated with *C. tinctoria* extract; Wt + DMSO: Wild type animals and without treatment; Wt + Glu: Wild type animals under glucotoxicity and without treatment; daf-2 + DMSO: daf-2 mutant animals and without treatment; daf-2 + Glu: daf-2 mutant animals under glucotoxicity; daf-16 + DMSO: daf-16 mutant animals without treatment; daf-16 + Glu: daf-16 mutant animals under glucotoxicity and without treatment).

Table 2.1.2. Detailed data and statistical analysis of the *C. elegans* longevity assays after treatment with *C. tinctoria* extracts.

Strain (n[a])	Median lifespan (days)± SEM	Mean lifespan (days)±SEM	Control strain (n)	Median lifespan (days)± SEM	Mean lifespan (days)±SEM	*P*-value[b]
daf-2+DMSO (159)	20±0.5	21.0±0.4	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **
daf-2+DMSO (159)	20±0.5	21.0±0.4	**daf-2+DMSO+GLU (112)**	19±0.9	18.7±0.6	***p*=0.001 ** **
daf-16+DMSO (224)	10±0.1	9.9±0.1	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **
daf-16+DMSO (224)	10±0.1	9.9±0.1	**daf-16+DMSO+GLU (221)**	11±0.2	10.5±0.1	***p*=0.001 ** **
WT+CT (193)	11±0.3	12.2±0.3	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **

WT+GLU (187)	12±0.2	11.9±0.2	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001***
WT+GLU+CT (153)	12±0.3	12.1±0.2	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001***
WT+GLU (187)	12±0.2	11.9±0.2	**WT+CT (193)**	11±0.3	12.2±0.3	*p*=0.210
WT+GLU (187)	12±0.2	11.9±0.2	**WT+GLU+CT (153)**	12±0.3	12.1±0.2	*p*=0.605

[a]Number of worms after censoring.
[b]The log-rank test (Mantel-Cox) was used to compare the groups.
$p \geq 0.05$ (NS), $p \leq 0.05$ (∗), $p \leq 0.01$ (∗∗), $p \leq 0.001$ (∗∗∗).

2.1.4. Discussion

The analysis of *C. tinctoria* extract revealed the presence of various phenolic compounds, including caffeoylquinic acids, quercetin, kaempferol, and catechin. Moreover, the extract demonstrated significant antioxidant potential through biochemical assays.

The toxicity of the extract was assessed in *C. elegans* through the clearance food assay, and the extract showed no toxicity. Additionally, under normal conditions, the extract did not induce any observable changes in the size of *C. elegans*. However, when exposed to glucotoxic conditions, where *C. elegans* typically increase in size and decrease their activity, the extract exhibited a notable effect by reducing the size of the animals while enhancing their activity levels. Moreover, there was a decrease in the lifespan of *C. elegans* in a glucotoxic environment, an effect that was neither reversed nor ameliorated by the *C. tinctoria* extract.

2.1.4.1. *C. tinctoria* phytochemical profile

In our extract, we detected quercetin, caffeoylquinic acids and their derivatives, along with catechins. The identification of these phenolic compounds in *C. tinctoria* flower extract aligns with findings from previous phytochemical studies in the literature (Lan et al., 2014; Li et al., 2014; He et al., 2020). However, scientific research has shown that the types and concentrations of phenolic compounds can differ even among different varieties of the same species, underscoring the intricate nature of *C. tinctoria* extracts (Wang *et al.*, 2022).

However, it is important to note that the chemical composition of plants can be influenced by various factors, including environmental and climatic variations (Sun *et al.*, 2023). Geographical conditions, influenced by factors such as altitude, humidity, soil composition, and climatic features, can result in distinct metabolic pathways for flavonoid compounds within the plants (Yonekura-sakakibara *et al.*, 2019; Yu *et al.*, 2020). Additionally, seasonality plays a crucial role in the cultivation and biomass production of

C. tinctoria, as factors such as water and nutrient availability can all impact the biosynthesis of metabolites in the cultivated plant (Abdel Aziz *et al.*, 2017; Li *et al.*, 2021; Li *et al.*, 2023).

Our extract was obtained from the challenging plateau terrains of the Kunlun Mountains region. Due to lower pollution levels and higher elevation, the Kunlun Mountains in Xinjiang offer a unique environment for *C. tinctoria*. The plants found in this area exhibit distinctive biological activities owing to their specific environmental conditions (Yao *et al.*, 2016).

The total flavonoid content of *C. tinctoria* can exceed 10% (Yang *et al.*, 2013), and in areas above 3000 meters above sea level, it can even reach 18.3% (Guzhalinuer *et al.*, 2013). When comparing the main flavonoid content of *C. tinctoria* between China and the USA, it was found that the total flavonoid contents of *C. tinctoria* in the USA were significantly lower than those in China (Yang *et al.*, 2013).

2.1.4.2. *In vitro* antioxidant activity of *C. tinctoria*

Reactive oxygen species (ROS) are implicated in the pathogenesis of various diseases, including Diabetes (Kalasz *et al.*, 2017). Free radicals are highly reactive molecules that can cause oxidative damage to biomolecules, while antioxidant metabolites can neutralize free radicals (Gulcin *et al.*, 2012). However, to comprehensively understand the mechanisms of action of an antioxidant, it is essential to use multiple assays that employ different reaction mechanisms (Ivanova *et al.*, 2020). In our experiment, we employed four different assays (DPPH, ICA, NO, and SO scavenging) to assess the antioxidant activity of *C. tinctoria* extract.

Our study revealed that the antiradical potency of the *C. tinctoria* extract was relatively lower compared to its iron-chelation capacity. Nonetheless, is noteworthy as the plant extract is composed of a complex array of compounds and the controls used were pure compounds. Overall, the *C. tinctoria* extract displayed higher antioxidant activity in all the assays conducted, indicating strong radical scavenging potential. Furthermore, numerous studies have reported the abundance of phenolic compounds in *C. tinctoria*, which are associated with its antioxidant properties (Chen *et al.*, 2016; Begmatov *et al.*, 2020).

Numerous studies have explored the antioxidant properties of *C. tinctoria* aqueous, methanolic, and ethanolic extracts and have demonstrated significant *in vitro* scavenging effects against several radicals, including DPPH, ABTS, and hydroxyl radical (Li *et al.*, 2019; Lan *et al.*, 2014; Yao *et al.*, 2016). Various components of *C. tinctoria*, such as total flavonoids, saponins, and polysaccharides, are known to play a pivotal role in its antioxidant activity (Luo *et al.*, 2018; Guo *et al.*, 2019; Yuan *et al.*, 2019).

Our study demonstrates that the antioxidant activity of the *C. tinctoria* extract is a result of a combination of free radical scavenging and iron-chelating activities, consistent with findings reported by other authors (Lan *et al.*, 2014; Li *et al.*, 2019).

However, non-standardized sample preparation, reaction conditions, analytical protocols, and expression of antioxidant action make it challenging to compare results between different laboratories (Xie & Schaich, 2014).

2.1.4.3. *In vivo* toxicity of *C. tinctoria* extracts

Understanding the safety of *C. tinctoria* is crucial, given its widespread use in traditional medicines. The plant is considered non-toxic in its country of origin (USA), and it is listed as a safe plant to use by the American Society for the Prevention of Cruelty to Animals® (ASPCA®, 2023) and by California Poison Control System (2023), indicating that *C. tinctoria* poses no harm to animals and humans.

Since our samples were from a different origin, we investigated the potential toxicity of *C. tinctoria* methanolic extracts in a food clearance assay using wild-type *C. elegans*. Results showed that none of the tested extract concentrations exhibited any toxicity *in vivo*, as there were no significant differences observed in survival, growth, and fecundity between the treated animals and those treated with the vehicle control. Additionally, measurements of body size were similar between treated with *C. tinctoria* extracts and controls, further supporting the absence of any toxic effects from the treatment.

Some studies (Dias *et al*; 2010a; Zhang *et al.*, 2022) were conducted to assess the potential toxicity of *C. tinctoria* at the animal level. In one study, mice were orally administered with an aqueous extract of *C. tinctoria* at a dosage of 500 mg/kg body weight, and no signs of toxicity or death were observed (Dias *et al*; 2010a). Furthermore, it was observed that the infusion of *C. tinctoria* did not exhibit any hepatotoxicity in rats (Dias *et al*; 2010a). In another 18-week feeding experiment, animals were administered aqueous extracts of *C. tintoria* via gavage at a dose of 0.1 mL/10 g bodyweight, daily, the results showed no harm to the animals, and no toxic effects on the liver and kidney were identified (Zhang *et al.*, 2022).

By contrast, Wang *et al.* (2016) investigated the toxicity of *C. tinctoria* extracts obtained using different polar solvents in ethanol extract and ethyl acetate. Their results revealed that ethanol extract and ethyl acetate extracted components could cause death when the Maximum Tolerance Dose (MTD) (19.565 g/kg and 16.414 g/kg, respectively) was exceeded, suggesting that *C. tinctoria* extracts may contain potentially toxic components with low polarity (Wang *et al.*, 2016).

In summary, the experimental findings suggest that *C. tinctoria* methanolic extract exhibits favorable safety profiles. However, caution and vigilance are necessary when using organic solvent extracts, as they may contain potentially toxic components.

2.1.4.4. Glucotoxicity model and the effect of *C. tinctoria*

There is raising evidence demonstrating that an excessive intake of glucose has an impact on the *C. elegans* lifespan (Franco-Juárez, *et al.*, 2021; Wang *et al.*, 2020) as worms also experience a reduction in functional ability as a result of a high glucose diet (Choi *et al.*, 2011; Franco-Juárez, *et al.*, 2021; Wang *et al.*, 2020). Also, previous studies highlighted *C. tinctoria* as a potential source of α-amylase and α-glucosidase inhibitors, which could potentially lower postprandial blood glucose levels (Cai *et al.*, 2016; Zhang *et al.*, 2019). However, nothing is known about the influence of *C. tinctoria* on the lifespan of *C. elegans* under glucotoxicity conditions. Therefore, using the *C. elegans* model, we explored the potential benefits of *C. tinctoria* in the context of glucotoxicity. Our findings reveal that *C. tinctoria*, can mitigate the damage caused by a high glucose diet like the increase of body size and the decrease of activity. However, the concentration tested was insufficient to mitigate the shortened lifespan induced by high dietary sugar intake.

It is widely recognized that high dietary sugar intake can contribute to conditions such as obesity, diabetes, and cardiovascular diseases (Aston, 2006; Jenkins *et al.*, 2021). However, there is limited information on the direct effects of a high-sugar diet on the aging process, particularly in terms of longevity. Our results demonstrate that glucotoxicity conditions reduce the lifespan and impair the health span of *C. elegans*. Moreover, *C. tinctoria* was found to reduce the *C. elegans* size and enhance the activity of *C. elegans*, thereby modulating responses induced by a high glucose diet. Furthermore, considering *C. tinctoria*'s reported inhibition of α-amylase and α-glucosidase (Cai *et al.*, 2016; Zhang *et al.*, 2019), it suggests its potential in regulating glucose metabolism.

The findings of our study reveal that while *C. tinctoria* extract attenuates some responses induced by glucotoxicity, it fails to mitigate the reduction in lifespan induced by glucose. It's important to note that we only tested one dose of the extract. Notably, this observation aligns with previous research indicating that metformin, a commonly prescribed antidiabetic drug, also decreases the lifespan of *C. elegans* under glucotoxic conditions (Cabreiro *et al.*, 2013). These results underscore the complexity of interventions aimed at addressing glucose-induced damage and emphasize the importance of further elucidating the mechanisms underlying these effects.

Excessive glucose intake may lead to enhanced oxidative stress by increasing cellular reactive oxygen species (ROS), thereby accelerating the aging process (De Souza-Ramalho *et al.*, 2009; Pitocco *et al.*, 2013; Mikrut *et al.*, 2016). Specifically, sustained hyperglycemia induces ROS formation by increasing advanced glycation end products, activating protein kinase C, and affecting the mitochondrial electron transport chain. This ultimately leads to damage to various macromolecules, including proteins, DNA, and lipids (Luo *et al.*, 2016; Zheng *et al.*, 2016). Therefore, removing the excess ROS induced by hyperglycemia is considered an effective strategy to alleviate diabetes complications (Lim & Park, 2023).

Numerous publications have explored the potential *in vivo* therapeutic benefits of *C. tinctoria* in hyperglycemic conditions (Dias *et al.* in 2010 and 2010a, Yao *et al.* in 2019, Li *et al.*, 2022, Zhang *et al.* in 2022, and Ma *et al.* in 2023). Beyond these precedents, our study strongly indicates that the flowers of *C. tinctoria* have a protective effect against glucose toxicity, highlighting the methanolic extracts as promising candidates for the development of anti-diabetic medications.

2.1.5. Conclusion

Overall, our findings contribute to the growing body of evidence supporting the medicinal properties of *C. tinctoria*, particularly in antioxidant and anti-diabetic applications. Further research is warranted to elucidate the underlying mechanisms and optimize extraction methods for harnessing the therapeutic potential of this botanical resource.

2.1.6. References

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Chapter 3: *Baccharis dracunculifolia* DC

This chapter encloses the following published article:

Laranjeira, I. M., Apolinário, E., Amorim, D., da Silva Filho, A. A., Dias, A. C. P., & Pinto-Ribeiro, F. (2024). *Baccharis dracunculifolia* DC consumption improves nociceptive and depressive-like behavior in rats with experimental osteoarthritis. *Foods,* 13(4), 535.

And the following manuscript in preparation:

Laranjeira, I. M., Apolinário, E., Amorim, D., da Silva Filho, A. A., Dias, A., & Pinto-Ribeiro, F. Exploring the phytotherapeutic potential of *Baccharis dracunculifolia* extract: Anti-Inflammatory effects in experimental acute inflammation.

3.1. *Baccharis dracunculifolia* DC consumption improves nociceptive and depressive-like behavior in rats with experimental osteoarthritis

3.1.1. Introduction

Pain, the main reason for seeking health care around the world, is the most common complaint of osteoarthritic (OA) patients (Katz, 2002). In addition, OA patients frequently exhibit clinical signs of comorbid emotional impairments, such as depression and anxiety (Fonseca-Rodrigues *et al.*, 2022; Katz, 2002), which are often overlooked by primary care doctors (Bair *et al.*, 2003).

Hawker *et al.* (2011) showed that OA pain causes fatigue and disability, which in turn leads to depression. In parallel, mood impairments alter central pain processing, and depressed patients experience more pain (Bair *et al.*, 2003) thus highlighting the existence of a synergistic relationship of negative effects between pain and negative emotions (Taylor *et al.*, 2017; Fonseca-Rodrigues *et al.*, 2022). While depression prevalence in primary care patients is approximately 10% (Kessler *et al.*, 2003) it increases by 4-fold in OA patients (40.7%) (Bair *et al.*, 2003). Depression is also more prevalent in women with (female/male ratio = 1.5:1) or without (female/male ratio = 2:1) chronic pain, and women report more severe clinical pain and feeling more depressed than men (Fillingim *et al.*, 2008). Importantly, patients suffering from chronic pain often display depression/anxiety (Fonseca-Rodrigues *et al.*, 2022) with recent assessments showing co-occurrence rates of 18–30% and 56–60% respectively (Bair *et al.*, 2003).

M1-polarized microglia are capable of generating pro-inflammatory cytokines, reactive oxygen species, and nitric oxide, which implies their potential role in contributing to the dysfunction of neural networks in the central nervous system (CNS) (Nakagawa and Chiba, 2014). In contrast, M2-polarized microglia express cytokines and receptors associated with inhibiting inflammation and facilitating the restoration of homeostasis (Nakagawa and Chiba, 2014; Chai *et al.*, 2022). M1 microglia are present in the brains of patients with major depressive disorder, and preclinical studies have shown that their presence in the ventral tegmental area, nucleus accumbens, amygdala, and rostroventromedial medulla contributes to depression progression and severity (Taylor *et al.*, 2017; Chai *et al.*, 2022; Wang *et al.*, 2022).

In recent years, the interest in alternative therapies has increased (Rodrigues *et al.*, 2009; Salmon, 2022). Also, the changing dietary patterns observed in recent decades (increased intake of highly processed fast foods) and their potential implications for public health (Elizabeth *et al.*, 2020) have amplified the trend of more health-conscious approaches to diets among consumers, increasing the consumption of foods recognized for their functional attributes—those that confer benefits on one or more physiological functions, contributing to individual health and well-being (Baker *et al.*, 2022; Galanakis, 2023).

Baccharis species are documented in the literature for their diverse applications, serving as sources of food, fodder for livestock, tools, and plants used for dyeing purposes (Filho *et al.*, 2008). Regarding *Baccharis dracunculifolia* DC (Asteraceae), traditional healing practices involve the use of teas produced from the leaves, employed to address issues related to the liver and stomach (Rezende *et al.*, 2014). Notably, several studies have revealed a connection between the chemical composition of *B. dracunculifolia* and Brazilian green propolis, extensively utilized for general consumption and phytopharmaceutical purposes (Guimarães *et al.*, 2012; Rodrigues *et al.*, 2020). Chemical substances found in the plant, such as flavonoids and coumaric acid derivatives, are present in Brazilian green propolis, as demonstrated in studies by Park *et al.* (2004) and Kumazawa *et al.* (2003). This correlation underscores the direct influence of the botanical source *B. dracunculifolia* on the composition and potential therapeutic properties of Brazilian green propolis (Guimarães *et al.*, 2012). Moreover, infusions and decoctions of *B. dracunculifolia* flowers are commonly used to alleviate inflammatory processes and to manage liver disorders and stomach ulcers (Nakanishi *et al.*, 2003). Indeed, this South American plant is an important source of active compounds showing antimicrobial (Ritter *et al.*, 2021), antigenotoxic, antimutagenic (Rodrigues *et al.*, 2009) immunomodulatory (Bonin *et al.*, 2020), anti-inflammatory, and free radical scavenging activity (Salazar *et al.*, 2018; Silva *et al.*, 2022) and could be a source of interesting functional food ingredients.

In this work, we aim to demonstrate that the oral administration of an extract of *B. dracunculifolia* improves OA-induced nociceptive and comorbid emotional impairments while decreasing M1 microglia in brain areas mediating pain and emotions.

3.1.2. Materials and methods

3.1.2.1. Plant material

B. dracunculifolia aerial parts were gathered from the garden of the Faculty of Pharmacy at the Federal University of Juiz de Fora (Juiz de Fora, Brazil). A voucher specimen CESJ 47482 has been deposited at the Leopoldo Krieger Herbarium in the Institute of Biological Sciences at the Universidade Federal de Juiz de Fora. The plant material was air-dried, triturated, and the resulting powder was macerated with a hydroalcoholic solution (8:2 *v/ v*, Ethanol P.A. Vetec, São Paulo, Brazil). The solution was then filtered and concentrated using a rotary evaporator (Buchi® RII, Flawil, Switzerland) under reduced pressure (Buchi® V-700 pump, Flawil, Switzerland) and bath at 50 °C. The extract was subsequently lyophilized (Christ® Alpha 2-4, B. Braun, Tuttligen, Germany) and stored in the dark at room temperature (Dias *et al.* 2007).

3.1.2.2. HPLC-DAD and UPLC-ESI-QTOF-MS analysis

The *B. dracunculifolia* extract was solubilized in methanol (3 mg/mL), filtered, and subjected to HPLC-DAD analysis to quantify the major phenolics present, following a previously described method (Ritter *et al*. 2021). The analysis was conducted using a Hitachi-Merck ELITE LaChrom system, comprising an L-2130 pump, an L-2200 autosampler, an L-2300 column oven (operating at 30 °C), and an L-2455 diode array detector (Merck-Hitachi, Tokyo, Japan). Chromatographic separation was achieved using a LiChrospher RP-18 end capped HPLC Column (5 μm particle size, 250 × 4.6 mm, Merck, Darmstadt, Germany), with water: formic acid (99.9:0.1; solvent A) and methanol: formic acid (99.9:0.1, solvent B) as mobile phases. The elution involved a gradient, starting with 10% of solvent B and maintaining the same concentration for 3 min. It then increased to 90% B at 35 min, maintaining 90% B until 45 min, decreasing to 10% B at 50 min, and maintaining 10% B at 60 min. Spectral data were collected in the wavelength range of 245–530 nm and chromatograms were recorded at 260, 280, and 350 nm. Caffeoylquinic acids and flavonoid derivatives were quantified using the external standard method, expressed as equivalents of chlorogenic acid and kaempferol pure standards (Sigma, Barcelona, Spain), respectively, as described by Dias *et al*. (2007).

For a more precise identification and confirmation of the compounds present in *B. dracunculifolia*, UPLC-MS analysis was conducted using an Acquity UPLC system equipped with a binary pump, in-line degasser, and an autosampler coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometer (ESI-Q-TOF/MS) (Waters Corp., Milford, MA, USA). *B. dracunculifolia* extract was dissolved in methanol and filtered using a 0.22 μm filter before analysis. Chromatographic separation was achieved using a Waters Acquity UPLCTM BEH C18 column (100 mm × 2.1 mm, particle size 1.7 μm), connected with a guard column (Vanguard 5 × 2.1 mm). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. The ESI-MS spectrometer was operated in both negative and positive modes with a scan range from m/z 100 to 1000. The source temperature was 120 °C; a desolvation temperature of 450 °C; capillary voltage set at 2.5 kV, and a cone voltage of 40 V. Nitrogen was used as desolvation gas (800 L/h) and cone gas (50 L/h). Collision energy was applied in ramp mode, ranging from 15–30 eV. For accurate mass measurements, data were centroided during acquisition, and 200 pg/mL of leucine-enkephalin (*m/z* 565.2771) dissolved in acetonitrile/0.1% formic acid (50:50, *v/v*), was continuously infused as an external reference (LockSpray™) into the ESI source with automatic mass correction enabled. Data were processed using Chromalynx™ application manager with MassLynx™ 4.1 software (Waters Corp., Milford, MA, USA). Compound identification was based on

MS spectra and data obtained by QTOF–MS analysis, compared with information available in the literature and several online databases (ChemSpider, MassBank, and Spectral Database for Organic Compounds).

3.1.2.3. *In vivo* assay of the pharmacological potential of *B. dracunculifolia*

3.1.2.3.1. Ethical considerations and handling

The experimental protocol received approval from the Institutional and National Ethical Commission (DGAV 23875/2019) and adhered to the European Community Council Directive 2010/63/EU regarding the use of animals for scientific purposes. Every effort was made to minimize animal suffering and to utilize only the necessary number of animals to generate reliable scientific data. The animals, specifically ovariectomized females, albino Wistar rats ($n = 6$ in each group), were housed in a controlled temperature environment at 22 °C, with 55% relative humidity, and a 12-hour light/dark cycle (lights off at 8 pm). They were kept in standard polycarbonate cages (45.4 × 25.5 × 20 cm) with a maximum of three animals per cage and provided *ad libidum* access to water and food (F0021; BioServ, Flemington, CA, USA).

General health parameters were assessed weekly, and all animals were handled daily by the researcher. On the day of the experimental sessions, the animals allowed to acclimate to the experimental room for an hour to habituate to the surroundings. All protocols were executed during the light phase of the cycle, with the exception of the sucrose preference test.

3.1.2.3.2. Anesthesia and euthanasia

For ovariectomy and induction of experimental OA, animals were anaesthetized with ketamine (0.75 mg/kg, Merial, Lyon, France) and medetomidine (0.5 mg/kg, Esteve Veterinaria, Léon, Spain) intraperitoneally (i.p.) (David.Pereira *et al.*, 2016). The size of the pupils, general muscle tone, and absence of nociceptive withdrawal reflexes were monitored to assess the depth of anesthesia. Body temperature was maintained within the physiological range using a warming blanket. Anesthesia was reversed using atipamezole hydrochloride (1 mg/kg, Pfizer, Oeiras, Portugal). For euthanasia, animals received a lethal dose of sodium pentobarbital (200 mg/mL, Ceva, Oeiras, Portugal).

3.1.2.3.3. Ovariectomy

Ovariectomy was performed to simulate post-menopause in women (Lasota and Danowska-Klonowska, 2004). Animals were positioned in ventral recumbency, and the lumbar spine area was shaved to remove hair, followed by cleaning with chlorohexidine (B. Braun). A longitudinal dorsal incision (1–2 cm) was made over the lumbar vertebrae, and the skin on each side was separated from the underlying muscle using blunt-end forceps. The ovaries, embedded in the fat pads. were gently withdrawn, exposing the

ovary, oviduct, and part of the uterus. A suture (4/0 Mersilk Soie Perma-hand (W501) Ethicon, Tuttligen, Germany) was tightly tied around the cranial portion of the uterus and uterine vessels. The exposed organs were removed, and the caudal portion of the uterus was reintroduced into the abdominal cavity. Muscle and skin were sutured (Muscle: 3/0 Mersilk absorbable (W571H); Skin: 2/0 Monocryl absorbable (W3448), Ethicon, Tuttligen, Germany), and the sutured area was cleansed with chlorhexidine (B. Braun).

3.1.2.3.4. OA induction

OA induction was carried out following the procedures outlined by Pinto-Ribeiro *et al.* (2013) and Amorim *et al.* (2014). A mixture of 3% carrageenan-kaolin (Sigma-Aldrich, St Louis, MO, USA) dissolved in sterile saline solution (0.9% NaCl pH 7.2, Unither, Amiens, France) was injected (0.1 mL) into the right knee joint. Control animals (SHAM) were injected with 0.1 mL of saline solution. Subsequently, the injected knee was flexed and extended ten times.

3.1.2.3.5. Drug preparation and administration

The *B. dracunculifolia* powdered extract was dissolved in 10 mM PBS (Applichem, Panreac, Barcelona, Spain) at pH 7.4 to achieve a final concentration of 50 mg/mL. The solution was placed on an ultrasound bath (Sonicator Branson 2510, Emerson Corporate, London, UK) for 30 min and subsequently stored in the dark at room temperature.

The concentration of the extract administered to each animal was adjusted weekly based on their body weight, ensuring that the final volumes administered (1 mL for the Bd50 group and 1.5 mL for the Bd100 group) contained 50 and 100 mg of extract per kg of rat, respectively (Rezende *et al.*, 2014). Administration occurred daily for 5 weeks through gavage. SHAM and OA animals were administered the vehicle solution.

3.1.2.4. Behavioral analysis

3.1.2.4.1. Pressure Application Measurement (PAM)

This method facilitated the accurate behavioral measurement of primary mechanical hypersensitivity in rodents experiencing chronic inflammatory joint pain. A force range of 0–1500 g was directly applied to the affected joint (Rénéric *et al.*, 2002). With the animal securely restrained, an increasing force was gradually exerted across the joint until it reached the maximum force necessary to elicit a response, such as paw withdrawal, vocalization, or wriggling movements. The results correspond to the force peak applied immediately before the response and are expressed in grams force (gf). The test was conducted twice (i) before and (ii) at the end of pharmacological treatment. Measurements were recorded as the limb

withdrawal threshold (LWT), measured twice on each knee joint at 1 min intervals. The mean LWTs were calculated per animal. To prevent bias, the analysis of PAM data was performed as follows:

PAM = (LWT after *B. dracunculifolia* treatment − LWT before *B. dracunculifolia* treatment)

3.1.2.4.2. Open Field Test (OFT)

The open field test (OFT) was employed to assess differences in locomotor and anxiety-like behaviors (Rénéric *et al.*, 2002). The OF apparatus comprised a square arena measuring 100 cm (W) × 100 cm (L) × 40 cm (H) placed in a dimly lit room, featuring a light gradient from the center to the periphery. A single rat was positioned in the center of the arena, and its behavior was recorded using a video camera. Anxiety-like behavior was evaluated base on the analysis of the following parameters: (i) number of squares crossed; (ii) number of rearings; and (iii) time spent in the center. Following each test, the area was cleaned with a 10% alcohol solution. Two blind researchers independently scored the recordings.

3.1.2.4.3. Forced Swimming Test (FST)

The evaluation of learned helplessness followed a protocol outlined by Rénéric *et al.* (2002). On the first day, animals underwent a pre-test session lasting 10 min, during which they were individually placed in a glass cylinder measuring 50 cm (H) × 29 cm (W), filled with water (21.5 ± 1.5 cm, 24 ± 0.5 °C), for 10 min. Twenty-four hours later, the animals were again placed in the cylinder for 5 min, and the session was recorded using a video camera. Behavioral measures were defined as follows: (i) immobility - lack of movement of the whole body; (ii) struggling - vigorous movements with the front paws in and out of the water; (iii) swimming - maintaining active swimming motions; (iv) latency to immobility - time taken by the animal to stop moving for the first time (Rénéric *et al.*, 2002). Two blind researchers scored the test sessions. Learned helplessness was characterized by an increase in immobility time at the expense of the time spent swimming/struggling and a decrease in the latency to immobility.

3.1.2.4.4. Sucrose Preference Test (SPT)

Anhedonia, assessed as a reduction in sucrose preference (SPT), was measured using a protocol adapted from Rodrigues-Fonseca *et al.* (2022). Prior to testing, animals were exposed to the sucrose solution (Labchem, Laborspirit, Portugal) for 2 hours. On the testing night, animals were housed individually, and two pre-weighted bottles were provided - one a 3% sucrose solution and the other sterilized water. After 12 hours, the bottles were removed and weighed. Sucrose preference was calculated using the following equation:

Sucrose_preference = [sucrose_intake/(sucrose + water)_intake] × 100

Results were adjusted based on the body weight of the animals.

3.1.2.5. Histological processing and analysis of the internal organs

At the conclusion of experimental procedures, the internal organs (thymus, lung, heart, spleen, liver, kidneys, and adrenals) were sampled, and the brain was excised and stored in 4% paraformaldehyde (PFA) (DAC, Applichem, Barcelona, Spain) for further processing.

Histopathological samples were stained with hematoxylin and eosin (Oliveira *et al.*, 2010). The samples underwent dehydration, followed by three washes with xylene and immersion in paraffin (Thermo Scientific, Leicestershire, UK). Blocks were sectioned into 4μm sections and mounted on a micro-slide glass (Superfrost Plus, Thermo Scientific, Leicestershire, UK), submerged in hexane (S.T. Chemical, Tokyo, Japan), and then in an ethylene/propylene mixture (Clear Plus, Falma Co., Tokyo, Japan), followed by three washes in 100% ethanol. The slides were transferred to a xylene solution (C8H10, Carlo Erba, Cedex, France), washed twice in ethanol, transferred to a 96% ethanol solution, and washed with tap water. The slides were then immersed in hematoxylin Harris solution (Millipore Corporation, Burlington, MA, USA), washed in tap water, immersed in a 0.5% ammonia solution (Sigma Aldrich, Darmstadt, Germany), rewashed in tap water, immersed in 96% ethanol followed by immersion in eosin Y solution (Thermo Scientific, Cheshire, UK). Slides were then dehydrated with 96% and absolute ethanol, immersed in xylene, and finally mounted with Entellan (Merck, Rahway, NJ, USA). After drying, all slides were examined under a light microscope (Olympus BX61, Olympus Co. Ltd., Tokyo, Japan).

3.1.2.6. Brain processing and immunohistochemistry for IBA-1

Following occision, the right brain hemisphere was excised, preserved in 4% PFA for a week, placed in a 20% sucrose solution for 48 hours, and sectioned using a vibratome (Leica, Carnaxide, Portugal). Coronal sections (50 μm thick) were stored in 12-well plates in 0.1M PBS at 4 °C (Amorim *et al.*, 2014). Brain sections were collected serially in 3 sets, and one set was used to assess microglial activation through IBA-1 immunohistochemistry. For IBA-1 immunohistochemistry (Amorim *et al.*, 2023), sections were washed thrice with 0.1M PBS, (Sigma Aldrich, Sintra, Portugal), incubated in a 3.3% hydrogen peroxide solution (Carlo Erba) in 0.1M PBS and washed once with 0.1M PBS and twice with PBS/T 0.3%. Subsequently, sections were incubated in 2.5% fetal bovine serum (FBS; Biochrom, Cambridge, UK), followed by an overnight incubation with the primary antibody IBA-1 (Abcam, Cambridge, UK) at room temperature. The next day, sections were washed in PBS/T (thrice), incubated in biotinylated swine anti-rabbit secondary antibody, and washed in PBS/T (thrice), followed by incubation with avidin-biotin

complex (Vector Laboratories, Peterborough, NH, USA). Then, sections were washed with PBS/T (thrice), PBS (thrice) and thrice in Tris-buffer (Ultrol Gade, Calbiochem, San Diego, CA, USA) and stained with a diaminobenzidine solution (Sigma Aldrich) in Tris-buffer. After the reaction, the sections were washed with Tris (twice) and PBS (twice). Sections were mounted on microscope glass slides (Superfrost Plus), dehydrated in increasing concentrations of ethanol (20%, 40%, 70%, 90%, and absolute ethanol), immersed in xylene (C8H10) and finally mounted with Entellan (Merck).

M1 microglial cells were quantified in cingulate (CC), prelimbic (PLC) and infralimbic (ILC) cortices, in the amygdala (AMY), and the dorsolateral (DlPAG) and lateral (LPAG) areas of the periaqueductal grey matter (PAG) based on their phenotype (Kreutzberg, 1996). A minimum of 3 sections per animal were randomly sampled and analyzed using the optical fractionator method (100 × 100 μm) with Stereo Investigator 10 software (Microbrigthfield Bioscience, Magdeburg, Germany). The analysis was conducted using a video camera (Sony, 3CCD, Exwavw HAD) coupled to a microscope (Axioplan2 Imaging, Zeiss, Olympus Co Ltd., Tokyo, Japan).

3.1.2.7. Experimental design

Upon arrival, animals were randomly distributed three per cage and quarantined. One week later, they were transferred to the housing facility, and daily handling was conducted for 15 min over a period of 2 weeks. The animals underwent ovariectomy surgery and OA induction, and were then subdivided into the ARTH, ARTH_Bd50, and ARTH_Bd100 groups.

Four weeks after OA induction, the treatment with *B. dracunculifolia* extract was initiated, and animals received daily treatment through gavage for 5 weeks. In the last week of treatment, mechanical hyperalgesia (PAM), anxiety- (OFT), and depressive-like behaviors (FST and SPT) were assessed. Subsequently, the animals were euthanized using pentobarbital, and internal organs and the brain were removed for further processing and analysis (Figure 3.1.1.).

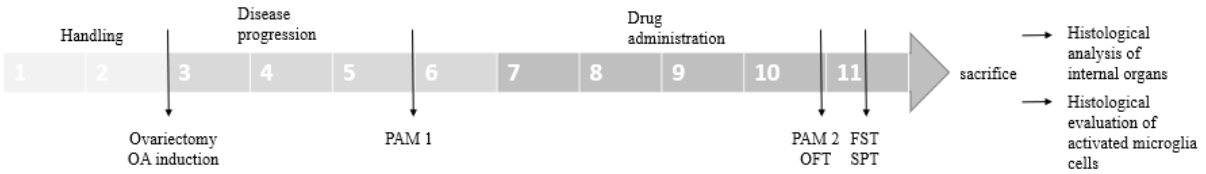


Figure 3.1.1. Schematic representation of the experimental time points. Animals were divided into 4 groups: (n = 6, each) - one control group (SHAM) and three experimental arthritis groups: one without treatment (ARTH); one treated with 50 mg/kg extract (ARTH_Bd50), and one treated with 100 mg/kg extract (ARTH_Bd100). Ovariectomy and OA induction were performed 2 weeks after the start of the experimental period. Mechanical hyperalgesia was assessed using the Pressure Application Measurement (PAM) one week before treatment administration and again 4 weeks later. Anxious-like behavior was

evaluated using the Open Field Test (OFT) in the 4th week of treatment, and depressive-like behavior was evaluated using the Forced Swimming Test (FST) and Sucrose Preference Test (SPT) in the 5th treatment week. At the conclusion of the experimental period, animals were euthanized, and the internal organs and the brain were excised for histopathological analysis and quantification of activated microglial cells.

3.1.2.8. Statistical/DATA analysis

Statistical analysis was carried out using the GraphPad Prism 5 software (La Jolla, CA, USA). Behavioral data and the quantification of Iba-1 positive cells were analyzed using a one-way ANOVA to compare groups ARTH, Bd50 and Bd100, followed by Bonferroni's post-hoc test. T-tests were employed to compare the groups SHAM and OA. Results are expressed as mean ± standard error (SEM). Correlation analysis was performed between the FST, OFT, and SPT. A significance level of $p < 0.05$ was considered significant.

3.1.3. Results

3.1.3.1. *B. dracunculifolia* extract phytochemical composition

Chromatographic data and the identification of *B. dracunculifolia* compounds were obtained using UPLC-ESI-QTOF-MS in both positive and negative modes. Additionally, HPLC-DAD data were utilized for qualitative and quantitative analysis. Pure standard compounds, such as cafeic acid, cumaric acid, chlorogenic acid, and kaempferol were employed for quantification when available. In cases where pure standards were not available, caffeoylquinic acids and flavonoids were quantified as equivalents of chlorogenic acid and kaempferol, respectively.

A representative *B. dracunculifolia* HPLC-DAD chromatogram is illustrated in Figure 3.1.2. The major compounds present in the *B. dracunculifolia* extract were initially tentatively identified based on typical UV-Vis spectra. Further confirmation of identification was achieved through UPLC-ESI-QTOF-MS, relying on molecular and fragment ions data. Detailed mass ion information and maximum absorption of each peak are provided in Table 3.1.1. Compound identification was validated using previously published data (references in Table 3.1.1.).

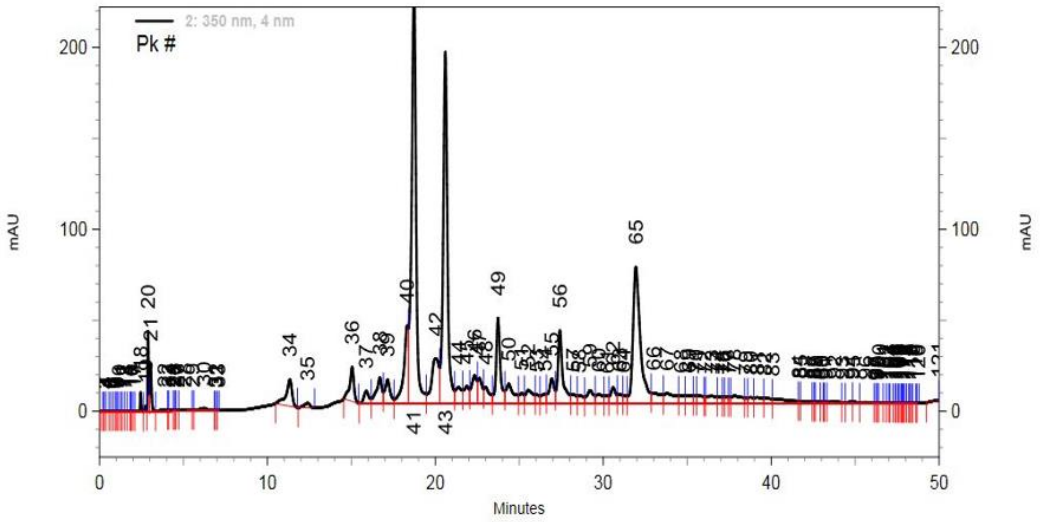


Figure 3.1.2. Typical HPLC-DAD chromatogram of *B. dracunculifolia* extract at 350 nm.

Table 3.1.1. HPLC data and quantification of the major identified compounds in *B. dracunculifolia* extract.

Peak	Compound	*m/z* Experimental	λ_{max} (nm)	Formula	mg/g	Reference
34	5-caffeoylquinic acid	353.0877	324	$C_{16}H_{18}O_9$	8.2	(Jaiswal *et al.*, 2014)
36	caffeic acid	179.0334	325	$C_9H_8O_4$	7.5	(Midorikawa *et al.*, 2001; Gardana *et al.*, 2007; Zhang *et al.*, 2017)
37	coumaric acid	163.0397	308	$C_9H_8O_3$	1.8	(Midorikawa *et al.*, 2001; Gardana *et al.*, 2007; Zhang *et al.*, 2017)
41	3,4-dicaffeoylquinic acid	515.1212	327	$C_{25}H_{24}O_{12}$	97.6	(Midorikawa *et al.*, 2001)
42	3,5-dicaffeoylquinic acid	515.1212	327	$C_{25}H_{24}O_{12}$	17.0	(Midorikawa *et al.*, 2001; Zhang *et al.*, 2017)
43	4,5-dicaffeoylquinic acid	515.1212	327	$C_{25}H_{24}O_{12}$	77.6	(Zhang *et al.*, 2017)
49	3,4,5-tricaffeoylquinic acid	677.1562	327	$C_{34}H_{30}O_{15}$	18.4	(Gardana *et al.*, 2007; Zhang *et al.*, 2017)

55	aromadendrin 4-methyl ether	301.0708	260, 341	$C_{16}H_{14}O_6$	1.5	(Costa *et al.*, 2018)
56	kaempferol	285.0751	264, 365	$C_{15}H_{10}O_6$	7.5	(Midorikawa *et al.*, 2001; Gardana *et al.*, 2007; Zhang *et al.*, 2017)
65	kaempferide	300.0634	264, 360	$C_{16}H_{12}O_6$	22.3	(Midorikawa *et al.*, 2001; Gardana *et al.*, 2007; Zhang *et al.*, 2017; Costa *et al.*, 2018)

Note: The peak numbers correspond to the ones indicated in Figure 3.1.2.

3.1.3.2. Animal welfare

Statistical analysis revealed no differences in organ weights between SHAM and ARTH groups (Figure 3.1.3A: liver, $t_{(8)} = 0.5746$, $p = 0.5814$; Figure 3.1.3C: adrenals, $t_{(8)} = 0.3481$, $p = 0.7368$ and Figure 3.1.3E: kidneys, $t_{(8)} = 1.348$, $p = 0.7368$). Similarly, no differences were found between OA groups (Figure 3.1.3B: liver, $F_{(3,14)} = 1.414$, $p = 0.2810$; Figure 3.1.3D: adrenals, $F_{(3,14)} = 0.4035$, $p = 0.6767$ and Figure 3.1.3F: kidneys, $F_{(3,14)} = 0.1666$, $p = 0.8485$). The histopathological examination revealed no abnormalities in the internal organs of our experimental animals (Figure 3.1.3G–J).

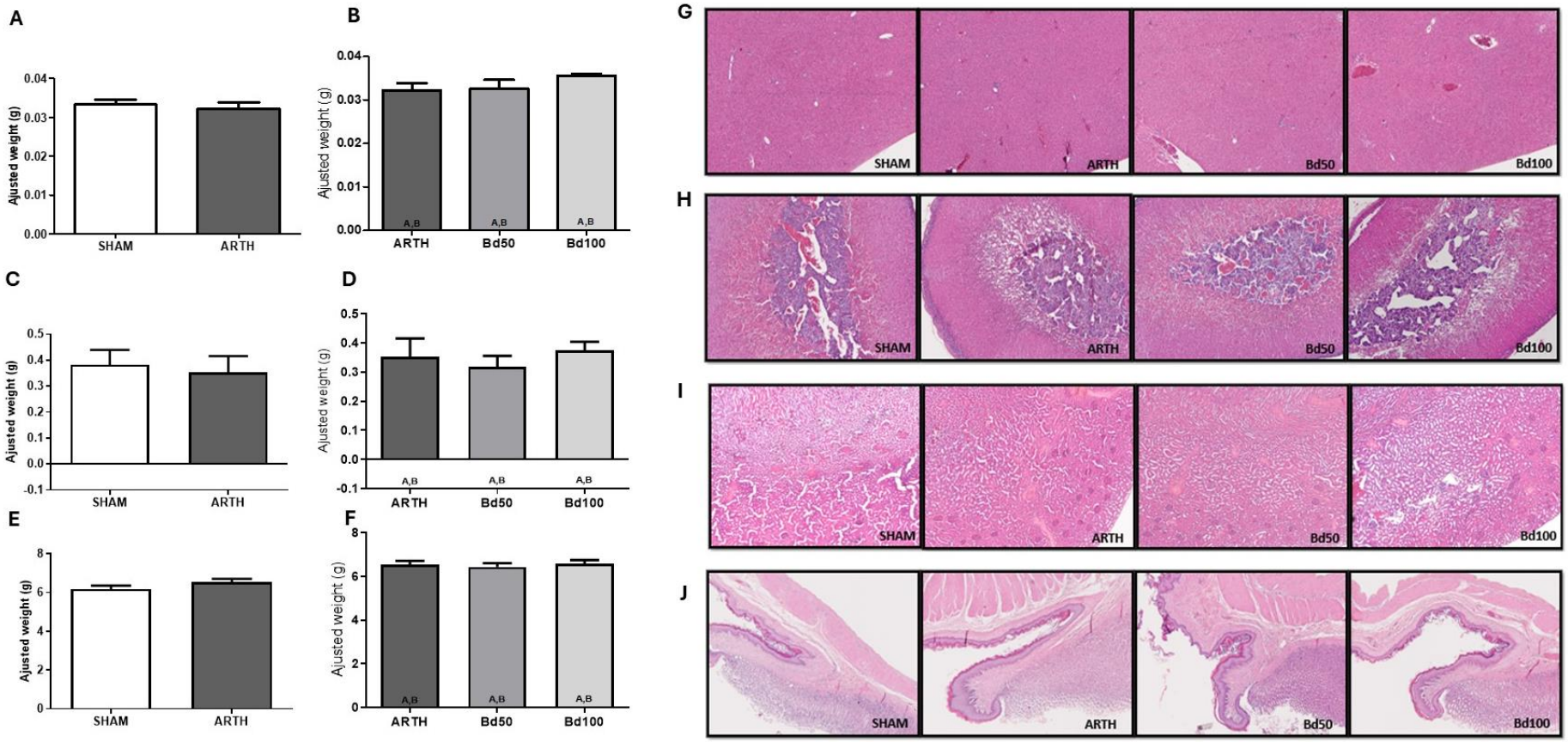


Figure 3.1.3. Weight (A–F) and photomicrographs (G–J) of internal organs. The weight of internal organs was adjusted to the animal's body weight. Liver ((A)—controls and ARTH; (B)—ARTH vs. ARTH-Bd50 and ARTH-Bd100); Adrenals ((C)—controls and ARTH; (D)—ARTH vs. ARTH-Bd50 and ARTH-Bd100); Kidneys ((E)—controls and ARTH; (F)—ARTH vs. ARTH-Bd50 and ARTH-Bd100). No significant differences were found between experimental groups in any of the organs evaluated. Photomicrographs of (G) Liver, (H) Adrenals, (I) Kidney and (J) Inferior esophagic sphincter (photomicrographs: 400× magnification). (SHAM: animals injected with saline in the right knee and treated with PBS; ARTH: animals injected with kaolin/carrageenan in the right knee and treated with PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and treated with 50 mg/kg of Bd extract; Bd100: animals injected with kaolin/carrageenan in the right knee and treated 100 mg/kg of Bd extract). Results are expressed as mean ± SEM, equal means are represented with letters (A, B).

3.1.3.3. Pressure Application Measurement (PAM)

ARTH animals exhibited a two-fold decrease in LWT compared to SHAMs (Figure 3.1.4A: $t_{(8)} = 3.164$, $p = 0.0031$). The comparison between OA groups showed that LWT varied among experimental groups (Figure 3.1.4A: $F_{(2,14)} = 4.566$, $p = 0.0335$). Post hoc tests revealed that Bd50 treatment significantly decreased LWT ($p < 0.05$).

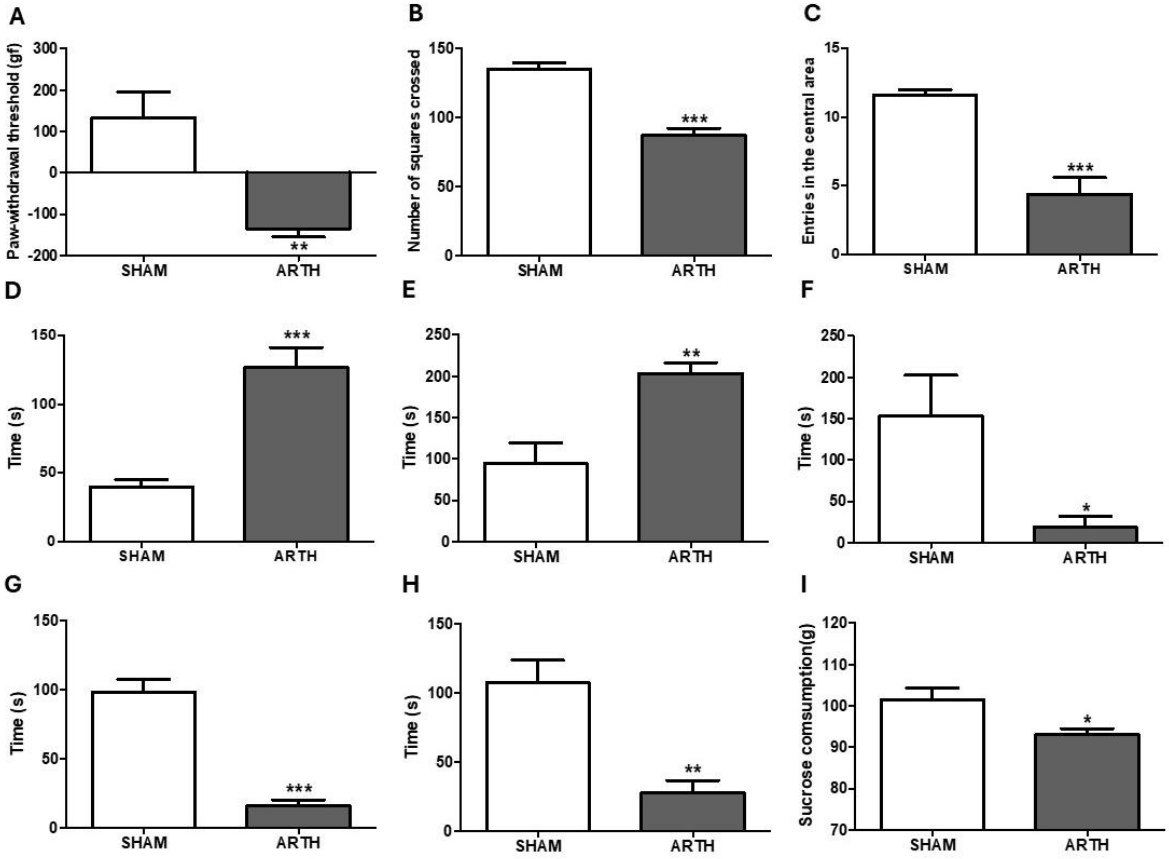


Figure 3.1.4. Evaluation of nociceptive and anxiety- and depressive-like behavior in control and arthritic rats. Mechanical hyperalgesia was assessed using the PAM test (A), and paw-withdrawal latency was significantly decreased in the arthritis (ARTH) group compared to control (SHAM) animals. In the OFT, ARTH animals displayed an anxiety-like phenotype characterized by a decreased exploratory drive with a lower number of squares crossed (B) and entries in the central area (C), and increased immobility (D) compared to SHAM. In the FST, ARTH animals exhibited learned helplessness as they spent more time immobile (E) and had increased latency to immobility (F). Additionally, ARTH animals spent less time swimming (G) and struggling (H) than SHAM. ARTH animals displayed an anhedonic-like behavior (I) as shown by the significant decrease in the preference for sucrose solution in the SPT. (SHAM: animals injected with saline in the right knee; ARTH: animals injected with kaolin/carrageenan in the right knee; PAM: Pressure Application Measurement; OFT: Open Field Test; FST: Forced Swimming Test; SPT: Sucrose Preference Test). Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$.

3.1.3.4. Open Field Test (FST)

OFT results are summarized in Figures 3.1.4. and 3.1.5. Significant differences were observed between SHAM and ARTH animals, as the total number of squares crossed (Figure 3.1.4B: $t_{(8)} = 6.915$, $p = 0.0001$) and entrances in the central area (Figure 3.1.4C: $t_{(8)} = 5.657$, $p = 0.0005$) were decreased in the ARTH group. Furthermore, ARTH animals spent more time immobile (Figure 3.1.4D: $t_{(8)} = 5.516$, $p = 0.0006$). *B. dracunculifolia* treatment did not improve ARTH animals' performance in the OFT (Figure 3.1.5B: number of crossed squares: $F_{(2,14)} = 1.194$, $p = 0.3365$; Figure 3.1.5C: number of entrances in the area center: $F_{(2,14)} = 1.311$, $p = 0.3056$; Figure 3.1.5D: immobility time: $F_{(2,14)} = 1.245$, $p = 0.227$).

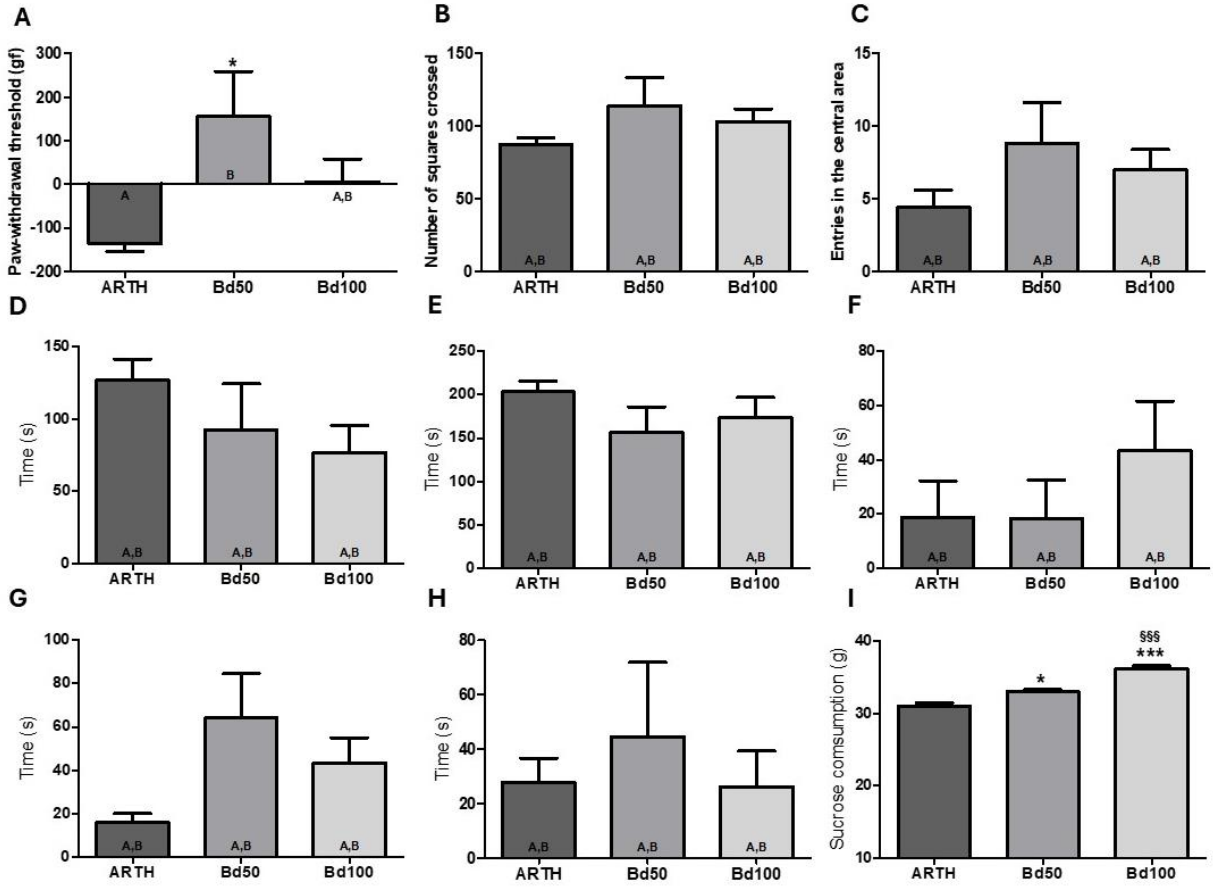


Figure 3.1.5. Evaluation of nociceptive and anxiety- and depressive-like behavior in control and Bd-treated rats. In the PAM test (A) mechanical hyperalgesia was partly (Bd100) or completely (Bd50) reversed by the Bd treatment. In the OFT, Bd treatments did not affect anxiety-like behavior (the number of squares crossed (B), the number of entries in the central area (C), and time spent inactive (D). In the FST, learned helplessness was not reversed after Bd treatment (time immobile (E); latency to immobility (F); time swimming (G) and time struggling (H). By contrast, Bd treatment reversed anhedonic-like behavior (I). (ARTH: animals injected with kaolin/carrageenan in the right knee and treated with PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and treated with 50 mg/kg of Bd extract; Bd100: animals injected with kaolin/carrageenan in the right knee and treated 100 mg/kg of Bd extract; PAM: Pressure Application Measurement; OFT: Open Field Test; FST: Forced Swimming Test; SPT: Sucrose Preference Test). Results are expressed as mean ± SEM. * $p < 0.05$, and *** $p < 0.001$, in comparison with the ARTH group and §§§ $p < 0.001$ in comparison with the Bd50 group, equal means are represented with letters (A, B).

3.1.3.5. Forced Swimming Test (FST)

The t-test revealed significant differences between the performance of SHAM and ARTH groups. In ARTH animals, latency to immobility (Figure 3.1.4F: $t_{(8)} = 2.641$, $p = 0.0297$) and the time spent active (Figure 3.1.4G: Swimming: $t_{(8)} = 8.285$, $p < 0.0028$ and Figure 3.1.4H, Struggling: $t_{(8)} = 4.260$, $p = 0.0028$) were decreased, while immobility (Figure 3.1.4E: $t_{(8)} = 3.858$, $p = 0.0048$) was increased. The administration of *B. dracunculifolia* did not reverse OA-induced emotional impairments (Figure 3.1.5F: latency: $F_{(2,14)} = 0.8387$, $p = 0.4561$; Figure 3.1.5E: immobility time: $F_{(2,14)} = 1.054$, $p = 0.3786$; Figure 3.1.5G: swimming: $F_{(2,14)} = 3.054$, $p = 0.0847$; and Figure 3.1.5H: Struggling: $F_{(2,14)} = 0.3151$, $p = 0.7356$).

3.1.3.6. Sucrose Preference Test (SPT)

In the SPT, the ARTH group exhibited a decreased preference for the sweet solution compared with the SHAM group (Figure 3.1.4I: $t_{(8)} = 2.690$, $p = 0.0275$). Interestingly, treatment with *B. dracunculifolia* significantly altered the results between experimental groups (Figure 3.1.5I: $F_{(2,14)} = 44.85$, $p < 0.0001$), with post-hoc tests showing a reversal of anhedonia.

3.1.3.7. Correlation data

In linear regression, a moderate level of agreement between the OFT and FST was observed (Figure 3.1.6A: $R = 0.5699$; $p = 0.033$; $n = 13$). The correlations between SPT and FST, and between SPT and OFT, were non-significant (Figure 3.1.6B: $R = -0.1894$; $p = 0.4680$; $n = 13$) or negligible (Figure 3.1.6C: $R = 0.2301$; $p = 0.3820$; $n = 13$), respectively.

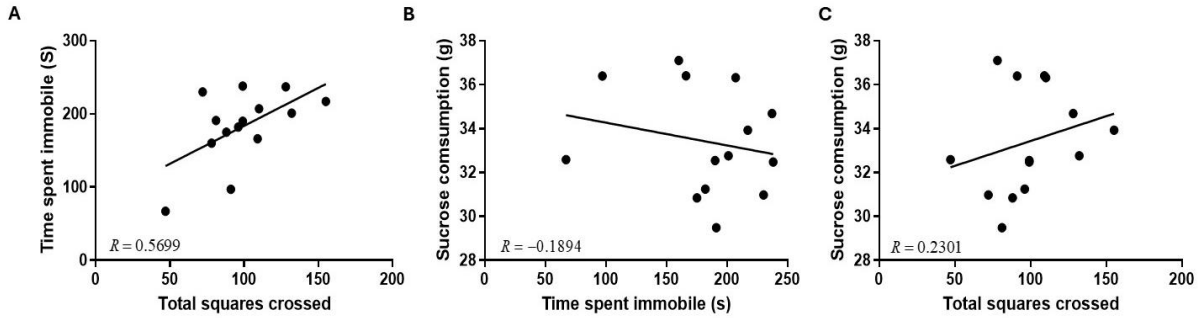


Figure 3.1.6. Correlation between the time spent immobile in the FST, the total number of squares crossed in the OFT, and sucrose consumption in the SPT test. (A) Correlation between the time spent immobile in FST and the total number of squares crossed in OFT; (B) Correlation between sucrose consumption in SPT and the time spent immobile in the FST; and (C) Correlation between sucrose consumption in SPT and the total number of squares crossed in the OFT. Correlation analysis was conducted using the ARTH-PBS, ARTH-Bd50 and ARTH-Bd100 groups. (ARTH: animals injected with kaolin/carrageenan in the right knee and treated with PBS; FST: Forced Swimming Test; OFT: Open Field Test; SPT: Sucrose Preference Test).

3.1.3.8. Immunohistochemistry staining

ARTH animals displayed an increased number of Iba-1 positive cells compared to the SHAM group in the PFC (Figure 3.1.7A: IL: $t_{(6)} = 4.386$, $p = 0.0046$; Figure 3.1.7B: PrL: $t_{(6)} = 3.573$, $p = 0.0117$; Figure 3.1.7C: Cg: $t_{(6)} = 8.199$, $p = 0.0002$), in the AMY (Figure 3.1.7G: $t_{(6)} = 5.528$, $p = 0.0015$), and the PAG (Figure 3.1.7H: DlPAG: $t_{(6)} = 9.788$, $p < 0.0001$; Figure 3.1.7I: LPAG: $t_{(6)} = 15.10$, $p < 0.0001$).

The number of Iba-1positive cells in the PFC of OA animals varied with the treatment (Figure 3.1.7D: IL: $F_{(2,11)} = 11.09$, $p = 0.0037$; Figure 3.1.7E: PrL: $F_{(2,11)} = 16.75$, $p = 0.0009$; Figure 3.1.7F: Cg: $F_{(2,11)} = 43.65$, $p < 0.0001$). Identical results were observed for the AMY (Figure 3.1.7J: $F_{(2,11)} = 21.30$, $p = 0.0004$) and the PAG (DlPAG, Figure 3.1.7K: $F_{(2,11)} = 9.788$, $p < 0.0001$ and LPAG, Figure 3.1.7L: $F_{(2,11)} = 45{,}25$, $p <$ 0,0001). Post-hoc tests showed treatment with *B. dracunculifolia* significantly decreased the number of Iba-1 immunoreactive cells in all studied areas.

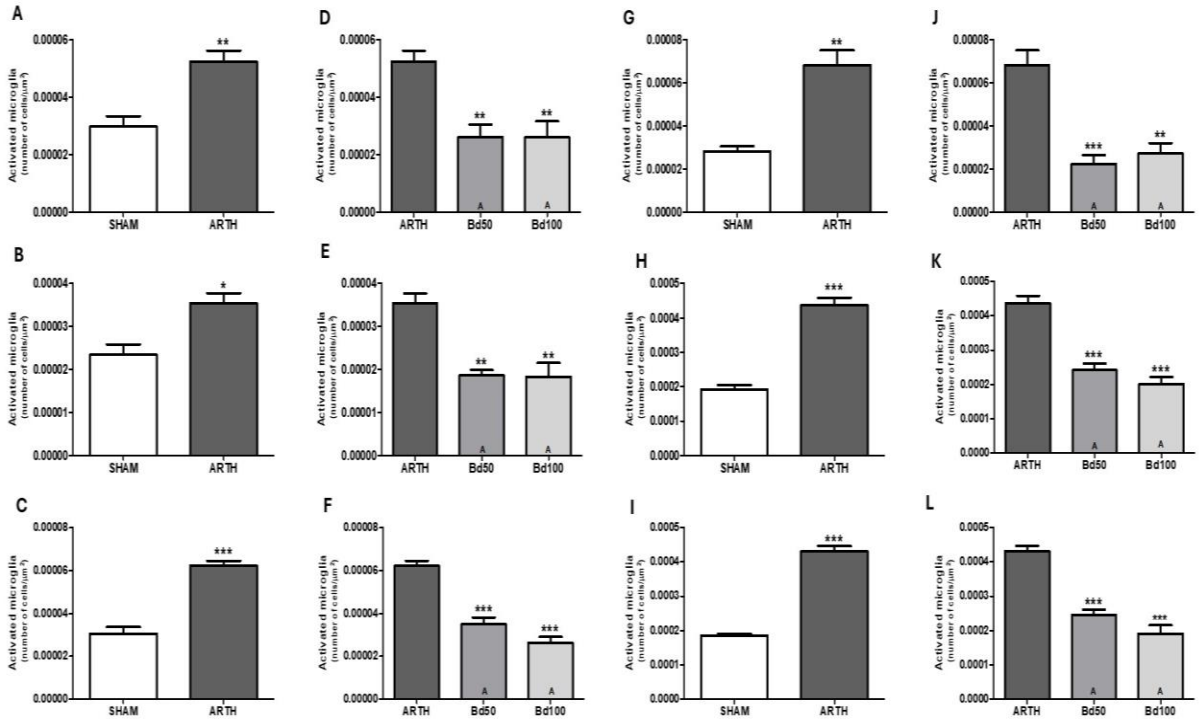


Figure 3.1.7. Number of activated microglia cells in the PFC (Prefrontal Cortex), AMY (Amygdala) and PAG (Periaqueductal Grey Matter) was assessed by immunohistochemistry staining with IBA-I. In the PFC, the number of active microglia cells in the ILC ((A,D) Infralimbic Cortex), PLC ((B,E) Prelimbic Cortex), and CC ((C,F) Cingulate Cortex) was increased in ARTH animals, and treatment with Bd reversed microgliosis in the mPFC (D-F). Similarly, in the AMY, ARTH animals displayed an increased number of active microglial cells ((G,J)) and treatment with Bd reversed this effect (J). Again, in the PAG, the number of active microglial cells was significantly increased in the DLPAG ((H,K): Dorsolateral Periaquedutal Grey Matter) and LPAG ((I,L) Lateral Periaquedutal Grey Matter), an effect reversed by treatment with Bd ((K,L)). (ARTH: animals injected with kaolin/carrageenan in the right knee and treated with PBS; Bd50: animals injected with kaolin/carrageenan in the right knee and treated with 50 mg/kg of Bd extract; Bd100: animals injected with kaolin/carrageenan in the right knee and treated 100 mg/kg of Bd extract; PAM: Pressure Application Measurement; OFT: Open Field Test;

FST: Forced Swimming Test; SPT: Sucrose Preference Test). Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$; equal means are represented with letters (A, B).

3.1.4. Discussion

In this study, we demonstrated that K/C-induced OA induces pain and the development of nociceptive, anxiety- and depressive-like behaviors in rats, primarily attributed to the increased presence of M1 microglia in brain areas associated with pain modulation and emotions. The observed behavioral enhancements following *B. dracunculifolia* treatment correlated with a reduction in the number of supraspinal M1 microglial cells. This underscores the potential of *B. dracunculifolia* extract consumption for controlling pain and associated mood disorders in OA. These findings suggest the extract's viability as a functional food or an additive to enrich common food products.

3.1.4.1. *B. dracunculifolia* extract phytochemical composition

The quantification of phenolics in *B. dracunculifolia* was conducted using HPLC-DAD and UPLC-MS (Table 3.1.1.). The phytochemical analysis of *B. dracunculifolia* extract revealed its richness in phenolic compounds, commonly associated with antioxidant and anti-inflammatory properties (Guimarães *et al.*, 2011; Albuquerque *et al.*, 2021). In this study, the phytochemical analysis of the ethanolic extract from *B. dracunculifolia* aerial parts indicated the presence of several caffeoylquinic acids, notably 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, which were present in higher concentrations. Additionally, some methoxyflavonoids (aromadendrin 4-methyl ether and kaempferide) and kaempferol were also identified. The primary compounds identified in our *B. dracunculifolia* extract align with other studies (Kumazawa *et al.*, 2003; Park *et al.*, 2004; Rezende *et al.*, 2014). Moreover, caffeoylquinic acids predominated, constituting up to 88% of the phenolics present in the extract.

Plant phenolics, particularly caffeoylquinic acids, can synergistically or additively protect against damage induced by free radicals during oxidative stress (Liu *et al.*, 2020; Boulebd *et al.*, 2023). They act as scavengers of reactive oxygen and nitrogen species, potentially reducing the risk of chronic diseases in humans (Liu *et al.*, 2020). These compounds offer various health benefits, including anti-inflammatory (Segheto *et al.*, 2018), anti-carcinogenic (Lodise *et al.*, 2019; Liu *et al.* 2020), anti-mutagenic (Akram *et al.*, 2020; Yoshimoto *et al.*, 2022), neuroprotective (Chen *et al.*, 2019; Albuquerque *et al.* 2021) and glucose-lowering effects (Wu *et al.*, 2014; Spínola and Castilho, 2017), and anti-obesity properties (Spínola and Castilho, 2017). Notably, several studies have demonstrated the antinociceptive effect of caffeoylquinic acids (dos Santos *et al.*, 2005; Kim *et al.*, 2019). Additionally, aromadendrin 4-methyl ether and kaempferide, also present in *B. dracunculifolia* extract, exhibit anti-inflammatory, antioxidant, and

gastroprotective bioactivity (Costa *et al.*, 2018). Numerous studies have shown the antinociceptive, anti-inflammatory, and antioxidant effects of kaempferol and its derivates (De Melo *et al.*, 2009; Abo-Salem, 2014; do Nascimento *et al.*, 2018).

3.1.4.2. Animals' well-being

In a prior study (Rodrigues *et al.*, 2009) the primary clinical manifestations of *B. dracunculifolia* toxicity included lethargy, reduced locomotor activity, and decreased exploratory behavior. Given that our animals had already exhibited these impairments, we chose to conduct a histopathological analysis of internal organs to evaluate toxicity (Sireeratawong *et al.*, 2016). As anticipated, considering our concentrations were 20 and 40 times lower than those reported by Rodrigues *et al.* (2009), no abnormalities were observed in our *B. dracunculifolia-treated* animals when compared to controls. Notably, a common complication associated with oral administration via gavage is the perforation of the esophagus or stomach; however, we confirmed the success of the intragastric administration.

3.1.4.3. Treatment with *B. dracunculifolia* decreases mechanical hyperalgesia

The K/C model induces mechanical hyperalgesia shortly after induction and is often employed for evaluating potential antinociceptive drugs (Radhakrishnan *et al.*, 2003). Pain originating from muscles or joints is diffuse, longer-lasting and more unpleasant, promoting patients to seek healthcare (Radhakrishnan *et al.*, 2003). Currently, joint replacement is the sole treatment for OA (Singh *et al.*, 2015). However, individuals undergoing total knee replacement face various complications, including infection, periprosthetic fracture, symptomatic implant loosening, and implant wear leading to mechanical failure (Singh *et al.*, 2015). These complications significantly diminish the benefits of total knee replacement and often necessitate surgery revision.

There is no single pharmacological treatment for OA, and patients typically undergo multiple treatments with nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics such as acetaminophen, or narcotic medications (Singh *et al.*, 2015), all of which exhibit important negative side effects (Singh *et al.*, 2015; Panchal and Sabina, 2022). Other treatments include intra-articular knee injections of corticosteroid or hyaluronic acid (HA) (Bennell *et al.*, 2012; Panchal and Sabina, 2022), which Briem *et al.* (2009) have shown to provide short-term symptom relief but may increase OA progression in the long run. Non-pharmacological therapies for knee osteoarthritis were found to decrease pain but not OA progression (Khanna *et al.*, 2009).

Daily treatment with *B. dracunculifolia* extracts partially (100 mg/kg) or completely (50 mg/kg) reversed mechanical hyperalgesia, and, importantly, with no side effects in our animals. Our data align with results

from Santos *et al.* (2010) demonstrating that *B. dracunculifolia* extract (50–400 mg/kg, p.o.) inhibited nociceptive responses in formalin-induced nociception and produced a long-lasting anti-hypernociceptive effect in acute inflammatory pain.

Medicinal plants offer several benefits to human health, in part due to their content in phenolic compounds that display various properties, including antioxidant, anti-inflammatory, and anti-carcinogenic effects (Santos *et al.*, 2010; Laranjeira *et al.*, 2023). Agents derived from plants, such as flavonoids, terpenes, quinones, catechins, alkaloids and anthocyanins, which can modulate the expression of pro-inflammatory cascades (Khanna *et al.*, 2009; Laranjeira *et al.*, 2023) show potential against arthritic-induced joint impairments but require extensive research in preclinical and clinical settings to prove their usefulness (Khanna *et al.*, 2009).

3.1.4.4. *B. dracunculifolia* treatment does not alter anxiety-like behavior

Mood disorders, including depression and anxiety, are highly prevalent in patients suffering from chronic pain (Fonseca-Rodrigues *et al.*, 2022; Amorim *et al.*, 2023). However, they are often overlooked by general practitioners, especially in OA patients (Amorim *et al.*, 2023). While patients with mood disorders report higher levels of pain and are more prone to developing chronic widespread pain, the mechanisms underlying the comorbidity between chronic pain and mood disorders remain unclear (Yalcin *et al.*, 2011).

Our arthritic animals displayed an anxious-like phenotype consistent with decreased exploratory behavior, distance travelled, and the number of entries in the central area, along with increased immobility in the OFT. Although it ameliorated, *B. dracunculifolia* administration did not reverse the anxious-like phenotype. This result contrasts with a report showing that rats with chronic mild stress receiving propolis (50 mg/kg) exhibited increases in the percentage of central quadrants crossed, time spent in the center and grooming (Reis *et al.*, 2014). It is possible that our extract does not display anxiolytic activity with the concentrations used herein, or that the composition between *B. dracunculifolia* extracts and propolis varied. Notably, *B. dracunculifolia* is one of the main sources of propolis, and its caffeoylquinic acid and apigenin have been shown to improve anxiety-like behavior (Bouayed, 2010).

OA patients often display motor impairments due to the degradation of joint/periarticular structures (Woo *et al.*, 2017). Accordingly, our OA animals also show some impairments in locomotor ability (OFT), which may bias the results of behavioral tests that require active movement, such as those assessing anxiety-like behavior. Therefore, the anxiolytic potential of the *B. dracunculifolia* extract should be tested using an anxiety animal model, such as the chronic unpredictable mild stress model, that does not induce locomotor deficits.

3.1.4.5. *B. dracunculifolia* treatment reversed depressive-like behavior

Previous studies suggest that the time frame may be critical for modeling neuropathic pain-induced affective impairments, with the expression of anxiety-like phenotypes requiring four weeks to develop and preceding the observation of depressive-like behavior, which was only noted six weeks post-induction (Yalcin *et al.*, 2011). The extension of our model to five weeks allowed us to demonstrate the development of both anxiety- and depressive-like behaviors. *B. dracunculifolia* treatment increased sucrose preference, especially at the dose of 100 mg/kg, demonstrating a clear anti-anhedonic effect of our treatment.

Anhedonia is related to the motivational aspects of major depressive disorder, and its evaluation has minimal requirements for motor coordination; thus, our findings are less likely to be confounded by potential motor deficits. Our data aligns with Reis *et al.* (2014), who reported that propolis extracts exerted antidepressant-like effects in different animal models of chronic stress, and with Lee *et al.* (2013), who demonstrated propolis antidepressant-like activity in mice subjected to stress. Besides the possibility of motor impairments masking our results concerning learned helplessness (FST), the duration of our treatment might not have been sufficient to demonstrate the effect of *B. dracunculifolia* treatment, as a major limitation of classical antidepressants is the need for 2–4 weeks of continuous treatment to elicit therapeutic effects (Ramaker and Dulawa, 2017).

3.1.4.6. OA animals display increased M1 microglial cells

Mounting evidence suggests that M1 microglial cells contribute to the plasticity of synaptic transmission in chronic pain states (Thakur *et al.*, 2012; Tozaki-Saitoh and Tsuda 2019). We observed a significant increase in the number of M1 cells in the brain of ARTH animals compared to SHAM, consistent with studies using the monoiodoacetate (MIA) model (Thakur *et al.*, 2012). Blocking M1 microglial cells has been shown to alleviate pain hypersensitivity in animal models of chronic pain, indicating that M1 microglia contributes to enhanced pain sensitivity and supporting the emerging perspective that central microglia are a key mechanism in the progression of chronic pain phenotypes (Malfait and Schnitzer, 2013; Taylor *et al.*, 2017; Tozaki-Saitoh and Tsuda, 2019).

Both concentrations of *B. dracunculifolia* treatment decreased M1 microglia, a response concomitant with reduced pain sensitivity and the reversal of anhedonic-like behavior. As the effects of *B. dracunculifolia* on nociceptive impairments resemble those of antidepressants, it is probable that *B. dracunculifolia* treatment also alters cytokine networks (Bielecka *et al.*, 2010) and inhibits proinflammatory cytokines (Bielecka *et al.*, 2010; Laranjeira *et al.*, 2023).

Importantly, M1 microglial cells and neuroinflammation play a significant role in the pathogenesis of psychiatric/neurodegenerative diseases (Bielecka *et al.*, 2010; Munshi *et al.*, 2020; Chai *et al.*, 2022; Li *et al.*, 2022). Thus, by decreasing M1 microglia, *B. dracunculifolia* extract may also prevent the sustained activation of microglial-dependent cascades known to alter neurotransmission, neuronal plasticity, and neurogenesis (Bielecka *et al.*, 2010; Chai *et al.*, 2022; Li *et al.*, 2022).

3.1.5. Conclusions

Our results demonstrate that the oral administration of *B. dracunculifolia* extract can reduce inflammation and modulate microglia M1 in the brain, representing a remarkable potential for application in the food industry and the development of nutraceuticals and functional foods. The findings suggest that incorporating this extract into food products could not only enhance its anti-inflammatory properties but also promote well-being through its antinociceptive and antianhedonic effects. These results present a valuable opportunity for creating innovative functional foods, offering benefits for both consumer health and the food industry, which is continually seeking natural and effective ingredients.

3.1.6. References

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3.2. Exploring the phytotherapeutic potential of *Baccharis dracunculifolia* extract: anti-inflammatory effects in experimental acute inflammation

3.2.1. Introduction

The skin plays a crucial role as a physical barrier against external challenges (Hay *et al.*, 2015), including microorganisms, parasites, irritants, and pollutants (Denisow-Pietrzyk, 2021). These agents have the potential to trigger inflammatory processes, leading to a range of dermatological disorders (Lim, 2021). Importantly, the skin's wound repair process is maintained by the coordinated action of various immune cells, including macrophages and neutrophils, which play essential roles in detecting and responding to pathogens and other stimuli (Aitcheson *et al.*, 2021; Nakabo *et al.*, 2022).

Inflammatory responses are mediated by a diverse array of cells and signaling molecules. Macrophages and neutrophils, as part of the innate immune system, are among the first responders to tissue injury or infection (Aitcheson *et al.*, 2021; Nakabo *et al.*, 2022). They play critical roles in phagocytosis, the process of destroying pathogens, as well as in the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12) (Aitcheson *et al.*, 2021; Ross *et al.*, 2021).

Cytokines are key signaling molecules involved in coordinating the inflammatory response (Freire and Van Dyke, 2013; Ross *et al.*, 2021). They regulate the recruitment and activation of immune cells, promote tissue repair, and modulate the intensity and duration of inflammation (Freire and Van Dyke, 2013; Ross *et al.*, 2021). Dysregulation of cytokine production can lead to excessive inflammation and tissue damage, contributing to the pathogenesis of various inflammatory diseases (Kany *et al.*, 2019).

Currently, clinical anti-inflammatory therapies include non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (Montinari *et al.*, 2019). However, these drugs carry the risk of adverse effects, particularly with prolonged use, including gastrointestinal, cardiovascular, renal, and hepatic complications (Ambriz-Pérez *et al.*, 2016; Montinari *et al.*, 2019).

Given the limitations and potential risks associated with conventional anti-inflammatory therapies, there is growing interest in alternative approaches with demonstrated efficacy and safety profiles (Calixto, 2019; Salmerón-Manzano *et al.*, 2020). Medicinal plants represent a rich source of bioactive compounds with diverse pharmacological properties, including antioxidant (Zhang and Tsao, 2016) and anti-inflammatory effects (Ambriz-Pérez *et al.,* 2016).

Baccharis dracunculifolia D.C. (Asteraceae), commonly known as "alecrim do campo," is a native Brazilian shrub renowned for its medicinal properties (Filho *et al.*, 2008). It has been traditionally used in folk medicine for its anti-inflammatory, antipyretic, and antiseptic effects (Parreira *et al.*, 2010). Numerous studies have investigated the therapeutic potential of *B. dracunculifolia* extracts and its bioactive constituents, highlighting its anti-inflammatory and antioxidant properties (Funari *et al.*, 2007; Guimarães *et al.*, 2012; Laranjeira *et al.*, 2024).

The aim of this study was to investigate the immunomodulatory and anti-inflammatory effects, both *in vitro* and *in vivo*, of a *B. dracunculifolia* aerial parts hydroethanolic extract. Specifically, our objective was to assess its ability to reverse inflammation induced by a topical challenge and decrease the production of inflammatory mediators.

3.2.2. Materials and methods

3.2.2.1. Plant material

Leaves of *B. dracunculifolia* were harvested from the botanical garden of the Faculty of Pharmacy at the Federal University of Juiz de Fora (UFJF), MG, Brazil. A voucher specimen (CESJ 47.482) was deposited at the Herbarium of the Botany Department of UFJF. The aerial parts were air-dried, then finely ground using a knife mill until a pulverized plant material (500g) was obtained. This biomass underwent maceration with a hydroalcoholic solution (8:2 v/v) containing Ethanol P.A. (Vetec, Rio de Janeiro, Brazil) for 3 days. Subsequently, the hydroalcoholic solution was filtered through filter paper and concentrated using a rotary evaporator (Buchi® RII, Flawil, Switzerland) under reduced pressure at 50°C. The resulting concentrated extract was further processed using a lyophilizer (Christ® Alpha 2-4, B. Braun, Melsungen, Hessen, Germany) to obtain a freeze-dried powder of *B. dracunculifolia*. This powder was properly stored in a sealed container in a dry, light-protected environment, at 4° Celsius, until further use.

3.2.2.2. Animals and ethical considerations

The experimental work comprised two distinct sets. In the first set, 17 adult female Wistar rats (*Rattus norvegicus*, var. *Albinus*; Charles Rivers, Barcelona, Spain) were utilized for the *in vivo* ear edema challenge. The rats were housed in polypropylene cages (45.4 x 25.5 x 20 cm) with *ad libitum* access to water and food (F0021; BioServ, Flemington, CA, USA) in the vivarium of the Life and Health Sciences Research Institute (ICVS). The vivarium maintained a controlled temperature environment at 22°C, with 55% relative humidity, and operated on a 12-hour light/dark cycle (lights off at 8 pm).

Weekly assessments of general health parameters were conducted, and all animals received daily handling by the researcher. On the day of the experimental sessions, the animals were allowed to acclimate to the experimental room for an hour to habituate to the surroundings. Notably, all protocols were performed during the light phase of the cycle.

These rats were divided into five groups: (i) naive animals (Ct), where no solution was applied to their ears, (ii) animals treated with *B. dracunculifolia* extract (Bd) applied topically, (iii) animals treated with croton oil (CO) applied to the ears, (iv) animals treated with both croton oil and *B. dracunculifolia* extract (CO+Bd) applied to the ears, and (v) animals treated with croton oil followed by dexamethasone (CO+DEX).

At the end of experimental procedure, rats were euthanized using appropriate methods: rats received an intraperitoneal administration of a lethal dose of pentobarbitone (200 mg/ml, Eutasil, Ceva, Oeiras, Portugal).

All experimental procedures adhered to ethical guidelines outlined in the European Community Council Directive 2010/63/EU (DGAV 23875/2019) for the use of animals in scientific research. All measures were taken to minimize pain and discomfort, and efforts were made to minimize the number of animals used while ensuring the generation of reliable scientific data.

3.2.2.3. Induction of ear edema

To induce acute dermal irritation in rat ears, croton oil (Sigma-Aldrich, Barcelona, Spain) was applied six hours prior to euthanasia using a methodology similar to that described by Rodrigues *et al*. (2016). Ears were randomly selected for the experiment. Briefly, one set of ears received 20 µl of pure acetone (control group, 7 ears), while another set received 20 µl of *B. dracunculifolia* dissolved in acetone (100 mg/ml) (Bd group, 7 ears). Additionally, a fresh acetone solution containing 5% croton oil was topically applied to a separate set of ears. Fifteen minutes after the application of croton oil, the ears received the following treatments on the inner surface: (i) 20 µl of pure acetone (CO group, 7 ears), (ii) 20 µl of *B. dracunculifolia* (CO+Bd group, 7 ears), or (iii) 20 µl of dexamethasone dissolved in acetone (1 mg/ml of active principle; Decadron, Climed, Portugal) (CO+DEX group, 7 ears).

3.2.2.4. Macroscopic assessment of ear edema.

Ear edema was evaluated by quantifying changes in ear thickness compared to control samples. Measurements were taken using an analog external micrometer (Analog external micrometer, 0 to 25 mm, resolution 0.001 mm, Mitutoyo, America) after 6 hours of applying the various compounds as

described by Laranjeira *et al.* (2023). Following this assessment, the animals were euthanized as described i section 3.2.2.2.

3.2.2.5. Histological processing of ear samples

Following euthanasia, 6 mm diameter ear biopsies were obtained from the ear central area using a metal punch. The samples were divided into two parts: one part was preserved in 4% paraformaldehyde (PFA) (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain), and the other part of the ear samples was snap-frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analysis.

Histopathological samples were processed and stained with hematoxylin and eosin (Oliveira *et al.*, 2010). The staining process involved several steps: first, the samples underwent dehydration in ascending grade ethanol/water solutions (ranging from 70% to 100%) using an automatic sample processor (Leica AutoStainer XL, Nussloch, Germany) for 19 hours. Subsequently, they were washed three times with xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group, France). Following this, the specimens were immersed in paraffin (Thermo Scientific, Cheshire, UK) at 62°C and allowed to solidify at −5°C. The paraffin-embedded blocks were then sectioned into 4 μm thick sections and mounted on micro-slide glass (Superfrost Plus, Thermo Scientific, Leicestershire, UK).

Prior to staining, the slides underwent deparaffinization using an automated slide processing apparatus (Leica AutoStainer XL, Nussloch, Germany). Subsequently, they were stained with hematoxylin Harris (Millipore Corporation, Massachusetts, MA, USA) for 1 minute, followed by immersion in tap water for 2 minutes. The slides were then washed with a 0.5% ammonia solution (Sigma Aldrich, Madrid, Spain) for 10 seconds, rinsed in tap water for 1 and a half minutes, and then washed with a 96% ethanol solution (for 45 seconds). Finally, the slides were immersed in eosin Y solution (Thermo Scientific, Cheshire, UK) for 30 seconds. Subsequently, the slides were dehydrated with 96% absolute ethanol, immersed in xylene, and then in an ethylene/propylene mixture before being mounted with Entellan (Merck, Rahway, NJ, USA). After drying, all slides were examined under a light microscope (Olympus BX61, Olympus Co. Ltd., Tokyo, Japan) for histopathological analysis.

3.2.2.6. Histopathological assessment of ear samples

Histopathological analysis of the ears was conducted to evaluate the presence or absence of acute inflammation following the application of croton oil and to assess the potential anti-inflammatory effect of *B. dracunculifolia*. The assessment involved two main parameters: (i) quantification of the number of infiltrations per ear, and (ii) measurement of the thickness of the dermis and epidermis following each treatment.

Measurements of epidermal and dermal thickness were conducted using an optical microscope (Olympus BX61, Tokyo, Japan) connected to a digital color camera (Olympus, DP70, Tokyo, Japan) via the CellSens Dimension software (Olympus Corporation, Münster, Germany) at a magnification of 100×. Three random measurements were taken for each ear sample (Laranjeira *et al.*, 2023).

The quantification of infiltrations, which refers to the presence or accumulation of cells within tissue samples indicating potential inflammation, was performed using a Carl Zeiss optical microscope (Axioplan 2 Imaging, Olympus Zeiss, Tokyo, Japan) connected to a computer with a camera (Sony Corporation, Tokyo, Japan) and utilizing the Stereo Investigator software (Microbrigthfield Bioscience, Madgedurg, Germany) at a magnification of 100×. The number of infiltrations was measured in three different samples from each ear.

3.2.2.7. Tissue myeloperoxidase (MPO) assay

MPO activity was assessed following the method outlined by Suzuki *et al.* (1983), with slight modifications. In brief, ear samples were homogenized for 45 seconds at 0°C in 2 mL of 80 mM sodium phosphate buffer (PBS, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), using an ULTRA-TURRAX tube drive control (IKA, Konigswinter Germany). Subsequently, the resulting homogenate was centrifuged at 12,000 g at 4°C for 15 minutes. After centrifugation, 25 µL of supernatant samples were added to 96-well microtiter plates (Sigma-Aldrich, Barcelona, Spain) containing 125 µL of 1.6 mM tetramethylbenzidine, 0.3 mM H_2O_2, and 8% *N,N*-dimethylformamide in PBS (pH 5.4). The plates were then incubated for 3 minutes at 37°C, and the reaction was halted by adding 100 µL of sodium acetate buffer (1M, pH 3.0, Sigma-Aldrich, Barcelona, Spain) at 4°C. Enzyme activity was determined colorimetrically at 630 nm using an iMark microplate reader (Bio-Rad, CA, USA). Triplicates were performed for each sample, and enzyme activity was expressed as mOD/mg tissue.

3.2.2.8. Tissue *N*-acetyl-β-D-glucosaminidase (NAG) assay

NAG activity was assessed according to the method outlined by Lloret and Moreno (1995), with slight modifications. Initially, ear samples were homogenized using the same procedure as described for the MPO assay. Following centrifugation, supernatant samples (100 µL) were transferred to 96-well microtiter plates (Sigma-Aldrich, Barcelona, Spain) containing 100 µL of 2.24 mM p-nitrophenyl-acetamide-µ-D-glucopyranoside in 0.1 M citrate buffer (pH 4.5). The mixture was then incubated for 60 minutes at 37 °C, and the reaction was terminated by adding 100 µL of glycine buffer (0.2 M, pH 10.4; Sigma-Aldrich, Barcelona, Spain). Enzyme activity was determined colorimetrically at 405 nm using an iMark microplate

reader (Bio-Rad, CA, USA). Triplicates were performed for each sample, and enzyme activity was expressed as mOD/mg tissue.

3.2.2.9. Evaluation of IL-1β, IL-6 and TNF-α levels

Ear samples were homogenized in 1 mL of extraction buffer consisting of 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.05% protease inhibitor cocktail (Sigma-Aldrich, Barcelona, Spain). Following homogenization, the resulting mixture was centrifuged at 12,000g at 4°C for 15 minutes. Supernatants obtained from the ear samples were utilized for cytokine quantification using commercial ELISA kits. Specifically, IL-1β (Thermo Scientific, MA, USA), IL-6, and TNF-α (BioVision, CA, USA) were quantified according to the manufacturer's instructions. Cytokine amounts in each sample were normalized to the protein concentration, which was determined using the Bradford method. The levels of cytokines were assessed in triplicate for each sample at 450 nm.

3.2.2.10. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA). For the analysis of the data a one-way analysis of variance (ANOVA one-way) followed by Bonferroni post-hoc test was performed. A significance level of $P < 0.05$ was considered statistically significant.

3.2.3. Results

3.2.3.1. *B. dracunculifolia* extract prevents croton oil-induced rat ear edema.

3.2.3.1.1. Assessment of ear edema and histopathological assessment of ear samples

Evaluation of ear edema challenge results are shown in Figure 3.2.1. Macroscopic ear thickness was significantly different among experimental groups (Figure 3.2.1A: ANOVA $_{one\text{-}way}$: $F_{(4,34)}=5.847$, $P = 0.0013$). Post hoc tests indicated that croton oil application significantly increased ear thickness compared to the control (Ct). Application of *B. dracunculifolia* extract (Bd) and dexamethasone (DEX) reversed this effect, while *B. dracunculifolia* extract alone did not impact ear thickness.

Similarly, analysis of Dermis thickness showed significant differences between experimental groups (Figure 3.2.1B: ANOVA $_{one\text{-}way}$: $F_{(4,29)}=18.52$, $P < 0.0001$). Post hoc tests showed croton oil significantly increased dermis thickness when compared to controls (Ct and Bd) and this effect was reversed by the application of the *B. dracunculifolia* extract and DEX.

Epidermis thickness was significantly different between experimental groups (Figure 3.2.1C: ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=20.75, P < 0.0001). The post hoc tests indicated croton oil significantly increased epidermal thickness when compared to controls (Ct and Bd). Notably, this effect was significantly prevented with the application of *B. dracunculifolia* extract (CO+Bd) and dexamethasone (CO+DEX).

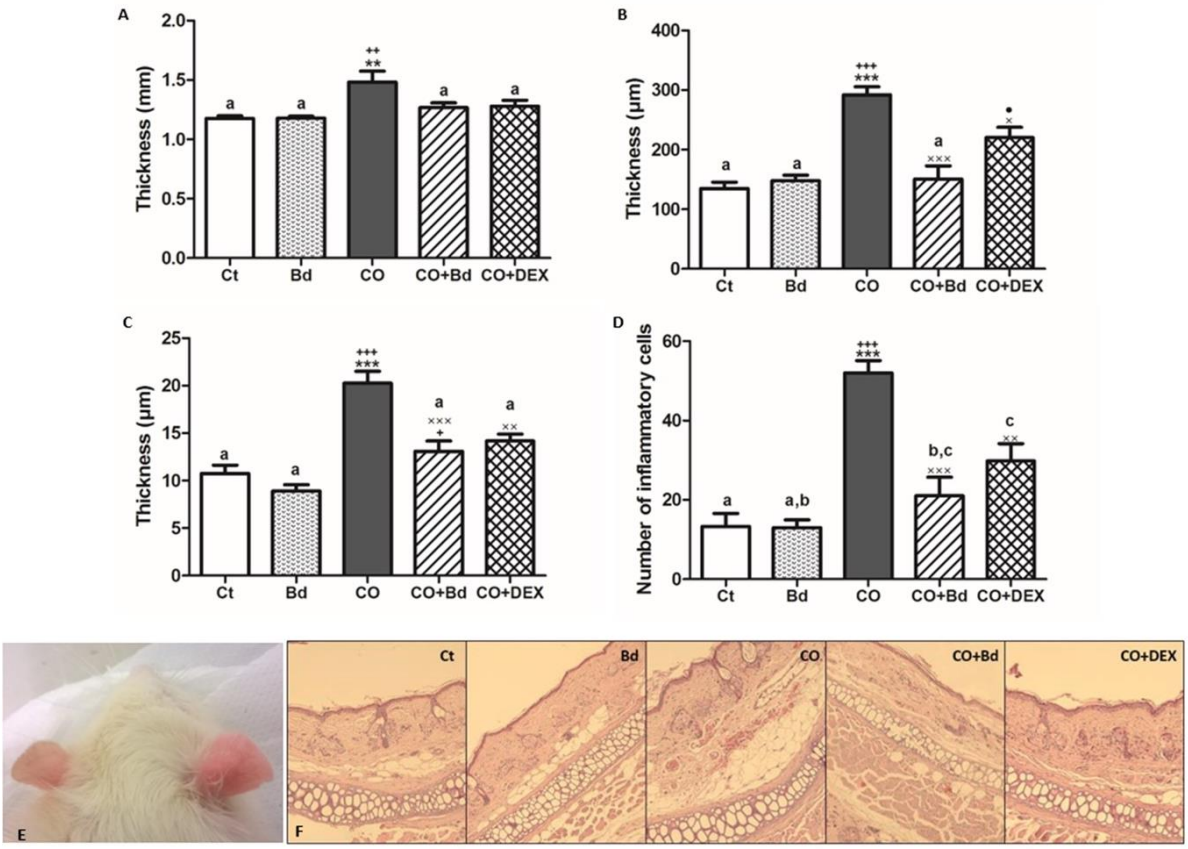


Figure 3.2.1. Effects of *B. dracunculifolia* extract and croton oil on rat ear morphology and inflammation. Macroscopic ear thickness (A), dermis thickness (B), epidermis thickness (C), and number of inflammatory cells in the epidermis and dermis of rats' ears (D) after the acute dermal irritation/corrosion test. (E) Example of ears treated with Bd extract (Bd - left ear) and croton oil (CO – right ear). (F) Photomicrographs were captured from ear sections of various experimental groups, including control animals (Ct), animals treated with *B. dracunculifolia* extract (Bd), animals treated with croton oil alone (CO), animals treated with croton oil followed by *B. dracunculifolia* extract application 15 minutes later (CO+Bd), and animals treated with croton oil followed by dexamethasone application 15 minutes later (CO+DEX). (Ct: control animals with no manipulation; Bd: *B. dracunculifolia* extract; CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone followed by application of *B. dracunculifolia* extract 15 minutes later; CO+DEX: croton oil 5% dissolved in acetone followed by application of dexamethasone 15 minutes later). Results expressed as mean ± SEM. $^{**}P$< 0.01 comparing to Ct; $^{***}P$< 0.001 comparing to Ct; $+P$< 0.05, $++P$< 0.01 comparing to Bd $+++P$< 0.001 comparing to Bd; $\times P$< 0.05, $\times\times P$< 0.01, $\times\times\times P$< 0.001 comparing to CO; $\bullet P$< 0.05 comparing to CO+Bd. Column values with the same letters are not significantly different (P> 0.05).

The number of infiltrations in the ears was significantly different between experimental groups (Figure 3.2.1D: ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=19.95, P < 0.0001). Post hoc tests showed croton oil significantly increased the number of inflammatory cells when compared to controls (Ct) and animals treated with the *B.*

dracunculifolia extract. Additionally, the application of *B. dracunculifolia* extract and of DEX, fifteen minutes after croton oil insult, significantly reversed/prevented its inflammatory effect.

Figures 3.2.1E and 3.2.1F illustrate photographs of rat ears at the end of the acute dermal irritation/corrosion test and photomicrographs of ear sections from control animals (Ct and Bd), animals treated with CO alone and animals treated with CO followed by *B. dracunculifolia* extract or DEX fifteen minutes later.

3.2.3.1.2. Effect of *B. dracunculifolia* on MPO and NAG activity

MPO (Figure 3.2.2A) and NAG activities (Figure 3.2.2B) exhibited significant differences between experimental groups (ANOVA one-way: $F_{(4,29)}$=87.81, P < 0.0001; ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=43.39, P < 0.0001, respectively). Post hoc tests revealed that croton oil application significantly increased MPO and NAG activities compared to controls (Ct and Bd). Importantly, treatment with *B. dracunculifolia* extract alone did not affect either enzyme activity. The application of *B. dracunculifolia* extract and DEX following croton oil exposure significantly mitigated the MPO and NAG activity.

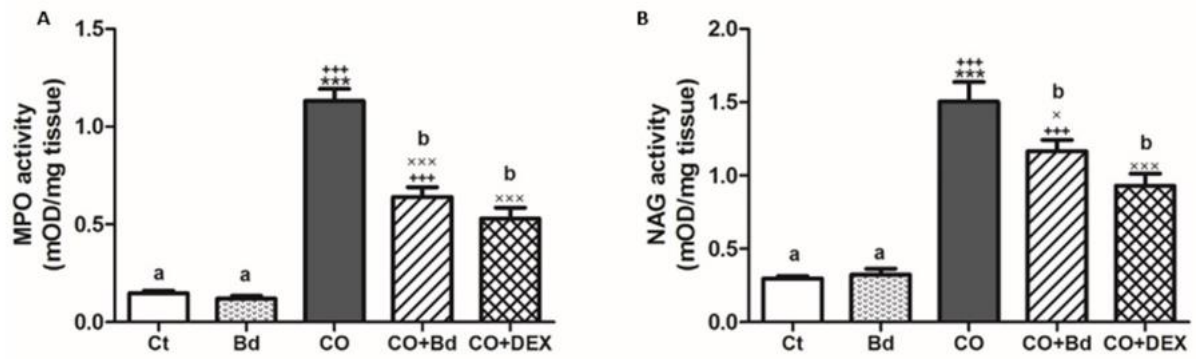


Figure 3.2.2. MPO activity (A) and NAG activity (B) during the acute dermal irritation/corrosion test. (Ct: control animals, no manipulation; Bd: topical aplication of *B. dracunculifolia* extract; CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone followed by application of *B. dracunculifolia* extract 15 minutes later; CO+DEX: croton oil 5% dissolved in acetone followed by application of dexamethasone 15 minutes later). Results are expressed as mean ± SEM. ***P< 0.001 comparing to Ct; +++P< 0.001 comparing to Bd; ×P < 0.05, ×××P< 0.001 comparing to CO. Column values with by the same letters are not significantly different (P > 0.05).

3.2.3.1.3. Effect of *B. dracunculifolia* on pro-inflammatory cytokines production

The analysis of IL-1β and IL-6 concentrations in the ear (Figure 3.2.3A and 3.2.3B, respectively) revealed significant differences among experimental groups (IL-1β: ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=103.2, P < 0.0001; IL-6 ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=46.72, P < 0.0001). Post hoc tests indicated that croton oil application significantly elevated the concentration of interleukins compared to the control (Ct), while treatment with Bd extract

and DEX reversed/prevented this effect. Notably, the application of Bd alone did not alter interleukin levels, remaining similar to the control. Tumor necrosis factor (TNF-α) quantification is depicted in Figure 3.2.3C. TNF-α concentration varied significantly among experimental groups (ANOVA $_{one\text{-}way}$: $F_{(4,29)}$=16.72, P < 0.0001). Post hoc tests revealed that croton oil increased TNF-α concentration compared to controls (Ct and Bd), whereas treatment with *B. dracunculifolia* extract and DEX, administered fifteen minutes after croton oil application, mitigated the TNF-α concentration level.

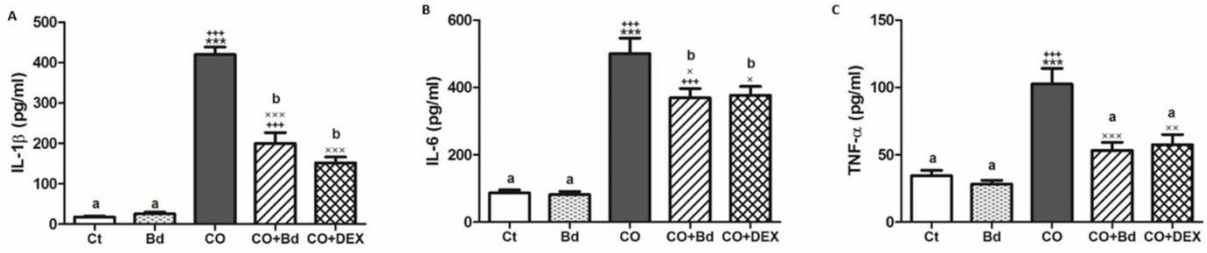


Figure 3.2.3. Quantification of interleukin-1β (A), interleukin-6 (B) and tumor necrosis factor (TNF-α) I in the acute dermal irritation/corrosion test. (Ct: control animals, no manipulation; Bd: application of *B. dracunculifolia* extract; CO: application of croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone followed by application of *B. dracunculifolia* extract 15 minutes later; CO+DEX: croton oil 5% dissolved in acetone followed by application of dexamethasone 15 minutes later). Results are expressed as mean ± SEM. *** P< 0.001 comparing to Ct; +++ P< 0.001 comparing to Bd; × P< 0.05, ×× P< 0.01, ××× P< 0.001 comparing to CO. Column values with by the same letters are not significantly different (P> 0.05).

3.2.4. Discussion

Ear edema models induced by topical administration of irritants mimic some of the features observed in human skin diseases (Rodrigues *et al.*, 2016; Silva *et al.*, 2015). In our model, the administration of croton oil induces an inflammatory reaction, including epidermal hyperplasia, a prominent inflammatory infiltrate, and increased vascularization (Di Meglio *et al.*, 2011).

Histopathological analysis of ears following the application of croton oil revealed an increase in ear thickness (both dermis and epidermis) and the number of inflammatory cells. This effect was mitigated by the topical application of *B. dracunculifolia* extract. Importantly, the application of *B. dracunculifolia* extract alone had no deleterious effects on ear macro- and microstructure, and its anti-inflammatory effects were comparable to those of dexamethasone, a drug commonly used in clinics to control edema and inflammation (Silva *et al.*, 2015).

Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and an ester of phorbol as its main components (Badilla *et al.*, 2007). Both compounds can activate protein kinase C (PKC) and subsequent cascades, including mitogen-activated protein kinases (MAPK) and phospholipase A2 (PLA2), which, through the release of platelet activation factor (PAF) and arachidonic acid (AA) (Heiman & Crews, 1985), trigger and sustain inflammation. This process increases local vascular permeability, vasodilation,

leukocyte migration, the release of histamine and serotonin (5-HT), and the synthesis of prostaglandins (PG) and leukotrienes (LT) (eicosanoids) (Stanley *et al.*, 1991; Badilla *et al.*, 2007; Rodrigues *et al.*, 2016). Topical application of cyclooxygenases (COX) and lipoxygenases (LOX) inhibitors, leukotriene B4 (LTB4) antagonists, and corticosteroids commonly reverse croton oil-induced inflammation (Murakawa *et al.*, 2006).

Considering the pathways involved in croton oil-induced inflammation, it is probable that the anti-inflammatory effect of the *B. dracunculifolia* extract is closely linked to its abundant phenolic compounds, particularly its high levels of caffeoylquinic acids and flavonoids (Funari *et al.*, 2007; Lemos *et al.*, 2007; Rodrigues *et al.*, 2016). Moreover, the phenolic compounds of this extract have been already identified through HPLC-DAD and UPLC by our group (Laranjeira *et al.*, 2024). Plant phenolics, particularly caffeoylquinic acids (CQAs), can synergistically or additively provide protection against damage induced by free radicals during oxidative stress, act as scavengers of reactive oxygen and nitrogen species, and reduce the risk of chronic diseases in humans (exhibiting anticarcinogenic, antimutagenic, glucose-lowering, and antiobesity properties) (Arakawa *et al.*, 2009; Puangpraphant *et al.*, 2011; Marković & Tošović, 2016). The antioxidant activity of CQAs is influenced by the number and position of esterification on the quinic moieties (Xu *et al.*, 2012). CQAs also play key roles in increasing plant protection against harmful UV light (Clé *et al.*, 2008; Lattanzio *et al.*, 2009), as well as in resistance of plants to bacteria (Lu *et al.*, 2021), viruses (Alcázar Magaña *et al.*, 2021; Wang *et al.*, 2023), and herbivores (Leiss *et al.*, 2009). Caffeoylquinic acids are produced as monoesters (monocaffeoylquinic acids, monoCQAs, which include chlorogenic acids) and diesters (dicaffeoylquinic acids, diCQAs) by members of plant families such as Asteraceae (Lattanzio *et al.*, 2009; Alcázar Magaña *et al.*, 2021).

In addition to caffeoylquinic acid derivatives, other phenolics belonging to the flavonoid class, such as the flavone apigenin, have been identified in our *B. dracunculifolia* extract (Laranjeira *et al.*, 2024). Several mechanisms of action have been proposed to explain the *in vivo* anti-inflammatory activity of flavonoids, including the inhibition of eicosanoid-generating enzymes (including PLA2, COX, and LOX) (Baumann *et al.*, 1980; Kim *et al.*, 2004; Sheppe and Edelmann, 2021). Inhibition of these enzymes by flavonoids reduces the production of arachidonic acid, prostanoids, and leukotrienes, which are crucial mediators of inflammation (Baumann *et al.*, 1980; Kim *et al.*, 2004). Furthermore, other studies have shown that certain flavonoids, especially flavone derivatives, exert their anti-inflammatory activity, at least in part, by inhibiting pro-inflammatory gene expression, such as PKC, MAPK, COX-2, NO synthase, and several pivotal cytokines (Kim *et al.*, 2004; Ou *et al.*, 2019; An *et al.*, 2022; Laranjeira *et al.*, 2023).

Indeed, our hypothesis was supported by our data, as treatment with *B. dracunculifolia* extract following the croton oil insult markedly reduced the levels of IL-1β, IL-6, and TNF-α in this experimental group. These cytokines are pivotal players in numerous inflammatory diseases (Sawa *et al.*, 2006; Li *et al.*, 2020; Liu *et al.*, 2021), influencing the growth and differentiation of dermal and epidermal cells, while also serving as chemotactic factors for T cells (Havran and Jamenson, 2010; Ho *et al.*, 2019), thus contributing to the perpetuation of inflammation (Ho and Kupper, 2019). Notably, the efficacy of *B. dracunculifolia* extract treatment was comparable to that of dexamethasone, a widely used glucocorticoid known to inhibit the expression of COX-2, iNOS, adhesion factors, complement factors, and pro-inflammatory cytokines, ultimately reducing inflammation (Silva *et al.*, 2015). So, the possible mechanism of action of *B. dracunculifolia* extract could be through the modulation of common inflammatory pathways.

In addition to the upregulation of pro-inflammatory cytokines, the infiltration of mononuclear cells into tissues characterizes acute cutaneous inflammation (Rodrigues *et al.*, 2016). In this context, the histopathological samples from animals treated with *B. dracunculifolia* extract after croton oil application exhibited a significant decrease in the number of inflammatory cells, a finding further supported by reduced expression of MPO and NAG in CO+Bd group. Since MPO and NAG serve as indirect markers of mononuclear cell content (including neutrophils, monocytes, and macrophages) in tissues (Krawisz *et al.*, 1984; Silva *et al.*, 2015). Our data suggest that *B. dracunculifolia* treatment interfered with cellular migration, likely stemming from decreased pro-inflammatory signaling as discussed earlier.

3.2.5. Conclusion

Our study clearly demonstrated that *B. dracunculifolia* exhibits potent anti-inflammatory activity upon topical administration. In the ear acute dermal irritation/corrosion test, *B. dracunculifolia* topical administration significantly prevented the development of ear edema, neutrophil, and macrophage activation, and reduced the levels of pro-inflammatory cytokines. Overall, our data suggest that the extract of *B. dracunculifolia* holds promise an adjuvant in the management of inflammation. However, additional studies are warranted to assess their efficacy in managing chronic inflammatory disorders.

3.2.6. References

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Chapter 4: *Genista tridentata* L.

This chapter encloses the following published articles:

Laranjeira, I. M., Dias, A. C. P., & Pinto-Ribeiro, F. L. (2023). *Genista tridentata* phytochemical characterization and biological activities: A systematic review. *Biology*, *12*(11), 1387.

Laranjeira, I. M., Gonçalves, J. N., Gonçalves, C., Silva, M., Mouta, N., Dias, A. C., & Pinto-Ribeiro, F. (2023). Anti-Inflammatory effect of *Pterospartum tridentatum* leaf extract in acute and chronic inflammation. *Applied Sciences*, *13*(7), 4494.

And the following manuscripts in preparation:

Laranjeira, I.M., Vilasboas-Campos, D., Silva, M., Rodrigues-Fonseca, D., Gonçalves, C., Teixeira-Castro, A., Maciel, P., Dias, A.C.P., & Pinto-Ribeiro, F. The multi-faceted benefits of *Genista tridentata* extract: From alleviating pain in diabetic rats to extending *C. elegans* lifespan in glucotoxic conditions

Laranjeira, I.M., Gonçalves, B., Rodrigues-Fonseca, D., Dias, A.C.P., & Pinto-Ribeiro, F. Topical application of *Genista tridentata* leaf extract: A promising strategy for diabetic wound management.

4.1. *Genista tridentata* phytochemical characterization and biological activities: A systematic review

4.1.1. Introduction

Wild edible plants are an important piece of the cultural and genetic heritage of distinct world regions, representing high importance, predominantly in rural and suburban areas (Pinela *et al*. 2017). Furthermore, they are interesting sources of bioactive compounds and need recognition as considerable contributors to human health promotion and disease prevention (Demasi *et al.*, 2021).

Genista tridentata (L.) Willk. (the recognized name for this species), also known as *Pterospartum tridentatum* (L.) Willk. (the commonly used name in both scientific literature and commercially available extracts). Among other synonyms, *Chamaespartum tridentatum* (P.) Gibbs is also used (Pinto *et al.*, 2020; Simões *et al.*, 2020). Commonly known as "prickled broom," it is a Leguminosae (Fabaceae) species belonging to the subfamily Papilionoideae (Coelho *et al.*, 2011; Ferreira *et al.*, 2012). In line with scientific literature and the Global Biodiversity Information Facility database (GBIF, 2023), the recorded countries of origin for the plant remain consistent, comprising Portugal, Spain, and Morocco. However, it is important to mention that the Plants of the World Online (POWO) database (POWO, 2023) also lists Algeria and Tunisia as potential countries of origin for this plant. This shrub can be found in the understory of *Arbutus unedo*, *Pinus,* and *Eucalyptus* forests, as well as in abandoned lands. It grows spontaneously up to 100 cm in acidic soils (Vitor *et al.*, 2004) and presents yellow flowers with a typical odor in alternate branches and coriaceous winged stems (Novais *et al.*, 2004). Traditionally, it is harvested in the spring between March and June.

G. tridentata are an aromatic plant that is very important in Portuguese gastronomy. The leaves are conventionally used as a condiment/spice for the seasoning of traditional rice and meat dishes (Grosso *et al.*, 2007). Moreover, fresh or shade-dried flowers of *G. tridentata* are also used in folk medicine, in infusions, decoctions, and tonics (Pinela *et al.*, 2011) as anti-inflammatory (Gião *et al.*, 2007; Bremner *et al*. 2009; Garcia-Oliveira *et al.*, 2022), diuretic and depurative of the liver (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Gonçalves *et al.*, 2013; Aires *et al.*, 2016). It is commonly used to ameliorate colds (Coelho *et al.*, 2011; Balanč *et al.*, 2016), in digestive disorders (Gião *et al.*, 2008; Coelho *et al.*, 2011; Serralheiro *et al.*, 2013; Balanč *et al.*, 2016), intestinal (Coelho *et al.*, 2007; Falé *et al.*, 2014) and urologic problems (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Gonçalves *et al.*, 2013; Balanč *et al.*, 2016; Garcia-Oliveira *et al.*, 2022), and rheumatism (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Gonçalves *et al.*, 2013). Additionally, it is also used for respiratory disorders (Gião *et al.*, 2007; Coelho *et al.*, 2011; Luis *et al.*,

2011; Ferreira *et al.*, 2012; Balanč *et al.*, 2016; Garcia-Oliveira *et al.*, 2022), headaches (Coelho *et al.*, 2011), to lower blood pressure (Coelho *et al.*, 2011; Ferreira *et al.*, 2012; Balanč *et al.*, 2016), against hypercholesterolemia (Coelho *et al.*, 2011; Luis *et al.*, 2011; Ferreira *et al.*, 2012; Serralheiro *et al.*, 2013; Falé *et al.*, 2014; Balanč *et al.*, 2016) and hyperglycemia (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Ferreira *et al.*, 2012; Gonçalves *et al.*, 2013; Aires *et al.*, 2016; Balanč *et al.*, 2016; Gonçalves *et al.*, 2018), and in weight loss programs (Coelho *et al.*, 2011).

This study aimed to systematically review the literature on the biological activities of *G. tridentata* extracts. It also focused on its phytochemical profile that could be relevant for understanding its use in folk medicine and its pharmacology potential, highlighting this nutraceutical potential.

4.1.2. Materials and methods

This systematic review was carried out according to the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Selçuk, 2019).

4.1.2.1. Search methods for identification of studies

4.1.2.1.1. Electronic searches

For the systematic review, 4 electronic databases (PubMed, Google Scholar, RCAAP, and ScienceDirect) were searched from inception up to 31 December 2022. The keywords used for database search were: "*Pterospartum tridentatum*" OR "*Genista tridentata*" OR "*Chamaeospartium tridentatum*".

4.1.2.1.2. Searching other resources

The authors did not proceed to the identification of studies through the screening of citations, nor were the authors contacted.

4.1.2.2. Criteria for considering studies for this review

4.1.2.2.1. Types of studies

For the evaluation of the biological activities of *G. tridentata,* we included only original research articles published in peer-reviewed journals.

4.1.2.2.2. Inclusion and exclusion criteria

The inclusion criteria were (i) original research articles, (ii) reports providing quantitative data, and (iii) reports focusing on the evaluation of the biological activities and phytochemical profile of *G. tridentata* extracts or essential oils. The exclusion criteria were (i) review articles, (ii) grey literature, (iii) reports

written in a language other than English, and (iv) reports with commercial pure compounds not directly derived from plant biomass.

4.1.2.2.3. Outcomes

The primary outcomes were synthesizing data concerning the biological activities of *G. tridentata* and its phytochemicals profiles.

4.1.2.3. Data collection and analysis

4.1.2.3.1. Selection of studies

After searching the database and removing duplicates, two independent researchers (I.M.L. and F.P.R.) screened the titles to select relevant papers, followed by screening the abstracts to evaluate their content. Any disagreements were resolved by consensus between the researchers.

4.1.2.3.2. Data extraction and management

Two authors (F.P.R. and I.M.L.) extracted data independently into an Excel file. Any discrepancies were resolved by discussion or, if necessary, by the third author (A.D.).

4.1.2.4. Quality assessment

The quality assessment of the included articles was performed using the ARRIVE GUIDELINES for animal research: Reporting *In vivo* Experiments (Sousa *et al.*, 2006), whenever applicable, as no specific guideline is in use for plant-based studies. Accordingly, we adjusted the guidelines for plant research by including two additional parameters, namely sample characterization and sample extraction.

4.1.3. Results

4.1.3.1. Literature search

A schematic representation of the literature screening process is provided in Figure 4.1.1. In the first triage, after the removal of the duplicates, 2001 results were identified. From these, 719 were excluded because of the language criteria, then 1282 were excluded as their topic was outside the scope of this review. The remaining 264 articles were screened by title and abstract, with 230 being excluded for the following reasons: 88 because of document type (review articles and grey literature), 79 did not provide data concerning biological or pharmacological activities; 1 was excluded because it reported mixed results (*G. tridentata* extract was mixed with other plant species); 13 were excluded because they reported results of isolated compounds not directly related to *G. tridentata* and, 14 were excluded because of missing data. Thus, 34 articles were included in the systematic review.

The reports included identification and analyses of the phytochemical compounds present in *G. tridentata* extracts and their bioactivities.

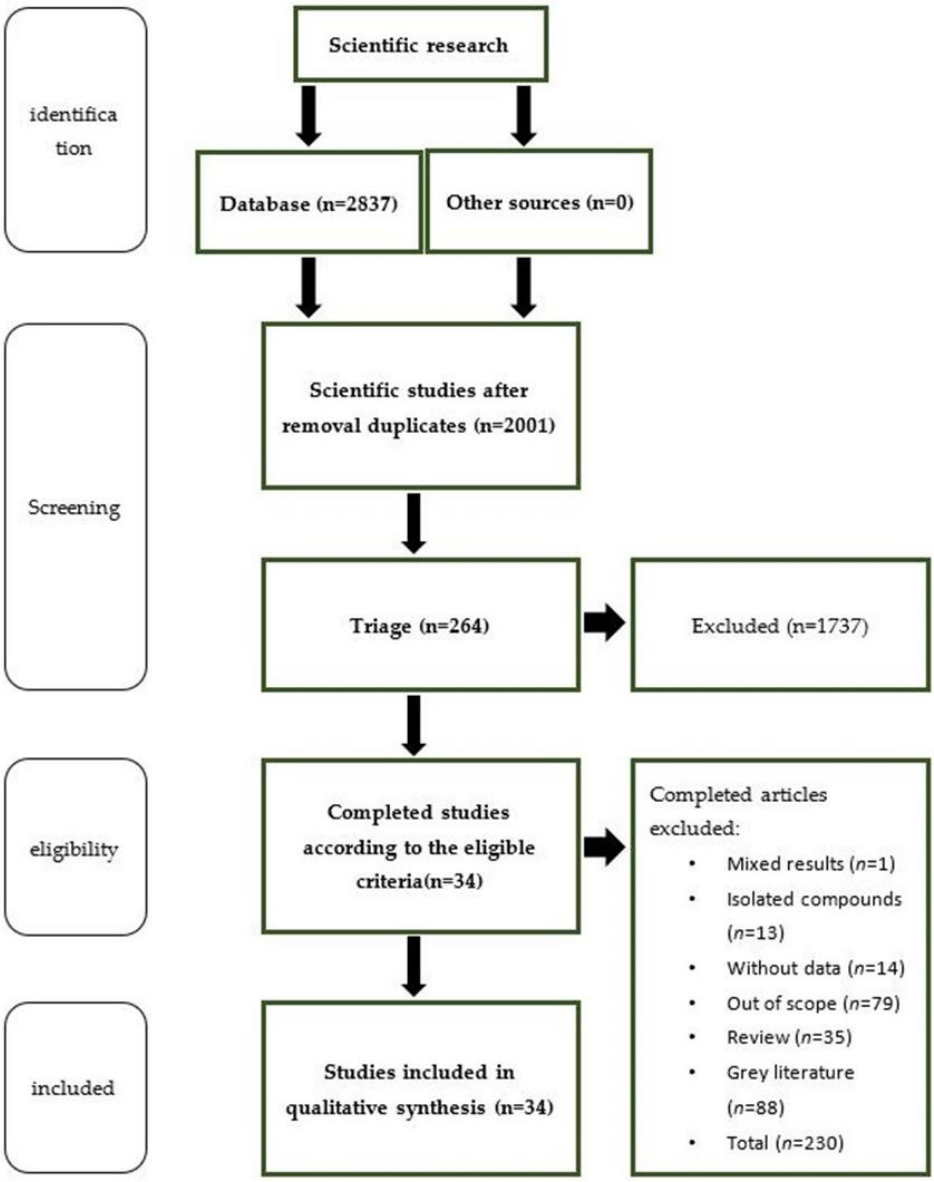


Figure 4.1.1. Schematic representation of the PRISMA flow chart depicting the literature screening process.

4.1.3.2. Extraction procedures, plant parts used, and sampling

Most extracts used in the studies were derived from aqueous extraction (61.8%) (Vitor *et al.*, 2004; Gião *et al.*, 2007; Grosso *et al.*, 2007; Gião *et al.*, 2008; Paulo *et al.*, 2008; Luis *et al.*, 2009; Barbosa *et al.* 2010; Coelho *et al.*, 2011; Gil *et al.*, 2011; Ferreira *et al.*, 2012; Faria *et al.*, 2013; Gonçalves *et al.*, 2013; Serralheiro *et al.*, 2013; Falé *et al.*, 2014; Roriz *et al.*, 2015, Aires *et al.*, 2016; Balanč *et al.*, 2016; Faria *et al.*, 2016; Gonçalves *et al.*, 2018; Caleja *et al.*, 2019; Gonçalves *et al.*, 2020; Garcia-Oliveira *et al.*, 2022) followed by methanolic (20.6%) (Bremner *et al.* 2009; Luis *et al.*, 2011; Roriz *et al.*, 2014; Martins *et al.*, 2015; Abreu *et al.*, 2016; Aires *et al.*, 2016; Gomes *et al.*, 2018), ethanolic (14.7%)

(Luis *et al.*, 2009; Gonçalves *et al.*, 2020; Simões *et al.*, 2020; Mota *et al.*, 2022) and acetone (2.9%) (Gonzalez-Hernandez *et al.*, 2003) extracts. One work used hydroglycolic extraction (Martinez *et al.*, 2012). One work did not disclose the type of solvent used to obtain the extract (Roriz *et al.*, 2014).

Considering the part of the plant used for extraction, 52.9% used flowers (Vitor *et al.*, 2004; Gião *et al.*, 2007; Grosso *et al.*, 2007; Paulo *et al.*, 2008; Barbosa *et al.* 2010; Coelho *et al.*, 2011; Luis *et al.*, 2011; Pinela *et al.*, 2011; Martinez *et al.*, 2012; Roriz *et al.*, 2014; Serralheiro *et al.*, 2013; Roriz *et al.*, 2014; Martins *et al.*, 2015; Roriz *et al.*, 2015; Gomes *et al.*, 2018; Gonçalves *et al.*, 2018; Caleja *et al.*, 2019; Gonçalves *et al.*, 2020; Garcia-Oliveira *et al.*, 2022), 20.6% aerial parts (Gonzalez-Hernandez *et al.*, 2003; Grosso *et al.*, 2007; Luis *et al.*, 2009; Gonçalves *et al.*, 2013; Faria *et al.*, 2016; Simões *et al.*, 2020), 14.7% leaves (Gonzalez-Hernandez *et al.*, 2003; Gião *et al.*, 2007; Gião *et al.*, 2008; Martinez *et al.*, 2012; Abreu *et al.*, 2016; Mota *et al.*, 2022), 5.9% stems and leaves (Grosso *et al.*, 2007; Luis *et al.*, 2011), 2.9% stems (Coelho *et al.*, 2011), and 2.9% leaves and flowers (Ferreira *et al.*, 2012). One work used *in vitro* culture (2.9%) (Gonçalves *et al.*, 2018), and four works (11.8%) did not disclose the part of the plants used to obtain the extracts (Gil *et al.*, 2011; Falé *et al.*, 2014; Aires *et al.*, 2016; Balanč *et al.*, 2016).

In terms of sampling location, 47.1% were collected in Portugal (Vitor *et al.*, 2004; Paulo *et al.*, 2008; Coelho *et al.*, 2011; Grosso *et al.*, 2007; Luis *et al.*, 2009; Luis *et al.*, 2011; Pinela *et al.*, 2011; Gonçalves *et al.*, 2013; Martins *et al.*, 2015; Abreu *et al.*, 2016; Aires *et al.*, 2016; Gomes *et al.*, 2018; Gonçalves *et al.*, 2018; Gonçalves *et al.*, 2020; Simões *et al.*, 2020; Garcia-Oliveira *et al.*, 2022; Mota *et al.*, 2022), 11.8% were collected in Spain (Gonzalez-Hernandez *et al.*, 2003; Bremner *et al.* 2009; Martinez *et al.*, 2012; Mota *et al.*, 2022), and 38.2% were obtained from herbal shops Gião *et al.*, 2007; Gião *et al.*, 2008; Barbosa *et al.* 2010 ; Gil *et al.*, 2011; Ferreira *et al.*, 2012; Faria *et al.*, 2013; Roriz *et al.*, 2014; Serralheiro *et al.*, 2013; Falé *et al.*, 2014; Roriz *et al.*, 2014 ;Roriz *et al.*, 2015; Faria *et al.*, 2016; Caleja *et al.*, 2019. One work (2.9%) (Balanč *et al.*, 2016) did not disclose how extracts were obtained.

Additionally, 35.3% of the samples were collected during the flowering period (Gonzalez-Hernandez *et al.*, 2003; Coelho *et al.*, 2011; Grosso *et al.*, 2007; Pinela *et al.*, 2011; Martinez *et al.*, 2012; Gonçalves *et al.*, 2013; Roriz *et al.*, 2014; Roriz *et al.*, 2015; Gonçalves *et al.*, 2018; Simões *et al.*, 2020; Garcia-Oliveira *et al.*, 2022) and 8.8% during the dormancy period (Serralheiro *et al.*, 2013 ;Aires *et al.*, 2016; Gonçalves *et al.*, 2018). The remaining 61.8% of the works do not disclose this information (Vitor *et al.*, 2004; Gião *et al.*, 2007; Gião *et al.*, 2008; Paulo *et al.*, 2008; Bremner *et al.* 2009; Luis *et al.*, 2009; Barbosa *et al.* 2010; Gil *et al.*, 2011; Luis *et al.*, 2011; Ferreira *et al.*, 2012; Faria *et al.*, 2013; Falé *et*

al., 2014; Martins *et al.*, 2015; Abreu *et al.*, 2016; Aires *et al.*, 2016; Balanč *et al.*, 2016; Faria *et al.*, 2016; Gomes *et al.*, 2018; Caleja *et al.*, 2019; Gonçalves *et al.*, 2020; Mota *et al.*, 2022).

4.1.3.3. Phytochemical characterization

The main compounds found are flavonoids, as well as hydroxycinnamic acids and hydroxybenzoic acids (Table 4.1.1., Figure 4.1.2). Additionally, extracts collected in the flowering period (May), as well as flowers, presented a more diverse phytochemical profile than extracts collected during the rest of the year.

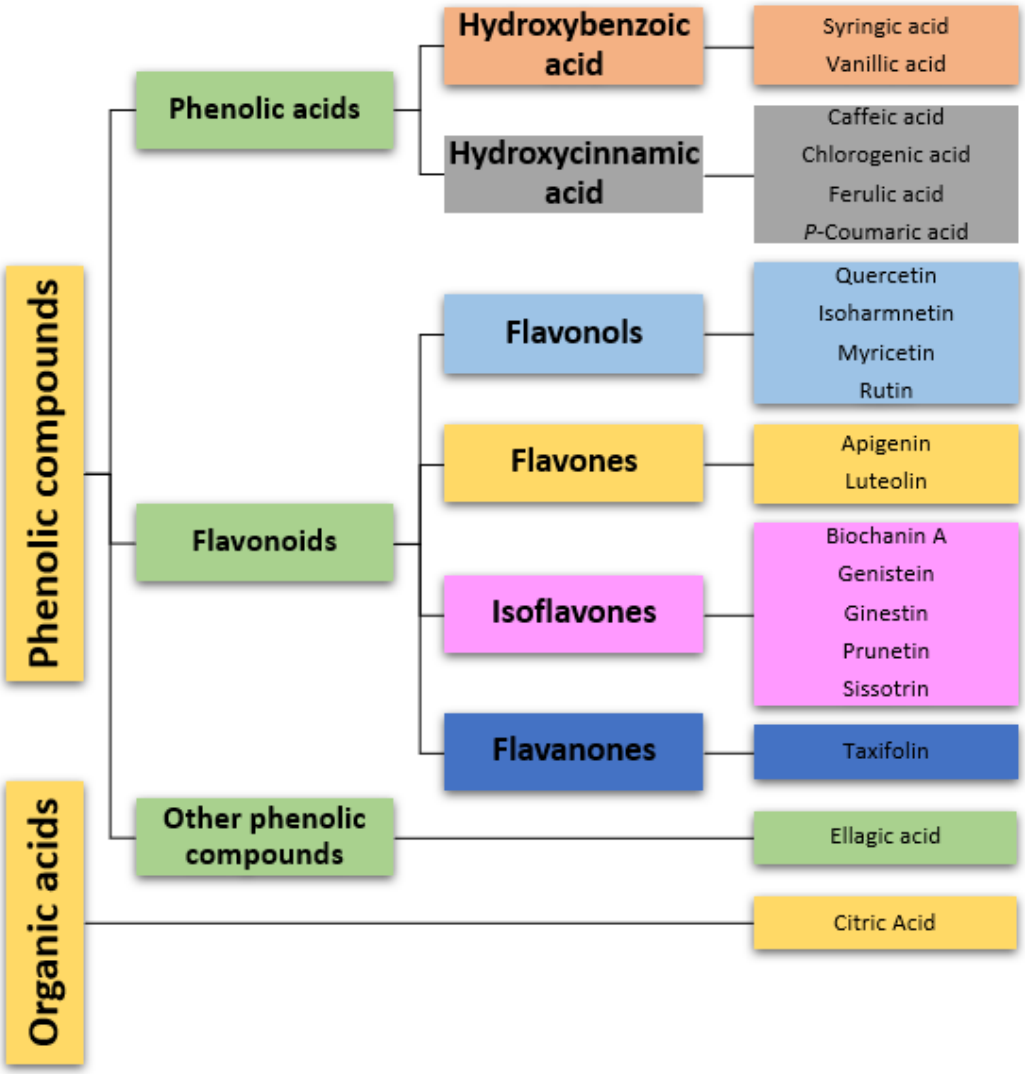


Figure 4.1.2. Classification of phytochemical compounds identified in *Genista tridentata*. Each distinct category is associated with a unique color.

Other classes of compounds found in the literature include essential oils (EOs) (Table 4.1.2.). Grosso *et al.* (2007) studied the EOs isolated by hydrodistillation and distillation-extraction of flowers, stems and leaves, and aerial parts of different populations. Another study (Barbosa *et al.* 2010) also characterized

EOs of *G. tridentata* using the same methodology. The results show that *G. tridentata* samples presented a yellowish oil with a yield of <0.05% (*v/ w*). The dominant components of the oils were phenylpropanoids, more abundant in aerial parts, and oxygen-containing monoterpenes in the flowers, stems, and leaves. Additionally, Faria *et al*. (2013, 2016) reported cis-theaspirane and trans-theaspirane as the main components.

Table 4.1.1. Major biologically active compounds were found in several samples of *G. tridentata* (X—detected; NA—not available; ND—not detected). The table's color scheme was aligned with Figure 4.1.2.

Authors	**Aires *et al.*, 2016**	**Luis *et al.*, 2011**		**Vitor *et al.*, 2004**	**Garcia-Oliveira *et al.*, 2022**	**Serralheiro *et al.*, 2013**	**Gonçalves *et al.*, 2018**		**Paulo *et al.*, 2008**	**Roriz *et al.*, 2015**	**Gonçalves *et al.*, 2020**	**Gonçalves *et al.*, 2018**						**Ferreira *et al.*, 2012**	**Falé *et al.*, 2014**	**Garcia-Oliveira *et al.*, 2022**	**Gonçalves *et al.*, 2020**	**Roriz *et al.*, 2014**
Extraction method	Methanolic							Aqueous												Ethanolic		NA
Part plant used	Crude	flowers	Stems and leaves	Flowers								Aerial parts				*In vitro* culture		Leaves + Flowers	NA	Flowers		Flowers
Sampling localization	Vila Real	Serra da Estrela	Serra da Estrela	Cinfães	Montesinho	Herbal Shop—DIÉTICA ®	Malcata	Gardunha	Cinfães	Herbal Shop—Ervital	Viseu	Malcata		Gardunha		Malcata	Gardunha	Herbal shop—Ervital	Herbal Shop—DIÉTICA ®	Montesinho	Viseu	Herbal shop—Ervital
Sampling period	NA				Spring 2019	NA	May	May	NA	Spring 2012	NA	February	May	February	May	NA				Spring 2019	NA	Spring 2012
5,5′-Dihydroxy-3′-methoxy-isoflavone-7-O-β-glucoside	ND	ND	ND	X	ND	ND	X	X	ND	X	ND	X	X	X	X	X	X	ND	ND	ND	ND	X
5,5′-Dihydroxi-3′-methoxyisoflavone	ND	ND	ND	ND	X	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND
7-Methylorobol	ND	ND	ND	ND	ND	ND	X	ND	X	X	ND	ND	ND	X	X	X	ND	ND	ND	ND	ND	X
Apigenin 5,7-dimethyl	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Apigenin 5,7-dimethyl ether 4′galactoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND
Biochanin A	X	ND	ND	ND	ND	X	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	X
Biochanin A O-acetylhexoside-O-hexoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
Biochanin A O-hexoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X

Biochanin A O-hexoside-O-hexoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
Biochanin A-glucoside	X	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND
Caffeic acid	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chlorogenic acid	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Citric acid	ND	ND	ND	ND	ND	ND	X	X	ND	ND	ND	ND	X	X	X	X	X	ND	ND	ND	ND	ND
Dihydroquercetin 6-C-hesoxide	ND	ND	ND	ND	X	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	X
Ellagic acid	ND	X	X	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND
Ferulic acid	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Genistein-8-C-glucoside	ND	ND	ND	ND	ND	X	X	X	ND	X	ND	X	X	X	X	X	X	ND	X	ND	ND	X
Ginestein	X	ND	ND	ND	ND	ND	X	X	X	X	X	X	ND	ND	X	X	ND	ND	ND	ND	X	X
Ginestein derivatives	X	ND	ND	ND	X	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	X	X	ND
Ginestin	X	ND	ND	X	ND	ND	ND	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
Isoquercitrin	ND	ND	ND	X	ND	X	X	X	X	X	X	ND	X	ND	X	X	X	ND	X	ND	X	X
Isorhamnetin-O-hexoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Luteolin-O-(O-acetyl)-glucuronide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Luteolin-O-glucuronide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Methylbiochanin A/methylprunetin	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
Methylbiochanin A/methylprunetin derivative	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X

Methylbiochanin A/methylprunetin O-hexoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
Myricetin-6-C-glucoside	ND	ND	ND	ND	X	X	X	X	X	X	ND	X	X	X	X	X	X	ND	X	X	-	X
p-Coumaric acid	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pentahydroxy-flavonol-di-O-glucoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Prunetin	ND	ND	ND	X	ND	ND	X	ND	X	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	X
Quercetin	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Quercetin 3-O-galactoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	X	ND	ND	ND	ND	ND	ND	ND
Quercetin deoxyhexosyl-hexoside	ND	ND	ND	ND	X	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	X
Quercetin O-hexoside	ND	ND	ND	ND	X	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	X
Quercetin-3-O-rutinoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	X
Quercetin derivates	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND	ND	ND	ND	X	X	ND
Quinic acid	ND	ND	ND	ND	ND	ND	X	X	ND	ND	ND	X	X	X	X	X	X	ND	ND	ND	ND	ND
Rosmarinic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Rutin	ND	ND	ND	ND	ND	ND	X	X	X	ND	ND	X	X	X	X	X	X	ND	ND	ND	ND	ND
Sissotrin	ND	ND	ND	X	ND	ND	X	X	X	X	ND	ND	ND	X	X	ND	ND	ND	ND	ND	ND	X
Syringic acid	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Taxifolin	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Taxifolin-6-C-glucoside	ND	ND	ND	ND	ND	X	X	X	ND	ND	ND	X	X	X	X	ND	ND	ND	X	ND	ND	ND
Vanillic acid	ND	X	X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 4.1.2. Composition (%) of the essential oils of *G. tridentata* isolated by hydrodistillation, collected in different years and locations. (AMF02: Flowers, collected in Arneiro das Milhariças in 2002; AMF03: collected in Arneiro das Milhariças in 2003; AML02: collected in Arneiro das Milhariças in 2002; AML03: collected in Arneiro das Milhariças in 2003; PAPN: collected in Pedra de Altar, Proença a nova; PSFPN: collected in Póvoa, Sobreira Formosa, Proença a nova; SCB: collected in Sarzeda, Castelo Branco; MCSB: collected in Milhasa do Corvo, Sarzeda, Castelo Branco; ND—not detected).

Components		**Flowers**			**Leaves + Stems**		**Aerial Parts**				
	Authors	**Grosso *et al.*, 2007**		**Barbosa *et al.*, 2010**	**Grosso *et al.*, 2007**						
	RI	**AMF02**	**AMF03**	**Herbal Shop**	**AML02**	**AML03**	**PAPN**	**PFSPNa**	**PFSPNb**	**SCB**	**MCSB**
trans-2-Hexenal	866	1.6	0.5	0.1	ND	1.6	ND	ND	1.7	3.2	ND
cis-3-Hexen-1-ol	868	1.6	1.2	ND	ND	5.3	ND	ND	0.8	3	ND
cis-2-Hexen-1-ol	882	1.5	1.2	ND	ND	0.8	ND	ND	0.6	1.2	ND
n-Hexanol	882	0.5	1.6	ND	ND	1.1	ND	ND	1.1	0.7	ND
n-Heptanal	897	11.8	4.8	0.9	ND	0.5	0.8	ND	ND	0.3	ND
n-Nonane	900	ND	ND	ND	ND	0.2	ND	ND	2.3	0.2	ND
Benzaldehyde	927	0.5	0.8	0.3	ND	0.6	1	ND	0.6	0.1	ND
α-Pinene	930	ND	0.3	0.3	ND	0.8	ND	ND	0.5	0.1	ND
n-Heptanol	952	0.5	1.6	ND	ND	1.5	ND	ND	ND	ND	1.3
1-Octen-3-ol	961	10.7	21	9.2	11.5	22.6	1.7	29.7	15	25.8	36.8
2-Pentyl furan	972	2.4	1.3	0.8	2.5	0.5	ND	ND	0.7	2.1	1.4
n-octanal	973	ND	ND	0.6	ND	ND	ND	ND	ND	ND	ND
3-Octanol	974	1.4	1.5	ND	1.9	ND	ND	ND	1.9	0.3	1.5
Benzyl alcohol	996	ND	ND	ND	0.3	0.4	ND	ND	ND	0.3	ND

Benzene acetaldehyde	1002	1.8	1.8	ND	0.3	1.2	ND	ND	0.4	1.4	0.6
p-Cymene	1003	ND	ND	0.3	ND	ND	ND	ND	ND	ND	ND
1,8-Cineole	1005	0.9	1	0.7	1.1	0.2	ND	ND	ND	ND	ND
Limonene	1009	0.9	1	ND	1.1	0.2	ND	ND	0.3	ND	ND
Acetophenone	1017	ND	1.4	ND	2.1	0.5	ND	ND	ND	ND	ND
n-Octanol	1045	0.5	0.4	0.7	2.1	0.3	0.6	ND	ND	ND	ND
p-Cymenene	1050	ND	ND	0.6	ND	ND	ND	ND	ND	ND	ND
Heptanoic acid	1056	0.5	1.2	ND	ND	0.4	ND	ND	ND	ND	2.1
Phenyl ethyl alcohol	1064	0.7	1.2	ND	2	1.7	ND	3.6	3.3	3.4	6.3
n-Nonanal	1073	14.5	6.1	6.5	4.6	0.9	10.5	4.1	0.2	0.9	1
Linalol	1074	2.9	0.5	7.1	ND	2	ND	5.2	ND	2.3	1
cis-Rose oxide	1083	2.9	0.5	ND	ND	ND	2	ND	5.2	2.3	1
Camphor	1095	ND	ND	0.7	ND	ND	ND	ND	ND	ND	ND
n-Undecane	1100	ND	ND	ND	ND	ND	ND	1	2.3	0.2	ND
trans-Rose oxide	1100	ND	ND	ND	2.1	0.7	ND	1	ND	ND	ND
trans-Pinocarveol	1106	ND	ND	0.3	ND	ND	ND	ND	ND	0.2	ND
2- *trans*,6 *cis*-Nonadienal	1106	2.1	0.3	0.2	ND	ND	ND	ND	ND	ND	ND
2- *trans*-Nonen-1-al	1114	0.5	0.4	ND	2.2	0.2	ND	ND	ND	ND	ND
Pentyl benzene	1119	1.5	ND	ND	ND	0.3	ND	ND	ND	ND	ND
Menthone	1120	ND	ND	0.2	ND	ND	ND	ND	ND	ND	ND

Benzyl acetate	1123	ND	ND	0.2	ND	ND	ND	ND	ND	ND	ND
Borneol	1134	ND	ND	1.1	ND	ND	ND	ND	ND	ND	ND
Lavandulol	1142	ND	ND	0.3	ND	ND	ND	ND	ND	ND	ND
Menthol	1148	ND	ND	0.5	ND	ND	ND	ND	ND	ND	ND
Terpinen-4-ol	1148	ND	ND	0.7	ND	ND	ND	ND	ND	ND	ND
Octanoic acid	1156	0.3	ND	0.5	0.5	ND	ND	ND	ND	ND	ND
α-Terpineol	1159	ND	ND	1.8	ND	ND	ND	1.2	0.8	0.3	ND
Safranal	1160	1.4	0.3	ND	ND	0.5	ND	ND	ND	ND	ND
Methyl chavicol (=estragole)	1163	ND	ND	0.9	ND	ND	ND	ND	ND	ND	ND
n-Decanal	1180	ND	0.3	0.4	ND	ND	ND	ND	ND	ND	ND
Pulegone	1210	ND	ND	1.4	ND	ND	ND	ND	ND	ND	ND
Geraniol	1236	0.3	1.6	0.6	4	9.2	3.2	1	-	1.4	2.8
Linalyl acetate	1245	ND	ND	1.4	ND	ND	ND	ND	ND	ND	ND
Trans-Anethole	1254	ND	ND	4.7	ND	ND	ND	ND	ND	ND	ND
n-Decanol	1259	0.3	1.6	0.6	4	0.2	3.2	3.4	2.5	3.2	1.9
2-Undecanone	1273	ND	ND	2.2	ND	ND	ND	ND	ND	ND	ND
Perilla alcohol	1274	ND	ND	ND	ND	3.4	ND	ND	ND	0.6	ND
Nonanoic acid	1274	ND	0.3	1.5	2.3	ND	ND	ND	ND	ND	ND
cis-Theaspirane	1279	1.6	2.2	ND	12.7	7.1	14.2	5.3	13.2	9	6.2
2 *trans*,4 *trans*-Decadienal	1285	0.8	1.3	ND	ND	0.1	ND	1.8	ND	2	ND

cis-Transpirane	1286	ND	ND	3.2	ND	ND	ND	ND	ND	ND	ND
Carvacrol	1286	ND	ND	0.3	ND	ND	ND	ND	ND	ND	ND
2-*trans*-4-*trans*-Decadienal	1286	ND	ND	1	ND	ND	ND	ND	ND	ND	ND
trans-Theaspirane	1300	2.4	1.9	3.9	12.1	6.8	17.2	6.3	13.6	10	5.5
Hexyl tiglate ester	1316	ND	ND	0.2	ND	ND	ND	ND	ND	ND	ND
Eugenol	1327	1.4	1.7	0.8	3.5	2.6	ND	3.1	3	3.2	3.6
α-Terpenyl acetate	1334	ND	ND	0.3	ND	ND	ND	ND	ND	ND	ND
α-Longipinene	1338	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND
Decanoic acid	1350	ND	ND	0.8	ND	ND	ND	ND	ND	ND	ND
trans-β-Dasmascenone	1356	ND	ND	0.8	ND	ND	ND	ND	ND	ND	ND
Geranyl acetate	1370	ND	ND	0.5	ND	ND	ND	ND	ND	ND	ND
α-Copaene	1375	ND	ND	ND	ND	ND	ND	0.9	ND	ND	ND
β-Bourbonene	1379	ND	ND	ND	ND	ND	ND	1.5	ND	1.1	ND
2-Pentadecanone	1390	ND	ND	0.8	ND	ND	ND	ND	ND	ND	ND
Longifolene	1399	ND	ND	ND	ND	ND	ND	1.4	ND	ND	ND
β-Caryophyllene	1414	ND	0.4	1.2	ND	ND	ND	2.7	ND	2	0.9
Geranyl acetonea	1434	ND	3.6	0.7	ND	ND	ND	1.2	ND	0.6	ND
allo-Aromadendrene	1456	ND	ND	0.7	ND	ND	ND	ND	ND	ND	ND
trans-β-Ionone	1456	ND	ND	1.1	ND	ND	ND	ND	ND	ND	ND
Germacrene-D	1474	ND	0.2	ND	ND	ND	9.7	3.3	ND	0.7	ND

α-Curcumene	1475	ND	ND	0.5	ND	ND	ND	ND	ND	ND	ND
γ-Cadinene	1500	ND	3.3	ND	ND	ND	ND	1.2	ND	1.1	1.9
σ-Cadinene	1505	ND	2.4	ND	ND	ND	ND	1.6	ND	2	1.9
Dodecanoic acid	1551	3.5	2.1	5.3	2.6	0.3	15	ND	ND	0.9	1.1
β-Caryophyllene oxide	1561	ND	ND	ND	ND	ND	ND	1.3	ND	1.2	2.9
n-Tetradecanal	1596	ND	ND	ND	ND	ND	ND	1.1	ND	2.7	1.5
n-Pentadecanal;	1688	ND	ND	ND	ND	ND	ND	ND	ND	0.8	ND
Tetradecanoic acid	1734	ND	ND	0.2	ND	ND	ND	ND	ND	ND	ND
Hexadecanoic acid	1779	ND	ND	0.7	ND	ND	ND	ND	ND	ND	ND
9,12-Octadecadienoic acid	1820	ND	ND	0.4	ND	ND	ND	ND	ND	ND	ND
% of identified components		71.8	75.1	71.8	78.4	76.8	77.1	82.9	64.8	88.5	82.2
Grouped components											
Monoterpene hydrocarbons		0.9	1.3	0.6	1.1	1	ND	ND	0.8	0.1	ND
Oxygen-containing monoterpenes		6.2	7	18.6	10.6	17.5	3.2	9.6	0.8	5.4	3.8
Sesquiterpene hydrocarbons		ND	6.3	2.5	ND	ND	9.7	12.6	ND	6.9	4.7
Oxygen-containing sesquiterpenes		ND	ND	7.1	ND	ND	ND	1.3	ND	1.2	2.9
Phenylpropanoids		1.4	1.7	6.4	3.5	2.6	ND	3.1	3	3.2	3.6
Oil yield (*v*/ *w*)		<0.05%	<0.05%	0.01%	<0.05%	<0.05%	<0.05%	<0.05%	<0.05%	<0.05%	<0.05%

4.1.3.4. Biological activities

Considering the bioactivities, 44.1% of the works are related to antioxidant activity (Vitor *et al.*, 2004; Gião *et al.*, 2007; Gião *et al.*, 2008; Luis *et al.*, 2009; Coelho *et al.*, 2011; Gil *et al.*, 2011; Luis *et al.*, 2011; Pinela *et al.*, 2011; Ferreira *et al.*, 2012; Serralheiro *et al.*, 2013; Roriz *et al.*, 2015; Abreu *et al.*, 2016; Gonçalves *et al.*, 2018; Caleja *et al.*, 2019; Gonçalves *et al.*, 2020; Garcia-Oliveira *et al.*, 2022), followed by antifungal (17.6%) (Abreu *et al.*, 2016; Aires *et al.*, 2016; Gomes *et al.*, 2018;Caleja *et al.*, 2019) and antibacterial (11.8%) (Martins *et al.*, 2015; Caleja *et al.*, 2019), anti-inflammatory (Bremner *et al.* 2009; Caleja *et al.*, 2019; Simões *et al.*, 2020; Garcia-Oliveira *et al.*, 2022; Mota *et al.*, 2022), and nematocidal (Barbosa *et al.* 2010; Faria *et al.*, 2013; Faria *et al.*, 2016) activities (14.7 and 8.8%, respectively). The cytotoxicity of the extract was evaluated in five works (Luis *et al.*, 2011; Ferreira *et al.*, 2012; Serralheiro *et al.*, 2013; Caleja *et al.*, 2019; Garcia-Oliveira *et al.*, 2022) (14.7%). Only two works studied the antihyperglycemic activity (5.9%) (Paulo *et al.*, 2008; Ferreira *et al.*, 2012). Other activities studied included anti-acetylcholinesterase (Serralheiro *et al.*, 2013) and anti-cholesterol (Falé *et al.*, 2014) activity (2.9% each).

4.1.3.4.1. Antioxidant activity

The antioxidant activity of *G. tridentata* was evaluated by numerous assays, from which the DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) scavenging assay and TBARS (thiobarbituric acid reactive substances) were the most used ones (Table 4.1.3.).

Gião *et al.* (2007) used the ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) test to evaluate the antioxidant activity, showing boiled infusions (leaves) exhibit the highest values (0.260 ± 0.030 gL^{-1} of ascorbic acid equivalent), in comparison to cold infusions (Powder: 0.164 ± 0.036 gL^{-1} of gallic acid equivalent; Leaves: 0.057 ± 0.025 gL^{-1} of gallic acid equivalent).

Three works refer to the antioxidant activity index, showing a high antioxidant activity of *G. tridentata* methanolic extracts of flowers (1.7 ± 0.06%) and moderate antioxidant activity of steam and leaves (0.7 ± 0.06%) (Luis *et al.*, 2011). Other studies identified a strong antioxidant activity of aqueous extracts (1.30 ± 0.05% (Luis *et al.*, 2009); 106.6 ± 0.7 μmol g^{-1} dw (Gil *et al.*, 2011)); however, they did not disclose the part of the plant used to obtain the extract.

Gonçalves *et al.* (2020) showed *G. tridentata* extracts were able to inhibit NO and O^{2-} in a concentration-dependent manner (infusion extract: NO: IC_{50} = 95.41 ± 0.96 μg/mL; O^{2-} IC_{50} = 23.31 ± 2.82 μg/mL; hydroethanolic extract: NO: IC_{50} = 281 ± 2.65μg/mL; O^{2-} IC_{50} = 26.76 ± 1.83 μg/mL).

When Pinela *et al*. (2011) and Roriz *et al*. (2014) evaluated the antioxidant activity of *G. tridentata* methanolic extracts of flowers by *β-carotene bleaching* assay, they obtained different results depending on the processing method (120 min: Freeze-drying: 0.14 ± 0.02 mg/mL; Shade-drying: 0.13 ± 0.01 mg/mL; methanolic maceration: 0.48 ± 0.09 mg/mL). Likewise, Ferreira *et al*. (2012) also showed antioxidant activity of aqueous extracts of leaves and flowers (30 min: 101.8 + 10.7%AA; 60 min: 169.5 + 17.2%AA).

Pinela *et al*. (2011) and Roriz *et al*. (2014) evaluated the reducing power of *G. tridentata* methanolic extracts of flowers to reduce Fe (III) to Fe (II) and obtained similar results (freeze-drying: 0.13 ± 0.00 mg/mL; shade-drying: 0.13 ± 0.00 mg/mL; methanolic maceration: 0.11 ± 0.00 mg/mL), independently of the extraction method used.

One study (Gonçalves *et al*., 2013) assessed the iron chelating activity of *G. tridentata* aqueous extracts of aerial parts, demonstrating that the hot infusions were the most effective although there was not a dose-dependent correlation between the concentration of the extract and the degree of inhibition (Cold: 0.4 mg/mL ext-1:94.55 ± 4.20%; 0.8 mg/mL ext-1:84.32 ± 1.17%; 1.6 mg/mL ext-1:68.32 ± 4.59%; Hot: 0.4 mg/mL ext-1:67.24 ± 2.34%; 0.8 mg/mL ext-1:63.48 ± 1.69%; 1.6 mg/mL ext-1:52.94 ± 0.62%).

Vitor *et al*. (2004) demonstrated that 3-*O*-glucoside isoquercitrin and prunetin, isolated from an aqueous extract of *G. tridentata*, were shown to prevent endothelial oxidative damage and have radical scavenging activity inhibiting O^{2-} generation.

Table 4.1.3. Antioxidant activity and phenolic and flavonoid content in several samples of *G. tridentata.* (AA—ascorbic acid; AAE—ascorbic acid equivalent; ext—extract; GAE-gallic acid equivalent; ip—inhibition percentage; CAE—caffeic acid equivalent; ClAE—chlorogenic acid equivalent; inf—infusion; Pt—Portugal; Sp—Spain; T—Trolox. TAC—total antioxidant capacity; in cases where the units are not indicated, the unit indicated in the first line must be considered; NA—not available; NR—not reported).

Authors	Extraction	Part Plant Used	Localization	Sampling Period	DPPH IC50	Lipidic Peroxidation (TBARS)	Total Phenol Content (mg $GAEg^{-1}$ dw)	Total Flavonoids Content (QE mg/g dw)
Aires *et al.*, 2016	Methanolic	Crude	Vila Real. Pt	NA	NA	NA	3.664 ± 0.04 mg g^{-1} dw	NA
Pinela *et al.*, 2011		Flowers	Trás-os-Montes. Pt	Spring 2010 (Freeze-drying)	NA	0.12 ± 0.02 mg/mL	523.42 ± 36.09 mg ClAE/g ext	58.12 ± 5.78
			Trás-os-Montes. Pt	Spring 2010 (Shade-drying)	NA	0.13 ± 0.04 mg/mL	519.81 ± 40.24 mg ClAE/g ext	85.58 ± 5.60
Luis *et al.*, 2011			Serra da Estrela. Pt	NA	26.1 ± 1.3 mg/L	NR	171.4 ± 0.7	NR
Roriz *et al.*, 2014			Herbal Shop—Ervital	Spring 2012	NR	1.18 ± 0.06 mg/mL	NR	NR
Luis *et al.*, 2011		Stems and leaves	Serra da Estrela. Pt	NA	69.7 ± 11.9 mg/L	NR	113.6 ± 1.5	NR
Gião *et al.*, 2007	Aqueous	Crude	Herbal Shop—Ervital	NA	NR	NR	0.308 ± 0.004 (g L^{-1} GAE)	NR
Coelho *et al.*, 2011		Flowers	Orvalho Mountain. Pt	May	3.6± 0.03 mMT/kg dw	NR	402.9 ± 17.07	NR
			Gardunha Mountain. Pt		3.2± 0.14 mMT/Kg dw	NR	337.7 ± 50.83	NR
			Malcata Mountain. Pt		3.5± 0.03 mMT/Kg dw	NR	309.5 ± 19.82	NR
Garcia-Oliveira *et al.*, 2022			Montesinho. Pt	Spring 2019	NR	IC50 (μg/mL): 5.3 ± 0.1	NR	NR
Serralheiro *et al.*, 2013			Herbal Shop—Dietética	NA	IC50 (μg/mL): 18.6 ± 0.7	NR	NR	NR
Caleja *et al.*, 2019			Herbal Shop—Ervital	NA	NR	8.4 ± 0.2 μg/mL	107 ± 2 (mg/g)	107 ± 2 (mg/g)
Gonçalves *et al.*, 2020			Viseu. Pt	NA	IC50 (μg/mL): 158 ± 1.45	IC50 (μg/mL): 83.48 ± 6.17	34.80 mg/g of dried extract	NR

Gonçalves *et al.*, 2013		Aerial Parts	Algarve. Pt	Spring 2012 (Cold)	NR	NR	314.89 ± 47.49 (µmol GAE gdw^{-1})	NR
				Spring 2012 (Hot)	NR	NR	529.35 ± 3.01 (µmol GAE gdw^{-1})	NR
Coelho *et al.*, 2011		Stems (dormancy period)	Orvalho Mountain. Pt	January	3.6 ± 0.07 mMT/Kg dw	NR	331.7 ± 35.05	NR
			Gardunha Mountain. Pt		3.2 ± 0.07 mMT/Kg dw	NR	394 ± 74.5	NR
			Malcata Mountain. Pt		3.5 ± 0.08 mMT/Kg dw	NR	320 ± 70.23	NR
		Stems (flowering period)	Orvalho mountain. Pt	May	3.6 ± 0.01 mMT/Kg dw	NR	335.9 ± 34.59	NR
			Gardunha Mountain. Pt		3.2 ± 0.07 mMT/Kg dw	NR	270.7 ± 70.8	NR
			Malcata Mountain. Pt		3.6 ± 0.05 mMT/Kg dw	NR	315.8 ± 73.5	NR
Gião *et al.*, 2007		Leaves	Herbal Shop—Ervital	NA	NR	NR	0.130 ± 0.026 (g L^{-1} GAE)	NR
Luis *et al.*, 2011		NA	Serra da Estrela. Pt	NA	NR	NR	222.69 ± 5.12	NR
Luis *et al.*, 2009			Serra da Estrela. Pt	NA	42.97 ± 1.69 (IC50 mg/L)	NR	-	NR
Gil *et al.*, 2011			Herbal Shop—Ervital	NA	NR	NR	44.1 ± 0.6 (CAE. mg g^{-1} dw)	NR
Roriz *et al.*, 2015			Herbal Shop—Ervital	Spring 2012	NR	NR	NR	33.40 ± 0.28 mg/g
Garcia-Oliveira *et al.*, 2022	Ethanolic	Flowers	Montesinho. Pt	Spring 2019	NR	IC50 (µg/mL): 3.19 ± 0.02	NR	NR
Gonçalves *et al.*, 2020			Viseu. Pt	NA	IC50 (µg/mL): 115 ± 0.70	IC50 (µg/mL): 113 ± 15.53	42.84 mg/g of dried extract	NR
Ferreira *et al.*, 2012		Herbal Shop—Ervital	NA	NA	NR	NR	15.5 ± 16.5	NR
Luis *et al.*, 2009		NA	Serra da Estrela. Pt	NA	60.39 ± 1.78 (IC50 mg/L)	NR	196.61 ± 3.94	NR

Caleja *et al.* (2019) and Gonçalves *et al.* (2020) evaluated the inhibition of free radical-induced membrane damage in erythrocytes, showing that *G. tridentata* aqueous extracts of flowers were able to protect the erythrocytes population for 120 min (60 m: 37.7 ± 0.9 μg/mL; 120 m: 69 ± 2 μg/mL) (Caleja *et al.*, 2019) and both, aqueous and hydroethanolic extracts of flowers, prevented hemolysis in a concentration-dependent manner (aqueous extract: IC_{50} = 21.73 ± 0.95 μg/mL; hydroethanolic extract: IC_{50} = 28.43 ± 2.26 μg/mL) (Roriz *et al.*, 2014; Garcia-Oliveira *et al.*, 2022). Additionally, flower extracts prevented the oxidation of hemoglobin in a concentration-dependent manner, with the results demonstrating that the aqueous extract was the most efficient (IC_{50} = 52.87 ± 2.22 μg/mL), followed by the hydroethanolic extract (IC_{50} = 54.03 ± 6.15 μg/mL) (Gonçalves *et al.*, 2020).

Moreover, in two studies (Gião *et al.*, 2008; Gonçalves *et al.*, 2020), aqueous extracts of *G. tridentata* were assayed for their capacity to protect deoxyribose from degradation. Results showed that the strongest antioxidant effect was associated with hot water extraction, compared to cold extraction.

4.1.3.4.2. Anti-Inflammatory activity

Bremner *et al.* (2009) assessed the anti-inflammatory activity of methanolic, petroleum ether, and ethyl acetate extracts of *G. tridentata* using the TNF-α inhibition assay in human monocytes. The results indicated an inhibition level between 80 and 60% for all extracts. Also, more recently, Simões *et al.* (2020) evaluated the anti-inflammatory activity of *G. tridentata* ethanolic extracts (roots, stems, and leaves) through the inhibition of LPS-NO (lipopolysaccharide-nitric oxid) production and demonstrated a downregulation of the Nos2 gene. The other two studies also demonstrated a decrease in the transcription of the pro-inflammatory genes IL1b, IL6, and Ptgs2 with stems and root extracts. LPS-NO production was decreased in flower-based *G. tridentata* aqueous extract (Caleja *et al.*, 2019; Garcia-Oliveira *et al.*, 2022) and in ethanolic extract (Garcia-Oliveira *et al.*, 2022). Mota *et al.* (2022) evaluated the myeloperoxidase (MPO) inhibition by ethanolic extract of *G. tridentata* and demonstrated an IC_{50} of 0.032 ± 0.004 mg/mL.

4.1.3.4.3. Antifungal and antibacterial activity

The results of the antifungal activity of *G. tridentata* are presented in Table 4.1.4. The results showed that *G. tridentata* extracts are effective against six *Candida* strains, namely *C. albicans*, *C. glabrata*, and *C. parapsilosis* species, while the majority of *C. tropicalis* strains were resistant (Martins *et al.*, 2015). The antifungal activity of aqueous extract of *G. tridentata* flowers was also demonstrated against *Aspergillus niger*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium aurantriogriseum* and *Penicillium verrucosum* (Caleja *et al.*, 2019; Garcia-Oliveira *et al.*, 2022).

Table 4.1.4 also shows the results for the screening of the antibacterial activity of *G. tridentata* against *S. aureus* and *E. coli*, *Salmonella Typhimurium*, *Bacillus cereus,* and *Listeria monocytogenes*. *G. tridentata* extracts display moderate to strong antibacterial activity against *S. aureus* (Aires *et al.*, 2016).

Table 4.1.4. Antifungal and antibacterial effect of *G. tridentata* extract. (IZ: inhibitory zone; When there was no evident halo, but inhibition of growth, the effect was also classified as (+) cell growth inhibition, (++) cell density reduction, (+++) cell density reduction and growth inhibition; MIC: minimal inhibitory concentration if MIC values ≤ 100 μg.mL^{-1}, moderate (++) when 100 < MIC ≤ 500 μg·mL^{-1}, not detectable (-) <4 g/L^{-1}; NR—not reported).

Authors	Extraction	Part Plant Used	Species	Strains	Method	Results
Martins *et al.*, 2015	Methanolic	Flowers	*Candida albicans*	ATCC90028	Disc diffusion assay	IZ: 10 mm
				575541		-
				557834		IZ: 10 mm
				558234		IZ: 9 mm
			Candida galabrata	ATCC2001		IZ: 11 mm
				D1		IZ: 11 mm
				513100		IZ: 9 mm
			Candida parapsilosis	ATCC22019		++
				AM2		++
				AD		-
				491861		-
				513143		-
			Candida tropicalis	ATCC750		+
				AG1		+++
				75		-
				12		-
				544123		-
				519468		-
				T2.2		-
Caleja *et al.*, 2019	Aqueous	Flowers	*Aspergillus niger*	ATCC 6275	Microdilution method	MIC: 8 mg/mL
			Aspergillus versicolor	ATCC 11730		MIC: 0.5 mg/mL
			Penicillium funiculosum	ATCC 36839		MIC: 0.5 mg/mL
			Penicillium verrucosum	Food isolates		MIC: 0.5 mg/mL
Garcia-Oliveira *et al.*, 2022			*Aspergillus niger*	ATCC 6275		MIC: 0.5 mg/mL
			Aspergillus versicolor	ATCC 11730		MIC: 0.5 mg/mL
			Aspergillus fumigatus	Human isolate		MIC: 1 mg/mL
			Penicillium funiculosum	ATCC 26839		MIC: 0.5 mg/mL
			Penicillium aurantiogriseum	ATCC 58604		MIC: 0.5 mg/mL
	Ethanolic	Flowers	*Aspergillus niger*	ATCC 6275		MIC: 0.25 mg/mL

			Aspergillus versicolor	ATCC 11730		MIC: 0.25 mg/mL
			Aspergillus fumigatus	Human isolate		MIC: 0.25 mg/mL
			Penicillium funiculosum	ATCC 26839		MIC: 0.25 mg/mL
			Penicillium aurantiogriseum	ATCC 58604		MIC: 0.5 mg/mL
Aires *et al.*, 2016	Methanolic	Crude	*Staphylococcus aureus*	ATCC 13565	Microdilution method	MIC: 312.5 $\mu g.mL^{-1}$ (moderate)
				MJMC021		MIC: 78.1 $\mu g.mL^{-1}$ (strong)
				MJMC024		MIC: 78.1 $\mu g.mL^{-1}$ (strong)
				MJMC026		MIC: 78.1 $\mu g.mL^{-1}$ (strong)
				MJMC025		MIC: 39.1 $\mu g.mL^{-1}$ (strong)
				MJMC027		MIC: 39.1 $\mu g.mL^{-1}$ (strong)
				MJMC029		MIC: 39.1 $\mu g.mL^{-1}$ (strong)
Abreu *et al.*, 2016				*S. aureus* CECT 97	Disc diffusion test	MIC < 4 g/L^{-1} (indifferent)
Gomes *et al.*, 2018		Flowers		ATCC 25923	Disc diffusion test	Inhibitory zone: 5 mm
Garcia-Oliveira *et al.*, 2022	Aqueous	Flowers		ATCC 6538	Microdilution method	MIC: 0.25 mg/mL
Caleja *et al.*, 2019			*Escherichia coli*	NR	Microdilution method	MIC: 0.5 mg/mL
			Salmonela ryphimurium	NR		MIC: 1 mg/mL
			Bacillus cereus	NR		MIC: 1 mg/mL
			Listeria monocytogenes	NR		MIC: 1 mg/mL
Garcia-Oliveira *et al.*, 2022			*Micrococcus flavus*	ATCC 10240		MIC: 2 mg/mL
			Enterobacter cloacae	ATCC 35030		MIC: 1 mg/mL
			Bacillus cereus	Clinical isolate		MIC: 1 mg/mL
			Listeria monocytogenes	NCTC 7973		MIC: 1 mg/mL
			Salmonella typhimurium	ATCC 13311		MIC: 1 mg/mL
	Ethanolic		*Staphylococcus aureus*	ATCC 6538		MIC: 0.25 mg/mL
			Micrococcus flavus	ATCC 10240		MIC: 1 mg/mL
			Enterobacter cloacae	ATCC 35030		MIC: 1 mg/mL
			Bacillus cereus	Clinical isolate		MIC: 0.5 mg/mL
			Listeria monocytogenes	NCTC 7973		MIC: 0.5 mg/mL
			Salmonella typhimurium	ATCC 13311		MIC: 0.5 mg/mL

4.1.3.4.4. Cytotoxicity

The cytotoxicity of *G. tridentata* aqueous extract was assessed using a human colorectal epithelial adenocarcinoma cell line (Caco-2) and a human cervical adenocarcinoma cell line (HeLa) (Serralheiro *et al.*, 2013). Additionally, the cytotoxicity of *G. tridentata* flower aqueous extract was also assessed using a human hepatocarcinoma cell line (HePG2) (Ferreira *et al.*, 2012). The results showed no toxicity in the cell lines evaluated.

Ferreira *et al.* (2012) also evaluated the toxicological effects of *G. tridentata* flower aqueous extract in mitochondrial respiratory rates (state 4 and state 3 respiration and FCCP-stimulated respiration) and respiratory indexes (respiratory control ratio and P/O ratios) in rat liver mitochondria. The results demonstrated no toxicity of the extracts since no identifiable interactions with respiratory enzymes were detected.

Caleja *et al.* (2019) and Garcia-Oliveira *et al.* (2022) also assessed the cytotoxicity of aqueous and ethanolic extracts of flowers using four human tumor cell lines: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung cancer), as well as a non-tumor cell primary culture PLP2 (porcine liver). The results indicate no cytotoxicity for non-tumor cells and for NCI-H460 and MCF-7 cells. Also, they verified an inhibition of 50% of cell growth in the HeLa and HepG2 cell lines (251 ± 6 μg/mL and 262 ± 11 μg/mL, respectively). Garcia-Oliveira *et al.* (2022) demonstrated an inhibition of 50% of cell growth in aqueous and ethanolic extract in the NCI-H460 (GI:142.7 ± 5.3 μg/mL and 160.5 ± 5.3 μg/mL, respectively), HeLa (GI:83.2 ± 6.5 μg/mL and 102.9 ± 10.6 μg/mL, respectively), MCF-7 (GI:129.1 ± 6.3 μg/mL and 146.8 ± 6.5 μg/mL, respectively), and HepG2 (GI:123.1 ± 19.1 μg/mL and 132.4 ± 8.5 μg/mL, respectively) cell lines.

4.1.3.4.5. Nematocidal/nematotoxicity activity

Barbosa *et al.* (2010) and Faria *et al.* (2013, 2016) evaluated the nematocidal activity of EOs of *G. tridentata,* highlighting a moderate to strong activity. One report showed a mortality rate of 100% against *Bursaphelenchus xylophilus* exposed for 24 hr to a 2 mg/mL of *G. tridentata* flower-based EO (Barbosa *et al.* 2010). Moreover, Faria *et al.* (2013) demonstrated a moderate (80–61%) nematotoxicity activity against *Bursaphelenchus xylophilus* when exposed to 2 μL/mL of *G. tridentata* EO. Likewise, Faria *et al.* (2016) demonstrated inhibition of *Meloidogyne chitwoodi* eggs hatching rates (>90%) when exposed to 2 $\mu L/mL^{-1}$ EO.

4.1.3.4.6. Other activities

Gonçalves *et al*. (2020) evaluated the *in vitro* capability of flower-based *G. tridentata* extracts to inhibit α-glucosidase. Aqueous and hydroethanolic extracts inhibited α-glucosidase in a concentration-dependent manner (IC_{50} = 130 ± 0.90 μg/mL; IC_{50} = 148 ± 2.54 μg/mL, respectively).

Currently, there is just one scientific report (Paulo *et al*., 2008) regarding the antihyperglycemic properties of *G. tridentata in vivo*. The antihyperglycemic effect of the aqueous extract was evaluated in normoglycemic rats using an oral glucose tolerance test. Rats were administered the *G. tridentata* extract (300 mg/kg). The extract exhibited a significant antihyperglycemic effect in the initial 30 min after the glucose challenge, although, at later time points, the blood glucose levels increased, and the extract demonstrated a paradoxical hyperglycemic effect. The same authors also analyzed blood glucose in normoglycemic rats after administration of sissotrin and isoquercitrin, demonstrating an opposite effect in glucose tolerance: isoquercitrin (100 mg/kg) significantly decreased blood glucose levels for 30 min while sissotrin (100 mg/kg) produced a significant increase in blood glucose levels for 60 min.

Acetylcholinesterase (AChE) enzymatic activity of *G. tridentata* flowers aqueous extracts was evaluated by Serralheiro *et al*. (2013). The results showed IC_{50} values for AChE activity of 1090 ± 4 μg/mL. After 4 h digestion, the remaining enzymatic activities for the gastric and pancreatic juices were 105.8 ± 5.5 % and 103.5 ± 17.3 %, respectively.

Falé *et al*. (2014) assessed the molecular mechanism of cholesterol reduction by *G. tridentata* aqueous extracts demonstrating a plasma total cholesterol reduction of 22% *in vivo* (humans; 10 g/L of dry infusion on the day of the experiment). This work also included *in vitro* studies to evaluate the inhibitory concentration of HMG-CoA (IC_{50}=329.04 ± 21.24 μg/mL) and the permeation in Caco-2 monolayers cells where they verified the bioavailability of some bioactive compounds in basolateral and intracellular compartments (isoquercitin—basolateral: 5.09 ± 0.54 and intracellular: 5.64 ± 0.31%; biochanin A—basolateral: 6.30 ± 0.52 and intracellular: 3.69 ± 0.06%), as well as the apparent permeability coefficient (Papp) (isoquercitin—13.09 ± 1.39 × 10^{-7} cm/s; biochanin A—16.20 ± 1.33 × 10^{-7} cm/s).

4.1.3.5. Quality assessment

The quality assessment of the works was performed using the ARRIVE quality guidelines, with each criterion being rated with a score between 0 and 1 (0—Absent; 0.5—Incomplete or not applicable; 1—Complete). About 56.25% of the studies were considered good quality, scoring higher than 15 out of 20, and 43.75% were considered moderate quality, rating between 12 and 15 out of 20. The mean score was determined for each work, and the total mean score for the quality of included works was 15.18 ± 1.14 (Table 4.1.5.).

Table 4.1.5. Evaluation of the quality of included articles using a modified version [§] of the ARRIVE guidelines for the reporting of *in vivo* experiments. (Criteria 1—Title; 2—Abstract; 3—Background; 4—Objectives; 5—Ethical statement; 6—Study design; 7—Inclusion/Exclusion criteria; 8—Experimental procedures; 9—Randomization; 10—Blinding; 11—Plant sample characterization [§]; 12—Plant extraction methods [§]; 13—Experimental outcomes; 14—Experimental animals; 15—Housing and husbandry; 16—Sample size; 17—Statistical methods; 18—Results; 19 –Interpretation/scientific implications; 20—Funding; Score between 0–1 (0—Absent (orange); 0.5—Incomplete or not applicable (yellow); 1—Complete (green)).

References	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Score	Rating
Simões *et al.*, 2020	1	1	1	1	0.5	1	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	17	Strong
Coelho *et al.*, 2011	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	1	0.5	0.5	0	1	1	1	1	16	Strong
Ferreira *et al.*, 2012	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	16	Strong
Vitor *et al.*, 2004	1	1	1	1	0.5	1	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	0.5	1	1	1	1	16	Strong
Grosso *et al.*, 2007	1	0.5	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	1	0.5	0.5	0.5	1	1	1	1	16	Strong
Pinela *et al.*, 2011	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	1	0.5	0.5	0	1	1	1	1	16	Strong
Gião *et al.*, 2007	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	16	Strong
Bremner *et al.* 2009	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0	1	1	0	13	Moderate
Garcia-Oliveira *et al.*, 2022	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	1	0.5	0.5	0.5	1	1	1	1	16	Strong
Gonçalves *et al.*, 2013	1	1	1	1	1	1	0	1	0	0	1	1	1	0.5	1	0.5	1	1	1	1	16	Strong
Aires *et al.*, 2016	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	16	Strong
Balanč *et al.*, 2016	1	1	1	1	0.5	1	0.5	1	0.5	0.5	0	1	1	0.5	0.5	0	1	1	1	1	15	Moderate
Gião *et al.*, 2008	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	16	Strong
Serralheiro *et al.*, 2013	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	0.5	1	1	1	1	1	16	Strong
Falé *et al.*, 2014	1	1	1	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	1	1	1	14	Moderate

Luis *et al.*, 2011																					15	Moderate
Gonçalves *et al.*, 2018																					14	Moderate
Paulo *et al.*, 2008																					13	Moderate
Luis *et al.*, 2009																					13	Moderate
Barbosa *et al.* 2010																					15	Moderate
Gil *et al.*, 2011																					16	Strong
Faria *et al.*, 2013																					14	Moderate
Roriz *et al.*, 2015																					15	Moderate
Faria *et al.*, 2016																					14	Moderate
Caleja *et al.*, 2019																					16	Strong
Gonçalves *et al.*, 2020																					17	Strong
Roriz *et al.*, 2014																					15	Moderate
Martins *et al.*, 2015																					15	Moderate
Abreu *et al.*, 2016																					16	Strong
Gomes *et al.*, 2018																					16	Strong
Mota *et al.*, 2022																					15	Moderate
Gonzalez-Hernandez *et al.*, 2003																					13	Moderate
Martinez *et al.*, 2012																					14	Moderate
Roriz *et al.*, 2014																					15	Moderate

4.1.4. Discussion

4.1.4.1. Phytochemical characterization

The phytochemical profile (Table 4.1.1.) clearly demonstrates that extract composition varies considerably with the extraction method, probably due to the solubility of the compounds. Methanolic and ethanolic solvents are less polar in nature, disrupting cell walls (nonpolar structures) and triggering the release of phenolic compounds; while water presents a high polarity index, it is best to extract compounds with higher polarity (Gonçalves *et al.*, 2020). As the extraction solvent influences the plant extract composition (Martins *et al.*, 2015; Gomes *et al.*, 2018), reports should carefully describe the extraction method to facilitate comparison between laboratories. The location and the vegetative stage also influence the phytochemical profile of extracts; consequently, it is important to be careful in the analyses of data from the works that do not indicate the sampling period. Plant extracts collected in the flowering period (May), as well as flowers, displayed a more diverse phytochemical profile than extracts collected during the rest of the year.

Furthermore, the *in vitro* extracts exhibited a different composition content than wild extracts (Gonçalves *et al.*, 2018). These variations could be explained because some compounds are not essential in *in vitro* plants as a defense mechanism against harmful environmental conditions, and consequently, they do not produce them (Gonçalves *et al.*, 2018).

Moreover, works performing a phytochemical characterization of *G. tridentata* are scarce, especially when using other extraction solvents, such as acetone. Another important parameter is the quantification of the phytochemical compounds identified, often overlooked by researchers.

Regarding EOs (Table 4.1.2.), the variability between samples from the same location and year indicates that the chemical composition of *G. tridentata* oils is not a consequence of climatic factors in distinct years but a consequence of other intrinsic factors related to the plant (ratio plant/organ, vegetative state, genetics) and its interaction with the environment (type of the soil, climate, harvest time during the day) (Takaishi *et al.*, 2008).

4.1.4.2. Biological activities

4.1.4.2.1. Plant bioactive compounds as antioxidants

There is no general procedure that can determine the antioxidant capacity of all samples precisely and quantitatively (Magalhães *et al.*, 2008). Standardized approaches to evaluate antioxidant activity should

follow specific requirements, and consequently, the procedures fall into two broad categories: radical scavenging activity and lipid peroxidation inhibition (Vicente and Boscaiu, 2018).

The variability of assays facilitates the establishment of *G. tridentata* potential (Pinto *et al.*, 2020). However, it is impossible to compare between essays since there is no standardization of procedures, and *G. tridentata* extracts are obtained with different solvents and from different locations. Additionally, not all evaluated works performed replicates of the antioxidant tests (Gonzalez-Hernandez *et al.*, 2003; Paulo *et al.*, 2008; Coelho *et al.*, 2011; Pinela *et al.*, 2011; Balanč *et al.*, 2016; Gonçalves *et al.*, 2018), so they should be repeated.

Furthermore, the anti-oxidative effects of plant extracts on deoxyribose and DNA were promising concerning practical applications of *G. tridentata* as an ingredient in the formulation of nutraceutical beverages and foods, as well as cosmetic formulations, since they add a protective effect.

The phytochemical characterization (Tables 4.1.1. and 4.1.3.) of *G. tridentata* extracts demonstrates a high level of phenolic compounds, especially flavonoids. Structurally, phenolic compounds possess an aromatic ring with one or more hydroxyl substitutes. The aromatic feature and highly conjugated system with multiple hydroxyl groups make these compounds good electron/hydrogen atom donors, neutralizing free radicals and other ROS (Soobrattee *et al.*, 2005).

The antioxidant activity of phenolics can be based on hydrogen atom transfer and/or single-electron transfer. Nevertheless, the antioxidant potential of a phenolic compound depends on the number and the position of hydroxyl groups in the molecule (Soobrattee *et al.*, 2005; de Guzman *et al.*, 2009).

Likewise, the degree of hydroxylation also influences the antioxidant activity. The longer distance separating the carbonyl group and the aromatic ring appears to improve the antioxidant capability. Additionally, a greater number of hydroxyl aromatic rings, such as in flavonoids, increased the antioxidant activity (Soobrattee *et al.*, 2005).

Importantly, flavonoids and their glycosylated derivates in *G. tridentata* extracts were stable during *in vitro* digestion, which indicates a strong antioxidant activity (Serralheiro *et al.*, 2013). Nevertheless, this analysis should be repeated since this single work evaluated gastric and pancreatic digestion, and their quality assessment indicates some deficiencies (Table 4.1.5.).

Some studies showed a significant synergistic effect between bioactive compounds and, consequently, an increase in antioxidant activity (Hajimehdipoor *et al.*, 2014; Pinto and Silva, 2021). Therefore, the high

antioxidant capacity of *G. tridentata* extracts is probably due to a synergistic interaction between their phytochemical components rather than due to a single chemical element.

4.1.4.2.2. Anti-inflammatory activity

Several health benefits attributed in traditional medicine to *G. tridentata* infusions or decoctions are linked to their anti-inflammatory activity (Coelho *et al.*, 2011; Dhifi *et al.*, 2016). Nevertheless, scientific reports focused on the anti-inflammatory potential of *G. tridentata* extracts are scarce and do not identify the mechanism of action. The phytochemical characterization of *G. tridentata* extracts demonstrated their richness in flavonoids, frequently correlated with anti-inflammatory activity (Ferrandiz and Alcaraz, 1991; Pinto *et al.*, 2020). Additionally, numerous studies demonstrated the anti-inflammatory potential of biochanin A (Ming *et al.*, 2015; Wang *et al.*, 2015; Zhang and Chen, 2015; Liu *et al.*, 2016), prunetin (Yang *et al.*, 2013; Piegholdt *et al.*, 2014), genistein (Du *et al.*, 2018; Wang *et al.*, 2019), rutin (Khajevand-Khazaei *et al.*, 2018; Caglayan *et al.*, 2019) and taxifolin (Cai *et al.*, 2018; Pan *et al.*, 2019) in decreasing inflammatory mediators as TNF-α, inhibiting the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-33) and of pro-inflammatory enzymes as nitric oxide synthases. Pinto *et al.* (2020) provided a comprehensive review that explores the potential mechanisms of action of several bioactive compounds also found in *G. tridentata* concerning their anti-inflammatory activity.

However, it would be important to evaluate the synergistic effects of the bioactive compounds, as well as the evaluation of extracts obtained with different solvents. Importantly, the *in vivo* evaluation of *G. tridentata* extracts could be central to understanding the mechanisms of action and establishing the pharmacological effects of the plant's extracts.

4.1.4.2.3. Antifungal and antibacterial activity

The antifungal activity of *G. tridentata* against *Candida* strains is probably associated with the existence of the flavonoids, quercetin, and genistein derivates. Quercetin inhibits growth (Herrera *et al.*, 2010; Yousefbeyk *et al*, 2014) and prevents microbial biofilm formation, possibly by reducing the cellular adhesion to abiotic surfaces, as described for *C. albicans* (Gao *et al.*, 2016). Also, genistein derivates inhibit *C. albicans* colony formation (Yoon *et al.*, 2006; Taveira *et al.*, 2010).

The antibacterial potential of *G. tridentata* can result from the higher content of phenolic compounds, flavonols, and isoflavones, which improves the antimicrobial power of extracts.

Additionally, the presence of taxifolin, genistin, and biochanin was previously reported as having an antibacterial activity (An *et al.*, 2011; Asmi *et al.*, 2017; Ahamed *et al.*, 2018), reducing bacteria

resistance mechanisms and conducting delayed protein synthesis in *S. aureus* involved in the production of enzymes and nucleic acids required for bacterial growth (Cushnie *et al.*, 2005; Aires *et al.*, 2016; Asmi *et al.*, 2017). As a result, membrane permeability increases, leading to a decline in bacteria survival. Also, flavonoids display a strong capacity to form complexes with bacteria cell walls, inhibiting bacteria growth (Cushnie *et al.*, 2005; Mahboubi and Haghi, 2008; Morteza-Semnani *et al.*, 2011).

Identically, rutin, isoquercitrin, and quercetin can act as antimicrobial agents, inhibiting nucleic acid and cytoplasmic membrane synthesis as well as bacterial metabolism (Cushnie *et al.*, 2005). Importantly, the above-mentioned phenolic compounds are more effective against Gram-positive bacteria (Kuspradini *et al.*, 2009), like *S. aureus*. Consequently, these compounds could have a bacteriostatic effect instead of a bactericidal action (Aires *et al.*, 2016).

Regardless of the need for additional studies to clarify the mechanisms of action, it is possible to conclude that *G. tridentata* can be valuable in treating or complementing commercial drugs due to its bacteriostatic and antifungal potential. However, the extraction solvent influences the plant extracts bioactivity (Martins *et al.*, 2015; Gomes *et al.*, 2018). Thus, based on that, it could be important to evaluate the antifungal and antibacterial effects of other extraction methods of *G. tridentata*.

4.1.4.2.4. Cytotoxicity

Firstly, it is important to clarify that human cell lines are preferred to primary cultures from animals, as they decrease species-related variations that can occur during the extrapolation of the results (Thabrew *et al.*, 2011). Secondly, data obtained by Serralheiro *et al.* (2013), Ferreira *et al.* (2012), and Caleja *et al.* (2019) demonstrated that *G. tridentata* aqueous extracts could be appropriately used in traditional medicine, in cooking and the preservation of aliments against oxidative stress.

4.1.4.2.5. Nematocidal/nematotoxicity activity

Although several studies showed that EOs display high nematocidal activities (Ismail *et al.*, 2020; Nguyen *et al.*, 2022), literature about *G. tridentata* EOs is still limited. The mechanism of action of EOs is complex and occurs through different pathways, and some authors suggest that the interference of EOs in the nematode nervous system (Oka *et al.*, 2000) is related to the neuromodulator octopamine (Kostyukovsky *et al.*, 2000) or GABA-gated chloride channels in insects (Priestley *et al.*, 2003). Moreover, as in bacteria or fungi, EOs might disturb the cell membrane of the nematode by altering its permeability (Oka *et al.*, 2000).

Importantly, the phytochemical profile of *G. tridentata* EOs (Table 4.1.2.) demonstrated the presence of carvacrol and geraniol. Some studies demonstrated the nematocidal activity of carvacrol, facilitated via tyramine receptor, that triggered a signaling cascade and, by interacting with a receptor-like SER-2, led to nematode mortality (Lei *et al.*, 2010). Similarly, the activity of geraniol in membrane disturbing, changing membrane-bound protein, and the intracellular signaling pathways (Tsuchiya *et al.*, 2001; Kaur *et al.*, 2011) has also been described. Although experimentally, EOs of *G. tridentata* display nematocidal activity; it is important to complement the *in vitro* tests with *in vivo* soil-based trials to validate the efficiency of this plant EOs.

The main advantages of the nematocidal/nematotoxicity activity of EOs are their minimal toxicity to mammalians and their low environmental persistence, making them safe and compatible as biological control agents (Isman *et al.*, 2011).

4.1.4.2.6. Other activities

Results obtained by Paulo *et al.* (2008) with the isolated compounds isoquercitrin and sissotrin revealed a time-dependent antihyperglycemic activity, indicating the presence of bioactive compounds that differently influence glucose uptake. While these results discourage the use of the plant in the control of glycemic blood levels, they highlight a possible post-prandial hypoglycemic effect of isoquercitrin. However, this work (Paulo *et al.*, 2008) presents several imperfections, especially regarding scientific work with animals, not revealing the number of animals used or blinding techniques.

Nevertheless, performing more studies to assess the antihyperglycemic activity of *G. tridentata* extracts is important to understand the mechanism of action of its bioactive compounds.

Serralheiro *et al.* (2013) demonstrated using a colorimetric assay that the *G. tridentata* aqueous extracts display anti-acetylcholinesterase activity. Acetylcholine (ACh) is an important excitatory neurotransmitter responsible for peristaltic movements (Holzer and Maggi, 1994; Fujii *et al.*, 2020) and is also responsible for the regulation and establishment of an adequate environment for enzymatic digestion and absorption and the lubrication of intestinal material (Hirota and McKay, 2006).

Serralheiro *et al.* (2013) suggested that the flavonoids present in the extract may play an essential role in the inhibition of ACh by interacting with their active bind sites. Falé *et al.* (2012) also showed small alterations in the ACh, namely in the aromatic amino acids, that affect enzymatic activity. The alterations occur in the presence of quercetin, romarinic acid, luteolin, and apigenin (Falé *et al.*, 2012), all of which are also present in *G. tridentata* aqueous extracts.

Falé *et al*. (2014) intended to understand the mechanism of action of *G. tridentata* extract in cholesterol in an *in vivo* experiment and found a reduction of 22% of plasma total cholesterol. Apart from the limited number of volunteers, the authors also do not refer to the control of other important variables such as diet and physical exercise.

They also performed *in vitro* studies to evaluate the Inhibitory concentration of HMG-CoA of *G. tridentata* extract HMG-CoA reductase. The downregulation of HMGCR is directly associated with cholesterol reduction via SREBP-2 activation as it acts in the upregulation of low-density lipoprotein receptor (LDLR) that increases the exclusion of cholesterol-rich low-density lipoprotein (LDL) particles from the blood circulation (Endo and Hasumi, 1989; Sato, 2010).

The same authors (Falé *et al*., 2014) also performed permeation studies in Caco-2 monolayer cells. The results demonstrated the bioavailability of some bioactive compounds, suggesting the biochanin A glucoside as a specific transporter of glycoside moiety that may be involved in the reduction of cholesterol (Falé *et al*., 2014). Sadri *et al*. (2017) also demonstrated that oral administration of biochanin A (10 and 15 mg/kg) in rats significantly decreased serum triglycerides, total cholesterol, and LDL cholesterol.

4.1.5. Conclusions

Genista tridentata extracts present a high extraction yield and have been described to possess high levels of flavonoids and phenolic compounds, suggesting a high potential for its application as a new source of natural antioxidants and preservatives for the food industry or in products with health benefits, such as nutraceuticals. Moreover, the results indicate that the plants can be collected at all seasons of the year, which represents an added benefit for the industry.

4.1.6. References

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4.2. Anti-inflammatory effect of *Pterospartum tridentatum* leaf extract in acute and chronic inflammation

4.2.1. Introduction

Inflammation is a protective mechanism of the organism against infection or injury to repair tissue structure and its physiological function, restoring homeostasis (Freire and Van Dyke, 2013), and can be divided into acute and chronic inflammation. The acute inflammatory response occurs immediately after injury and comprises both the eradication of the infectious agents and a repair phase in order to return to homeostasis (Freire and Van Dyke, 2013; Furman *et al.*, 2019). Non-resolving acute inflammation leads to an increased and persistent inflammatory response, becoming chronic inflammation (Furman *et al.*, 2019).

Current clinical anti-inflammatory therapies include, amongst others, non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (Tabas and Glass, 2013). However, the pharmacological options available display considerable potential for gastric, cardiovascular, renal, and hepatic adverse effects (Tolba, 2017), especially in cases of prolonged usage. Therefore, the interest in alternative therapies with anti-inflammatory effects and proven safety has been increasing.

Medicinal plants present many benefits to human health, which are related to the presence of secondary compounds, namely, flavonoids, terpenes, quinones, catechins, alkaloids, and anthocyanins, which display several advantageous properties, including antioxidant, anti-inflammatory, and anti-carcinogenic effects (Khanna *et al.*, 2007; Rahman *et al.*, 2022). Although plant-derived products are promising, extensive investigation is necessary in preclinical and clinical settings to demonstrate their utility (Khanna *et al.*, 2007; Tungmunnithum *et al.*, 2018).

Pterospartum tridentatum (L.) Willk. (previously known as *Chamaespartum tridentatum* P. Gibbs. and *Genista tridentate* L. Willk), or "prickled broom" or "carqueja" in Portugal, is an endemic Leguminosae (Fabaceae) species of Europe belonging to the subfamily Papilionoideae (Coelho *et al.*, 2011; Ferreira *et al.*, 2012). This is an aromatic plant, commonly used in Portuguese gastronomy, and the leaves are conventionally used as a condiment/spice for seasoning of traditional rice and meat dishes (Ribeiro *et al.*, 2000).

The use of *Pterospartum tridentatum* in traditional medicine as anti-inflammatory (Gião *et al.*, 2007; Bremner *et al.*, 2009) and its extracts have been described to possess high levels of flavonoids and phenolic compounds (Luís *et al.*, 2011; Aires *et al.*, 2016)

We aimed to assess the effect of *P. tridentatum* extracts in acute and chronic inflammation and correlated it with its phytochemical composition. Additionally, we aimed to evaluate the molecular interactions between the major *P. tridentatum* phytochemical compounds and proteins involved in the inflammatory response, thus elucidating its possible mechanism of action.

4.2.2. Materials and methods

4.2.2.1. Plant material

Leaves of *Pterospartum tridentatum* (Figure 4.2.1A) were collected in Esposende (41°34′30″ N; 8°46′0″ W) (Figure 4.2.1B), Portugal, in May 2019. The plant material was identified at the Biology Department (University of Minho), and a voucher sample was deposited in the Herbarium of the same Department with the reference 12/2019. The plant material was dried (room temperature) and triturated. The resulting powder was macerated with a methanolic solution (7:3 *v/v*) (Methanol P.A., Merck) for seven days (100 g of biomass in 1 L of solvent). Subsequently, the solution was filtered and concentrated with a rotary evaporator (Buchi® RII) under reduced pressure (Buchi® V-700 pump, Buchegg, Switzerland) and bath at 50 °C. The extract was lyophilized (Christ® Alpha 2-4, B. Braun, Gottingen, Germany) for two days and stored (dark at room temperature) until further use, following the protocol of Dias and colleagues (Dias *et al.*, 2007).

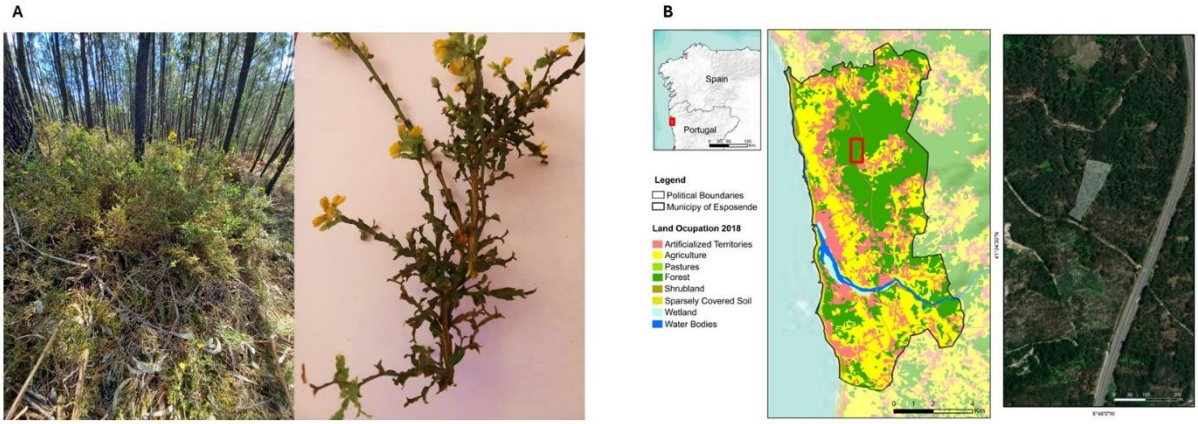


Figure 4.2.1. *P. tridentatum* collected specimen (A) and spatial representation of the *P. tridentatum* collection area (marked in red box) (B).

4.2.2.2. High-Performence Liquid Chromatography (HPLC)

HPLC analysis was performed in a Hitachi-Merck HPLC-DAD system (high-performance liquid chromatography with diode array detection), controlled by Merck pc-software (Merck, New York, NY, USA). *P. tridentatum* extract (3 mg/mL) was dissolve in pure methanol and filtered thru a 0.2 µm nylon syringe filter (Sigma, Barcelona, Spain). The injection volume was 20 µL.

The solvent system was a gradient based of methanol (HPLC grade, Merck, Darmstadt, Germany) with 0.1% of formic acid (P.A. grade, Merck, Germany) (solvent A) and ultra-pure water with 0.1% of formic acid (solvent B). The optimal elution profile consisted of a six-step gradient: starting with 15% of solvent A and installing a gradient to obtain 15% A at 3 min, 90% (solvent A) at 35 min, 90% (solvent A) at 45 min, 15% (solvent A) at 50 min, and 15% (solvent A) at 60 min.

The detection used a diode array detector. Spectral data from all peaks were accumulated in the wavelength range 245–530 nm, and chromatograms were recorded at 260, 280, and 350 nm.

The UV-visible spectra and retention times were used to characterize the presence of phenolic compounds in the samples compared to commercial standard reference compounds, according to Dias and colleagues (Dias *et al.*, 2007). The commercial standards (minimum 98% purity) used were taxifolin, rutin, isoquercetin, quercetin, genistin, and genistein, all from Sigma-Aldrich (Barcelona, Spain). The compounds in the chromatogram were quantified by the external standard method. Isoflavone and quercetin derivatives were quantified as genistein and quercetin equivalents, respectively.

4.2.2.3. *In vivo* experiments

4.2.2.3.1. Ethical considerations and handling

The experimental protocol was approved by the Institutional and National Ethical Commission (DGAV 23875/2019) and followed the European Community Council Directive 2010/63/EU concerning the use of animals for scientific purposes. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Male Wistar Han outbred *Rattus norvegicus* (Charles Rivers, Barcelona, Spain) weighting between 313–358 g were kept in a controlled temperature environment, mean temperature 22 °C, 55% relative humidity, 12 h light/dark cycle (light cycle starting at 08:00 AM), in standard polycarbonate cages (45.4 × 25.5 × 20 cm), two animals per cage, with access to water and food (F0021; BioServ, Flemington, NJ, USA) *ad libidum.*

The general health parameters were evaluated weekly. Inittially, animals were handled daily by the researcher for 15 days. All subsequent protocols were performed during the day (light phase). When possible, the experimenters were blind to the experiment.

4.2.2.3.2. Induction of ear oedema

Acute dermal irritation of rat ears was induced using croton oil, 6 h before euthanasia, with a methodology similar to Rodrigues *et al.* (2016), but this involved switching from acetone to ethanol as the vehicle due to the latter presenting superior solubility of the extract.

For this experiment, 26 animals were first divided in two groups: (1) a control group, to which 20 μL of pure ethanol (ETOH) was administered to the inner surface of the ear tip; and (2) an acute inflammation group (CO), to which 20 μL of a fresh solution of croton oil 5% was administered to the inner surface of the ear tip. Fifteen minutes later, the groups were subdivided into: (1) controls receiving a second dose of 20 μL of pure ethanol (ETOH-ETOH, $n = 5$); (2) controls receiving 20 μL of *P. tridentatum* extract dissolved in ethanol (100 mg/mL) (ETOH + PtL, $n = 5$), and the acute inflammation group was treated with either (3) 20 μL of pure ethanol (CO-ETOH, $n = 5$), (4) 20 μL of *P. tridentatum* extract dissolved in ethanol (100 mg/mL) (CO + PtL, $n = 6$), or (5) 20 μL of Medrol dissolved in ethanol (1 mg/mL of active principle; Medrol, Sigma, Portugal) (CO + ME, $n = 5$).

4.2.2.3.3. Macroscopic assessment of ear oedema

Ear oedema was calculated as ear thickness variation from controls, measured using a micrometer (Analog external micrometer, 0 to 25 mm, resolution 0.001 mm, Mitutoyo, Aurora, IL, USA). Ear thickness was measured after 6 h of the second application of the various compounds followed by occision.

4.2.2.3.4. Histological processing of ear samples

After occision, ear biopsies (6 mm diameter) were collected using a metal punch and preserved in 4% paraformaldehyde (PFA) (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain).

Histological processing followed a protocol described by Oliveira *et al.* (Oliveira *et al.*, 2010). Briefly, each sample was placed in cassettes (Biocassette, Bio Optica, Milano Italy), identified, and placed in 4% PFA (DAC, Applichem Panreac, Barcelona, Spain) at room temperature.

Samples were dehydrated in ascending grade ethanol/water solutions (from 70 to 100%) using an automatic sample processor (Leica AutoStainer XL, Nussloch, Germany) for 19 h followed by washing thrice with xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group, France). Afterward, the specimens were immersed in paraffin (Thermo Scientific, Cheshire, UK) at 62 °C and allowed to solidify at −5 °C. Specimens were cut into 4 μm thick sections and mounted on a Superfrost glass slide (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK). Before staining, the slides were deparaffinized in an automated slide processing apparatus (Leica AutoStainer XL, Nussloch, Germany), and they were stained

with hematoxylin Harris (Millipore Corporation, Massachusetts, MA, USA) for 1 min followed by a bath in tap water for 2 min. Then, slides were washed with 0.5% of ammonia solution (Sigma Aldrich, Spain) for 10 s, washed with tap water for 1 and half minutes, and washed with 96% ethanol solution (45 s). Slides were then immersed in eosin Y solution (Thermo Scientific, Cheshire, UK) for 30 s and dehydrated with 96% ethanol (1 min), absolute ethanol (2 min each time), and xylene (first wash for 1 min and the second for 2 min). Slides were mounted using resinous medium (Entellan, Merck, Darmstadt, Germany), and they were covered with lamellae (24 × 60 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK). Finally, the slides were allowed to air dry for seven days.

4.2.2.3.5. Histopathological assessment of ear samples

Histopathological analysis of ears was performed to analyze the existence/absence of acute inflammation after the application of croton oil and the anti-inflammatory effect of *P. tridentatum* extracts. The assessment was based on the thickness of the dermis and epidermis and the presence of inflammatory cells after each treatment.

Measurement of dermis and epidermis thickness was performed using an optical microscope (Olympus BX61, Tokyo, Japan) interconnected to a digital color camera (Olympus, DP70, Tokyo, Japan) through the CellSens Dimension software (Olympus Corporation, Münster, Germany) at 100× amplification. For each ear sample, nine random measurements were made.

Slides were then photographed using the CellSens Dimension Olympus software (Olympus Corporation, Münster, Germany) on an optical microscope (Olympus, BX61) coupled to a digital color camera (Olympus, DP70, Münster, Germany), and the number of inflammatory cells was counted.

4.2.2.3.6. Experimental osteoarthritis (OA) induction

For the induction of experimental OA, the animals were anesthetized with a mixture of ketamine (0.75 mg/kg, Imalgene, Merial, Oeiras, Portugal) and medetomidine (0.5 mg/kg Dorbene, Esteve, Carnaxide, Portugal).

For the induction of OA (Pinto-Ribeiro *et al.*, 2013), a mixture of 0.1 mL of 3% carrageenan 3% kaolin (Sigma-Aldrich, StLouis, MO, USA) dissolved in a sterile saline solution (NaCl 0.9% pH 7.2, Unither, Amiens, France) was injected into the right knee joint with a hypodermic needle (P20, 0.3 × 13 mm, Microlance, UK). The animals of the sham group were injected with 0.1 mL of vehicle (NaCl 0.9% pH7.2, Unither, Amiens, France). To guarantee the distribution of the mixture and the start of the degradation of the cartilage, the animal's paw was flexed and extended ten times.

Afterwards, the anesthesia was reversed using atipamezole hydrochloride (1 mg/kg, Antisedan, Pfizer, Oeiras, Portugal, i.p.) according to the protocol of David-Pereira *et al.* (David-Pereira *et al.*, 2016). Animals were observed until eating and grooming, and welfare was evaluated daily (grooming, dehydration/weight loss, and locomotor abnormalities).

4.2.2.3.7. Drug preparation and administration

Powdered extract of *P. tridentatum* was dissolved in PBS 10 mM (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 1% Tween 20 (Applichem, Panreac, ITW companies, Barcelona, Spain), pH 7.4), placed on an ultrasound bath (Sonicator Branson 2510, Emerson Corporate, London, UK) for 30 min and stored in the dark at room temperature.

The administered concentration of the extract was adjusted weekly according to the animals' body weight to the final concentrations of 100 mg/kg and 300 mg/kg of *P. tridentatum*, respectively. Before administration, the extract solution was sonicated for 15 min.

Administration of the treatment was performed daily for three weeks through gavage. Controls animals were administered the vehicle solution.

4.2.2.3.8. Pressure Application Measurement (PAM)

Mechanical hyperalgesia in the knee was evaluated using the pressure PAM method (Amorim *et al.*, 2014). Briefly, with the animal securely held, an increasing force was gradually applied across the joint of interest until it reached the maximum force required to elicit a response: paw withdrawal, vocalization, or wriggling movements. The results correspond to the force peak applied immediately before the response and are presented in grams force (gf) (Barton *et al.*, 2007; Malfait *et al.*, 2013).

The measurements were recorded as the limb withdrawal threshold (LWT). LWT was measured twice on each knee joint at 1 min intervals. The mean LWTs were calculated per animal.

4.2.2.3.9. Perimeter of knee

Oedema of the knee joint in rats was evaluated by measuring the perimeter of the right knee in each rat, as described by Adães and colleagues (2015). The measurement was performed at specific times throughout the experiment: (1) before the induction of OA; (2) two weeks after surgery; and (3) after treatment. The measurement was made in the region of the kneecap. The edema results are expressed in centimeters.

4.2.2.3.10. Experimental design

Experiment 1

To test the effect of PtL extract in acute inflammation, animals were challenged through the application of an irritant, croton oil (CO) applied topically in the ear inner surface. For this purpose, animals were first administered either ethanol (ETOH) or croton oil (CO) followed by treatment 15 min later. Treatment consisted in the application of either vehicle (ETOH), and *P. tridentatum* extract (ETOH + PtL and CO + PtL), or an anti-inflammatory drug (glucocorticoid; CO + Medrol). Assessment of the anti-inflammatory efficacy of treatments was evaluated 6 h later by measuring ear thickness followed by occision. Ear samples collected through the punch method were preserved in PFA 4%, processed, and histopathologically evaluated.

Experiment 2

Before the induction of experimental OA, baseline assessments of mechanical hyperalgesia and perimeter of knees were performed in all animals. Animals were allocated to one of two experimental groups, OA (n = 19), or SHAM (n = 4). SHAM and OA surgeries were performed on the same day, and animals were henceforth re-tested weekly. Two weeks post-OA induction, OA animals were subdivided into four groups to receive administration of either the extract (PtL) at a concentration of either 100 mg/kg (OA_PtL100, n = 5), or 300 mg/kg (OA_ PtL300, n = 5), or PBS 1 mg/kg (OA-VEH, n = 5), or a nonsteroidal anti-inflammatory drug (Ibuprofen—Brufen, Mylan, EUA) at 15 mg/kg (OA_AI, n = 4), daily, for a period of three weeks. Sham rats received the vehicle (PBS) alone (SHAM_VEH, n = 4).

4.2.2.4. Computational models—molecular docking

The crystal structure of peroxisome proliferator-activated receptor alpha and gamma (PPARα (2P54) and PPARγ (6TSG), respectively), mPGES-1 (4YL1), prostaglandin H (2) Synthase-1 (1Q4G), protein kinase CK2α (6TGU), inhibitor-bound human 5-lipoxygenase-activating protein (2Q7M), and the crystal structure of mPGES-1 bound to inhibitor (5T36) were obtained from the Protein Data Bank (https://www.rcsb.org/pdb accessed in 08/01/2023). The initial crystal structures were processed with Pymol tools 2.5.2 by removing non-polar water molecules and adding polar hydrogen. The original charges of the crystal structures were saved, and the files were exported as pdbqt. The three-dimensional structure of isoquercetin, quercetin, and genistein were downloaded from NCBI (https://pubchem.ncbi.nlm.nih.gov/ accessed in 08/01/2023), which were then optimized with Pymol 2.5.2 software and processed with AutoDock 4.2 to form .pdbqt files for docking studies.

The probable interaction between bioactive compounds and crystal structures of the mentioned proteins was explored by AutoDock 4.2 with the grid box centered at the protein active center. The docking calculations were performed 250 times runs by the Lamarckian genetic algorithm. The protein–ligand complexations that possess the minimum energy scoring of −7 Kcal/mol were selected for further visualization of the docked conformation with Discovery Studio Software (Biovida Dassault Systèmes, Discovery Studio Software, version 4.5, San Diego, CA, USA) (Hendriani *et al.*, 2017).

4.2.2.5. Statistical analysis

Statistics were performed using the GraphPad Prism 5 software (GraphPad Software Inc., version 5, LaJolla, CA, USA). For comparing groups in the acute dermal irritation test a T-test between experimental groups (ETOH and ETOH + PtI; ETOH and ETOH + CO) was performed, followed by a one-way analysis of variance (ANOVA one-way) between the CO group and treatments (ETOH + CO, CO + PtL and CO + Medrol), followed by the Bonferroni post hoc test.

For the analysis of chronic inflammation data (PAM and Knee oedema), a two-way ANOVA was performed, followed by Bonferroni post hoc test. $p < 0.05$ was considered statistically significant.

4.2.3. Results

4.2.3.1. Identification of phenolic compounds by HPLC-DAD

The methanolic powder extract of leaves of *P. tridentatum* was subjected to HPLC-DAD analysis (Figure 4.2.2). Phenolic compounds were identified by direct comparison of the chromatogram with commercial standards (Table 4.2.1., peaks 14–17 and 22) or based on retention time and UV-Vis absorption spectra (Table 4.2.1., peaks 19, 20, 23, 24). Peaks 19, 20, and 24 were identified as isoflavone derivatives based on their UV spectra (λmax at 260–262 nm) and similarity with genistein spectra. Peak 23 has a spectra similar to quercetin (λmax at 256, 370 nm), but with a slightly different RT. So, this compound was identified as a quercetin derivative. The identification of these compounds agrees with previous works (Roriz *et al.*, 2015; Aires *et al.*, 2016). Taken together, these compounds represent the major phenolics found in our *P. tridentatum* extract.

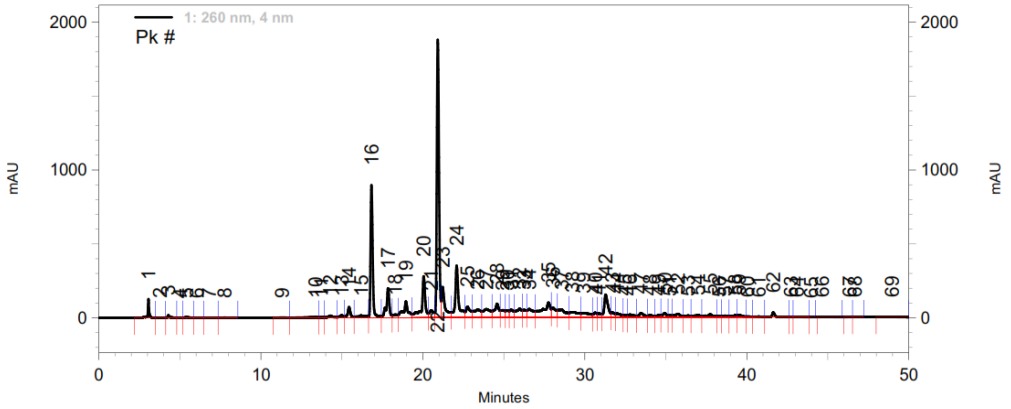


Figure 4.2.2. HPLC-DAD-UV (260 nm) phenolic profile of the methanolic extract from leaves of *P. tridentatum.*

Table 4.2.1. Phenolic composition of the *P. tridentatum* extract.

Peak	Retention Time (min)	Compound	µg/mg dwb
14	15.447	taxifolin	12
15	16.186	rutin	8
16	16.827	isoquercetin	135
17	17.853	genistin	33
19	18.953	i.d.	29
20	20.053	i.d.	53
22	20.920	genistein	225
23	21.220	q.d.	52
24	22.087	i.d.	65

i.d.—isoflavone derivative; q.d.—quercetin derivative.

4.2.3.2. Macroscopic assessment of ear edema

Macroscopic evaluation of ear thickness reveals the application of *P. tridentatum* extract alone (ETOH + PtL) did not alter this parameter when compared to the control group (ETOH) (Figure 4.2.3A: $t_{(8)} = 0.4174$, $p = 0.6874$). Croton oil application (CO + ETOH) significantly increased macroscopic ear thickness when compared to the control group (ETOH) (Figure 4.2.3A: $t_{(8)} = 11.22$, $p < 0.0001$). Further analysis showed significant differences in macroscopic ear thickness after treatment (Figure 4.2.3A: $ANOVA_{one\text{-}way}$: $F_{(2,13)}$ =

49.60, $p < 0.0001$), with post hoc tests demonstrating the application of *P. tridentatum* methanolic extract, and Medrol counteracted ear thickness increase after CO application.

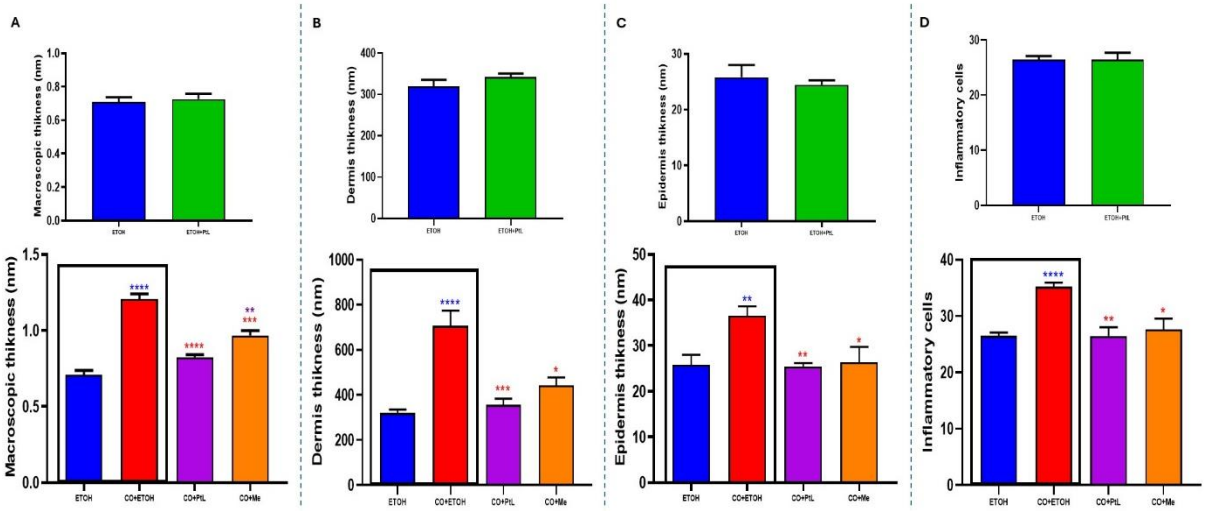


Figure 4.2.3. Changes in macroscopic ear (A), dermis (B) and epidermis thickness (C) and the number of inflammatory cells in the rats' ears (D) after the croton oil challenge test. Differences between the ETOH and ETOH + PtL and the ETOH and CO + ETOH groups were assessed using a Student's T-test, while differences between CO + ETOH, CO + PtL, and CO + Me were assessed using an one-way ANOVA. (ETOH: control animals, application of pure ethanol; CO + ETOH: croton oil 5% dissolved in pure ethanol; ETOH + PtL; application of *P. tridentatum* extract dissolved in pure ethanol; CO + Me: croton oil 5% dissolved in pure ethanol followed by application of Medrol 15 min later; CO + PtL: croton oil 5% dissolved in pure ethanol followed by application of *P. tridentatum* extract 15 min later). Results are expressed as mean ± SEM. Symbols are colored according to their respective experimental group. Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2.3.3. Histopathological assessment of ear samples

The application of *P. tridentatum* extract alone (ETOH + PtL) did not significantly alter dermis (Figure 4.2.3B: t(8) = 1.285, p = 0.2347) and epidermis (Figure 4.2.3C: t(8) = t(8) = 0.5749, p = 0.5812) thickness when compared to the control group (ETOH). Croton oil application (CO + ETOH) significantly increased dermis (Figure 4.2.3B: t(8) = 5.481, p = 0.0006) and epidermis (Figure 4.2.3C: t(8) = 3.560, p = 0.0003) thickness in comparison with the ETOH group.

Similarly to what was observed previously, ear thickness was significantly decreased after topical application of *P. tridentatum* extract (CO + PtL, ANOVAone-way: F(2,13) = 16.05, p = 0.0003) and Medrol (CO + Me, ANOVAone-way: F(2,13) = 7.686, p = 0.0063).

Additionally, the application of *P. tridentatum* extract alone (ETOH + PtL) did not significantly alter the number of inflammatory cells in the ear samples (Figure 4.2.3D: t(8) = 0.00, p > 0.9999) when compared to the control group, while the application of croton oil significantly increased it (Figure 4.2.3D.: t(8) = 8.976, p < 0.0001). Finally, further analysis showed significant differences after treatment (Figure 4.2.3D:

ANOVAone-way: F(2,13) = 9.728, p = 0.0026), with post hoc tests showing that the application of *P. tridentatum* extract and Medrol significantly decreased the number of inflammatory cells.

4.2.3.4. Mechanical hyperalgesia

To evaluate the development of mechanical hyperalgesia, the PAM test was performed. Statistical analysis of PAM showed mechanical hyperalgesia in the ipsilateral knee varied differently throughout the experimental period depending of the experimental group (Figure 4.2.4.; $ANOVA_{two\text{-}way}$: Interaction: $F_{(16,90)}$ = 6.941, $p < 0.0001$; Time: $F_{(4,90)}$ = 23.11, $p < 0.0001$; Group: $F_{(4,90)}$ = 36.63.4, $p < 0.000$). Post hoc tests showed OA animals displayed greater levels of mechanical hyperalgesia when compared to shams during weeks one to four. Post hoc tests also showed both extract treatments significantly decreased mechanical hyperalgesia during weeks three to four when compared to non-treated OA animals. Additionally, the treatment with the commercial non-steroidal anti-inflammatory drug partially reverts mechanical hyperalgesia when compared to non-treated OA animals.

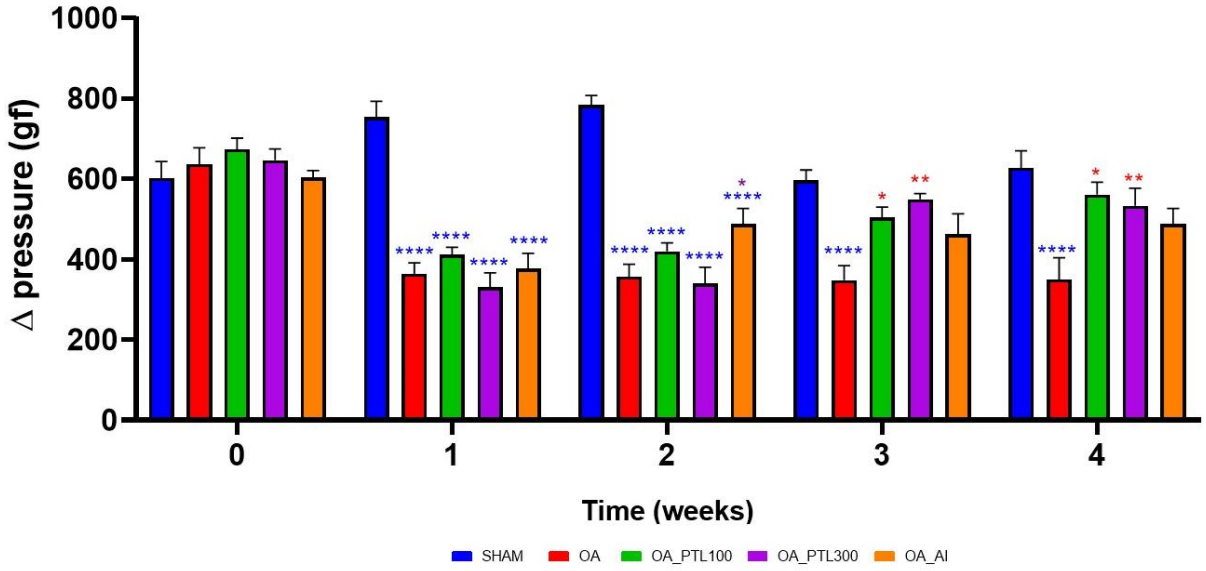


Figure 4.2.4. Assessment of mechanical hyperalgesia in the knee throughout the experimental period. Symbols are colored according to their respective experimental group. Differences between the sham, OA, OA_PtL100, OA_PtL300, and OA_AI groups were assessed using a two-way ANOVA. Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (*n*SHAM = 4; *n*OA = 4; *n*OA_PTL100 = 5; *n*OA_PTL300 = 5; *n*OA_AI = 5) (SHAM—control animals that underwent all surgical procedures but were not induced OA; OA—osteoarthritic animals; OA_PTL100—osteoarthritic animal that from week two were treated with a concentration of 100 mg/kg of *Pterospartum tridentatum* extract; OA_PTL300—osteoarthritic animals that, from week two, were treated with a concentration of 300 mg/kg of *Pterospartum tridentatum* extract; OA_AI—osteoarthritic animal that, from week two, were treated with a commercial nonsteroidal anti-inflammatory drug).

4.2.3.5. Perimeter of knee

To evaluate inflammation of the knee, the knee's perimeter was assessed (Figure 4.2.5.). Statistical analysis showed the edema in the ipsilateral knee varied differently throughout the experimental period depending on the experimental group ($ANOVA_{two\text{-}way}$: Interaction: $F(8,54) = 1.694$, $p = 0.01210$; Time: $F(2,54) = 51.98$, $p < 0.0001$; Group: $F(4,54) = 4.383$, $p = 0.0038$). Post hoc tests showed OA animals displayed increased knee perimeter when compared to shams during weeks one to four. Moreover, both extract treatments partially reverted the knee swelling when compared to non-treated OA animals. Treatment with the commercial nonsteroidal anti-inflammatory drug also partially reverted knee oedema when compared to non-treated OA animals, but only in the fourth week.

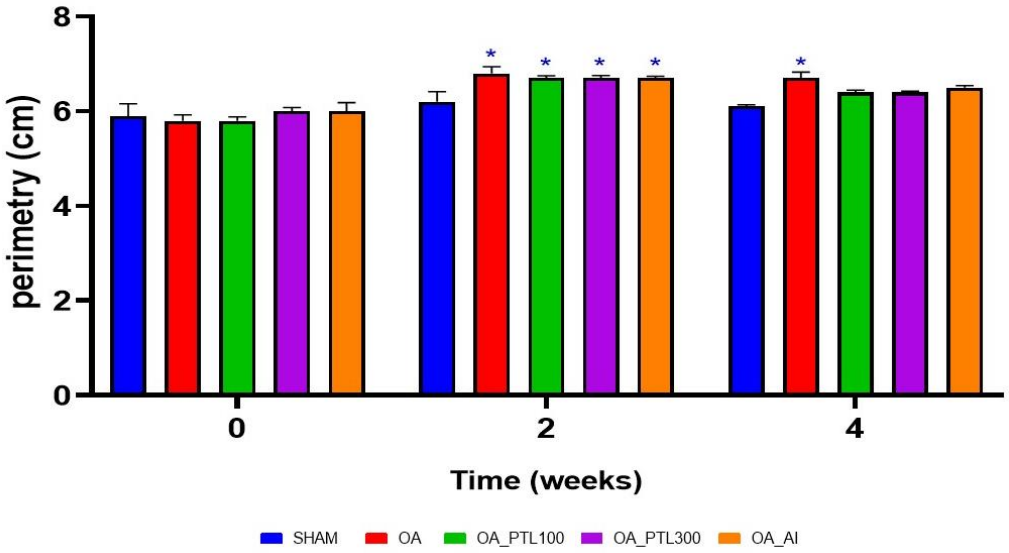


Figure 4.2.5. Assessment of knee perimeter throughout the experimental period. Symbols are colored according to their respective experimental group. Differences between the sham, OA, OA_PtL100, OA_PtL300, and OA_AI groups were assessed using a two-way ANOVA. Results are expressed as mean ± SEM. * $p < 0.05$ *n*SHAM = 4; *n*OA = 4; *n*OA_PTL100 = 5; *n*OA_PTL300 = 5; *n*OA_AI = 5) (SHAM—control animals that underwent all surgical procedures but were not induced OA; OA—osteoarthritic animals; OA_PTL100—osteoarthritic animals that from week two were treated with a concentration of 100 mg/kg of *Pterospartum tridentatum* extract; OA_PTL300—osteoarthritic animals that from week two were treated with a concentration of 300 mg/kg of *Pterospartum tridentatum* extract; OA_AI—osteoarthritic animals that from week two were treated with a commercial nonsteroidal anti-inflammatory drug).

4.2.3.6. Molecular docking analysis

The molecular docking studies of quercetin, isoquercetin, and genistein were performed against the crystal structure of PPARα (PDB 2P54) and PPARγ (PDB 6TSG), respectively), mPGES-1 (PDB 4YL1), prostaglandin H (2) Synthase-1 (PDB 1Q4G), protein kinase CK2α (PDB 6TGU), inhibitor-bound human 5-lipoxygenase-activating protein (PDB 2Q7M), and the crystal structure of mPGES-1 bound to inhibitor

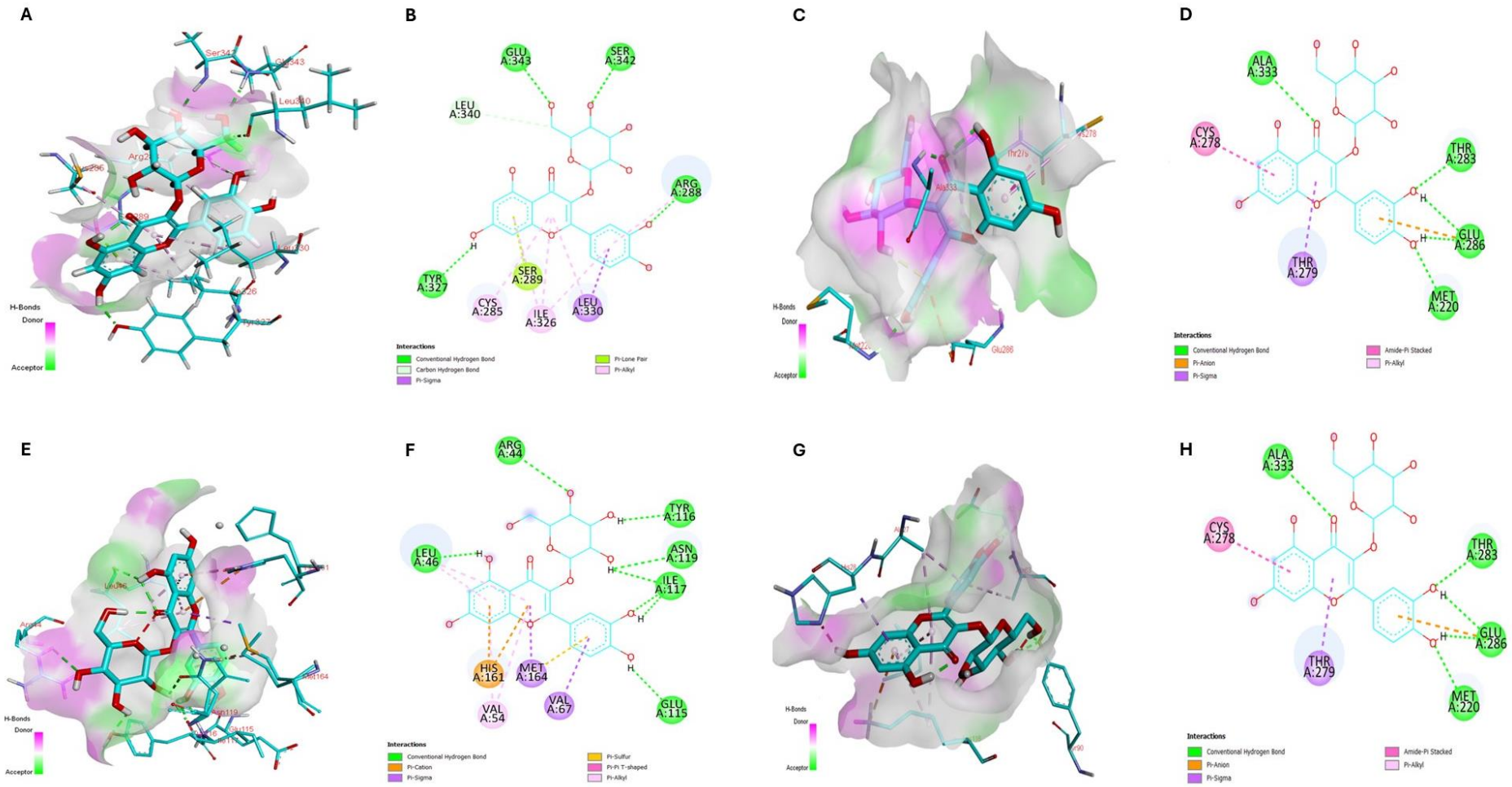
(PDB 5T36) to determine their binding affinities. The results are synthesized in Table 4.2.2. and Figure 4.2.6.

Isoquercetin demonstrated strong molecular interactions with PPARy, PPARa, CK2a, and 5-LO proteins (binding energies of −7.24, −7.27, −8.66, and −7.96 kcal/mol, respectively). Genistein and quercetin had docking for Ck2a (binding energies of −9.97 and −9.78, respectively) and COX-1 (binding energies of −9.01 and −8.78, respectively).

Table 4.2.2. Molecular docking results of isoquercetin, genistein, and quercetin against crystal structure of peroxisome proliferator-activated receptor alpha and gamma (PPARα (PDB 2P54) and PPARγ (PDB 6TSG), respectively), mPGES-1 (PDB 4YL1), prostaglandin H (2) Synthase-1 (PDB 1Q4G), protein kinase CK2α (PDB 6TGU), inhibitor-bound human 5-lipoxygenase-activating protein (PDB 2Q7M), and the crystal structure of mPGES-1 bound to inhibitor (PDB 5T36).

Protein	**Compound**	**Binding Energy (kcal/mol)**	**Inhibition Konstant (μM)**	**Intermolecular Energy (kcal/mol)**
PPARγ (PDB 6tsg)	Isoquercetin	−7.24	4.91	−10.82
	Quercetin	−6.23	26.93	−10.11
	Genistein	−5.81	55.18	−9.09
COX-2 (PDB 4yl1)	Isoquercetin	−4.66	385.08	−8.24
	Quercetin	−4.79	310.18	−8.66
	Genistein	−4	1160	−7.28
COX-1 (PDB 1q4g)	Isoquercetin	−5.98	41.07	−9.56
	Quercetin	−9.01	0.24772	−10.80
	Genistein	−8.78	0.36957	−9.97
PPARα (PDB 2p54)	Isoquercetin	−7.27	4.68	−10.85
	Quercetin	−5.66	71.17	−9.54
	Genistein	−6.24	26.76	−9.52
CK2α (PDB 6tgu)	Isoquercetin	−8.66	0.44756	−12.08
	Quercetin	−9.97	0.04941	−11.76
	Genistein	−9.78	0.06758	−10.98
5-LO	Isoquercetin	−7.96	1.46	−11.54

(PDB 2q7m)	Quercetin	−6.92	8.43	−10.80
	Genistein	−6.87	9.19	−10.15
mPGES-1 (PDB 5t36)	Isoquercetin	−4.62	410.41	−8.2
	Quercetin	−1.52	5457	−5.6
	Genistein	−5.04	201.89	−8.32


A
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H-Bonds
Donor
Acceptor
Interactions
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Pi-Sigma
Pi-Lone Pair
Pi-Alkyl
Pi-Anion
Amide-Pi Stacked
Pi-Cation
Pi-Sulfur
Pi-Pi T-shaped
GLU A:343
SER A:342
LEU A:340
ARG A:288
TYR A:327
SER A:289
CYS A:285
ILE A:326
LEU A:330
ALA A:333
CYS A:278
THR A:283
GLU A:286
THR A:279
MET A:220
ARG A:44
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GLU A:115

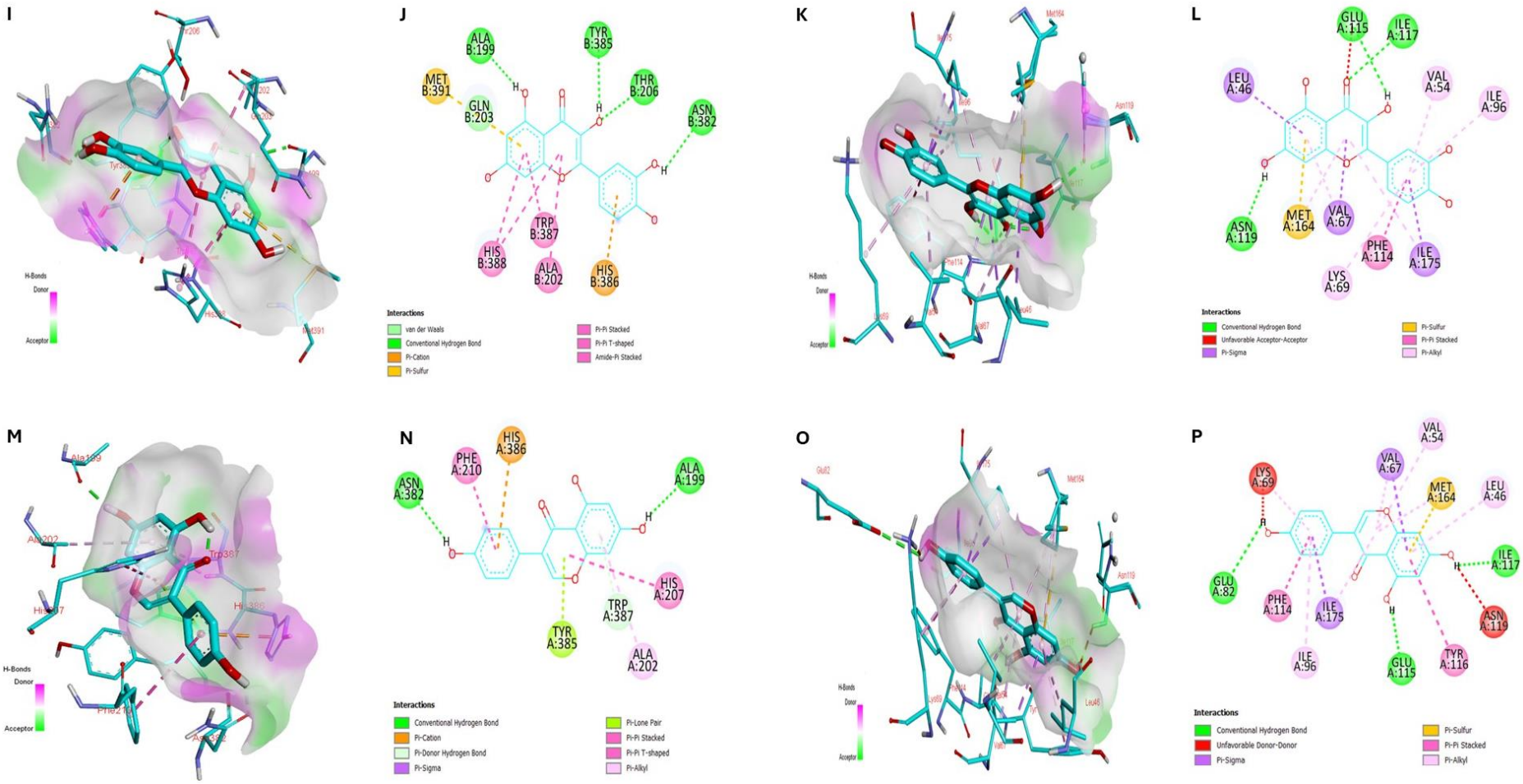


Figure 4.2.6. Schematic representation of the interaction of isoquercetin ligand on the active site of PPARγ (PDB 6tsg) receptor in 3D (A) and 2D (B) structure; PPARα (PDB 2P54) receptor in three-dimensional (C) and two-dimensional (D) structure; CK2α (PDB 6TGU) receptor in three-dimensional (E) and two-dimensional (F) structure; 5-lipoxygenase-activating protein (PDB 2Q7M) receptor in 3D (G) and 2D (H) structure; interaction of quercetin and genistein ligand on the active site of prostaglandin H (2) synthase-1 (PDB 1Q4G) in three-dimensionsal (I,M, respectively) and two-dimensional (J,N, respectively) structure and CK2α (PDB 6TGU) receptor in three-dimensional (K,O, respectively) and two-dimensional (L,P, respectively) structure.

4.2.4. Discussion

P. tridentatum is known as displaying anti-inflammatory activity, with fresh or shade-dried flowers of *P. tridentatum* also being used in traditional medicine, in infusions, decoctions, and tonics (Pinela *et al.*, 2011) as anti-inflammatory (Gião *et al.*, 2007; Bremner *et al.*, 2009) , diuretic, and depurative of the liver (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Gonçalves *et al.*, 2013 ; Aires *et al.*, 2016).

Herein, we evaluated the effects of the leaves methanolic extract of *P. tridentatum* using various *in vivo* tests in rodents, as well as computational models to assess the molecular interaction of its biological compounds with proteins involved in inflammatory cascades.

Our data shows the topical application (100 mg/mL) and oral administration (100 and 300 mg/kg) of *P. tridentatum* extract were able to significantly decrease ear and knee oedema, respectively, as well as mechanical hyperalgesia. Moreover, the molecular docking analysis evaluated the binding of the compounds (isoquercetin, quercetin, and genistein) with amino acid residues at the mentioned proteins active site and measured binding free energy parameter, inhibition constants, hydrogen bonding, and interactions. The free energy value can predict a compound's binding affinity and its ability to inhibit the protein. A negative free energy value indicates a stronger affinity between the ligands and receptors (Hendriani *et al.*, 2017). Based on the results in Table 4.2.2., isoquercetin, quercetin, and genistein had lower inhibition constant, negative free binding, and negative intermolecular interaction energies, indicating stronger affinity with the receptors and that they could potentially inhibit the activation of inflammatory cascade.

Regarding the *in vivo* experiment of acute inflammation, the ear oedema induced by topical administration of croton oil mimics several characteristics observed in human skin diseases (Rodrigues *et al.*, 2016). In the used model, the application of croton oil provokes an inflammatory reaction, specifically epidermal hyperplasia, increased vascularization, and increased inflammatory infiltrates (Di Meglio *et al.*, 2011). After croton oil application, the ears' histopathological analysis revealed an increase in ear thickness (macroscopic and microscopic), as well, regarding the number of inflammatory cells. This effect was prevented by the topical application of *P. tridentatum* extract, which did not affect ear macro- and microstructure when applied alone. Moreover, its effects were the same as Medrol application, a synthetic glucocorticoid used to treat local inflammation (Barnes, 2006).

In the chronic inflammation model, the K/C-induced experimental osteoarthritis causes inflammation in the knee joint and subsequent chronic pain (Barnes *et al.*, 2006; Amorim *et al.*, 2014). The PAM data showed the animals developed mechanical hyperalgesia in the knee, confirming the correct induction of

the model. This effect was sustained from the first week after induction and lasted until the end of the experiment. Importantly, our results showed both concentrations of the extract (100 and 300 mg/Kg) were able to decrease mechanical allodynia from the third week onwards (one week after the start of treatment). Moreover, the extract was more efficient than the commercial nonsteroidal anti-inflammatory drug used as a control for drug administration (ibuprofen). As with the PAM, the knee perimeter is an essential measure to confirm experimental OA, as this test, by measuring the knee perimeter, measures oedema and indirectly inflammation (Barton *et al.*, 2007; Neugebauer *et al.*, 2007). Previous studies showed that K/C-induced osteoarthritis causes inflammation and swelling in the knee (Amorim *et al.*, 2014; Mussawy *et al.*, 2021). Our outcomes are in line with previous results, since, after OA induction, the knee perimeter of rats increased (Amorim *et al.*, 2014). Similarly, to what was observed in the PAM test, both the extract treatments and the nonsteroidal anti-inflammatory drug treatment decreased knee perimeter in OA animals. Furthermore, both extract concentrations displayed identical efficiency.

The phytochemical characterization of *P. tridentatum* demonstrated its richness in phenolic compounds, which are frequently associated with anti-inflammatory activity (Ferrandiz and Alcaraz, 1991; Bremner *et al.*, 2009; Pinto *et al.*, 2020). In the present study, the HPLC-DAD analysis of *P. tridentatum* leaves methanolic extract demonstrated the presence of various phytochemical compounds, predominantly quercetin, quercetin derivates, isoquercetin, and genistein. These outcomes agree with the phytochemical studies found in the literature, which describe the occurrence of these types of compounds in extracts of *P. tridentatum* (Roriz *et al.*, 2014; Roriz *et al.*, 2015 Caleja *et al.*, 2019).

Phenolic compounds can act alone or by synergy to protect against damage caused by oxidative stress. In their structure, phenolic compounds display an aromatic ring that presents one or more hydroxyl substitutes, including their functional derivates. This aromatic feature in association with a highly conjugated system with multiple hydroxyl groups makes these compounds good electron or hydrogen atom donors and enhances their ability to neutralize free radicals and other reactive oxygen species (Zhang and Tsao, 2016).

As previously shown, isoquercetin and genistein, phenolic compounds present in *P. tridentatum* extracts, could be linked to the scavenging of reactive oxygen species (Elnoury, 2019; Yang *et al.*, 2021; Goh *et al.*, 2022). Nevertheless, our results in molecular docking analysis showed isoquercetin can also act as an agonist of PPARα and PPARγ, inhibiting NF-κB signaling and activation of macrophages, consequently decreasing inflammatory cytokine production (tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and Il-1β (Decara *et al.*, 2020). Furthermore, isoquercetin showed an important capability to inhibit 5-LO,

which decreases TNF-α, IL-6, and MCP-1 production, and it is also involved in inflammation (Mohd Amin *et al.*, 2020). Additionally, isoquercetin and genistein demonstrated very strong molecular interactions with CK2α, which is known to inhibit NF-κB activation in macrophages and subsequently IL-1, IL-6, and IL-10 cytokine secretion (Glushkova *et al.*, 2018; Larson *et al.*, 2020). Finally, the inhibition of COX-1 by quercetin and genistein can act by decreasing the levels of arachidonic acid, thus decreasing the production of bioactive prostanoids (Calvello *et al.*, 2017; Schmid *et al.*, 2021).

In the literature, studies about the effect of *P. tridentatum* on inflammation are scarce and do not identify the mechanism of action, but some reports support our results. Bremner *et al.* (2009) evaluated the anti-inflammatory activity of *P. tridentatum* (methanolic, petroleum ether, and ethyl acetate extracts), and their results demonstrated the inhibition of TNF-α levels in human monocytes. Likewise, Simões *et al.* (2020) assessed the anti-inflammatory activity of *P. tridentatum* ethanolic extracts and demonstrated a downregulation of the Nos2 gene, along with a decrease in the transcription of the pro-inflammatory genes IL1b, IL6, and Ptgs2.

4.2.5. Conclusions

Our work highlights the potential of *P. tridentatum* extracts as a source of compounds that can be used as adjuvants in the management of pain and inflammation. However, further studies should evaluate the long-term effect of the extracts to check their contribution to preventing OA progression and its efficiency in other experimental models of inflammation. In addition, it would also be interesting to test a larger range of extract concentrations to determine the lowest concentration that maintains anti-inflammatory effects.

4.2.6. References

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4.3. The multi-faceted benefits of *Genista tridentata* extract: from alleviating pain in diabetic rats to extending *C. elegans* lifespan in glucotoxic conditions

4.3.1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Chaudhury *et al.*, 2017). It is a global health concern, with escalating prevalence rates and substantial impacts on morbidity and mortality worldwide (Zimmet *et al.*, 2016). According to the International Diabetes Federation (IDF), the number of adults living with DM was 537 million in 2021, with projections estimating a rise to 783 million by 2045. Alongside the physical manifestations of DM, such as polyuria, polydipsia, and polyphagia, patients are predisposed to a myriad of complications, including neuropathy, nephropathy, retinopathy, and cardiovascular diseases (Giri *et al.*, 2018).

Amongst these, diabetic neuropathy stands out as a major concern (Rosenberger *et al.*, 2020) as around one third of individuals diagnosed with DM will experience diabetic neuropathic pain (Abbott *et al.*, 2011). Moreover, the risk of painful neuropathic symptoms in type 2 diabetes was twice that of type 1 diabetes (Abbott *et al.*, 2011). This condition encompasses symptoms such as allodynia (pain triggered by typically non-painful stimuli) and hyperalgesia (heightened sensitivity to painful stimuli) (Jolivalt *et al.*, 2016), thereby substantially reducing the quality of life for those affected (Molvær *et al.*, 2023). Also, DM exerts profound effects on various organs, particularly the pancreas, liver, kidneys, and immune system (Giri *et al.*, 2018), contributing to significant morbidity and mortality. The pathological changes associated with T2DM involve alterations in metabolic homeostasis, oxidative stress, and inflammation, leading to tissue damage and dysfunction (Oguntibeju, 2019). Given the adverse impact of diabetic neuropathy on patient well-being, there is a pressing need for effective therapeutic strategies to alleviate pain and improve quality of life in individuals with diabetes (Molvær *et al.*, 2023).

Herbal remedies, long utilized in traditional medicine, have garnered attention for their potential in managing various disorders (Salmon, 2022), including neuropathic pain associated with DM (Arora *et al.*, 2021; Palai *et al.*, 2023). *Genista tridentata* (L.) Willk., previously known as *Chamaespartum tridentatum* P. Gibbs and *Pterospartum tridentatum* L. Willk, is a Leguminosae (Fabaceae), subfamily Papilionoidae, commonly referred to as "prickled broom" or "carqueja" in Portugal (Coelho *et al.*, 2011; Ferreira *et al.*, 2012). With its aromatic qualities, *G. tridentata* is frequently utilized in Portuguese cuisine, where its leaves are traditionally employed as a seasoning for rice and meat dishes (Ribeiro *et al.*, 2000). In addition to its culinary applications, *G. tridentata* has a history of use in traditional medicine for its suggested anti-

inflammatory (Bremner *et al.*, 2009; Garcia-Oliveira *et al.*, 2022) and antidiabetic properties (Coelho *et al.*, 2011; Ferreira *et al.*, 2012). Extracts from this plant have been noted for their high levels of flavonoids and phenolic compounds (reviewed by Laranjeira *et al.*, 2023a). Furthermore, extracts from *G. tridentata*, have shown promise due to their diverse pharmacological properties, including anti-inflammatory and antioxidant effects (Garcia-Oliveira *et al.*, 2022; Mota *et al.*, 2022; Laranjeira *et al.*, 2023).

Our study aims to explore the potential antinociceptive effects of *G. tridentata* methanolic extracts in diabetic rats. Prior to conducting the *in vivo* study in the rat model, we conducted cytotoxicity assessments of the extracts using HepG2 cells and evaluated their impact on the toxicity and lifespan of *C. elegans*. Subsequently, our focus shifted to rats, where we evaluated mechanical and thermal allodynia and hyperalgesia, key hallmarks of diabetic neuropathy. We also assessed the efficacy of *G. tridentata* treatments in mitigating these nociceptive behaviors. Additionally, we aimed to examine the influence of DM on rat's internal organs and determine whether *G. tridentata* extracts could ameliorate the histopathological changes induced by DM.

4.3.2. Material and methods

4.3.2.1 Production of *G. tridentata* extract

Aerial parts of *G. tridentata* were collected on the North of Portugal (Esposende) during the flowering season in May 2019. A voucher specimen 12/2019 was deposited at the Herbarium of the Biology department of University of Minho. The plant material was air-dried, the leaves were separated and subsequently triturated using a blender. The resulting powder underwent a maceration process with a methanolic solution (7:2 v/v) for 5 days. Following this, the solution was filtered and concentrated using a rotary evaporator (Buchi® RII, Tuttligen, Germany) under reduced pressure (Buchi® V-700 pump, cidade, país) with a bath set at 30 °C. The extract was then subjected to lyophilization (Christ® Alpha 2-4, B. Braun, cidade, país) for 3 days and stored in the dark, at room temperature.

4.3.2.2. *G. tridentata* cytotoxicity

4.3.2.2.1. Cell culture

The HepG2 (hepatocellular carcinoma) cell line was obtained from European collection of cell cultures (ECACC). These cell lines were cultured in 25 cm2 polystyrene flasks (Nunc Cell Culture, Thermofisher Scientific, Antwerpen, Belgium) with complete DMEM (Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, 1% antibiotic–antimycotic solution, and 1.5 g/L sodium bicarbonate; Sigma-Aldrich, Barcelona, Spain) at 37 °C in a humidified incubator with 5% CO2 (Binder

CB150, Labstuff.eu e. K., Malente, Germany). Sub-culturing was performed thrice a week using 0.05% trypsin (Sigma-Aldrich, Barcelona, Spain), and cells were seeded at a density of 5×10^5 cells per ml. For all subsequent assays, DMEM without FBS was used.

4.3.2.2.2. Cell viability/cytotoxicity assay

To assess the potential cytotoxicity of *G. tridentata* extracts, HepG2 cells were seeded in 96-well culture plates (Sigma-Aldrich, Barcelona, Spain) at a density of $1.5x10^5$ cells/ml (200 µl/well) in complete DMEM. After 24 hours, the medium was removed, and the cells were exposed to different concentrations of *G.tridentata* extracts ranging from 100 to 500 µg/ml for 4 h and 24 h. Cells in DMEM without extracts and cells treated with 0.5% and 10 % DMSO in DMEM served as normal growth and death controls, respectively. Cell viability was assessed using the MTT assay previously described by Oliveira *et al.*, (2018). The cell viability was expressed as a percentage of the control (cells cultured only in DMEM).

4.3.2.3. Exploring *G. tridentata* effects in *C. elegans* under glucotoxicity conditions

4.3.2.3.1. *C. elegans* strains, maintenance, and synchronization

The *C. elegans* strains employed in this study were supplied by the *Caenorhabditis* Genetics Center (CGC). Standard procedures as outlined by Brenner (1974) were followed for the cultivation of animals on nematode growth medium (NGM) agar plates, maintaining a temperature of 20°C. To sustain the *C. elegans* population, the OP50 strain of *Escherichia coli* was cultured on plates, grown overnight in Luria Broth (LB; Sigma-Aldrich, Barcelona, Spain) media at 37°C and 180 rpm. For synchronization of *C. elegans*, a hypochlorite treatment was employed, utilizing a solution comprising approximately 2.6% NaClO and 0.5 M NaOH. This treatment lasted for 5 minutes, ensuring the synchronization of the *C. elegans* population for subsequent experimental procedures.

4.3.2.3.2. Toxicity evaluation of *G. tridentata* extracts in *C. elegans* – Food clearance assay

To evaluate the toxicity of extracts, the *C. elegans* Bristol strain N2 was utilized in a 96-well plate assay conducted in a liquid culture, following the procedure outlined by Teixeira-Castro *et al.* (2015). Each well had a total volume of 60 µL, comprising 15 µL of egg suspension (containing 20-25 eggs), 25 µL of *G. tridentata* plant extract at concentrations ranging from 0.125 to 2 mg/mL, and 20 µL of OP50 bacteria with an optical density (OD) of 0.6-0.7 at 595 nm.

The OP50 bacteria were cultivated overnight at 37°C and 180 rpm in Luria Broth (LB) media, then pelleted through centrifugation, inactivated via three cycles of freeze/thawing, frozen at -80°C, and subsequently resuspended in complete S-medium. The worms were cultured with continuous shaking at

180 rpm and 20°C using a Shell Lab incubator shaker for a duration of seven days. The consumption of OP50 was measured daily at 595 nm using a NanoQuant Plate™ (Tecan). The drug vehicle utilized was the control DMSO (1%), while DMSO at 5% served as the positive control for toxicity.

4.3.2.3.3. Glucotoxicity induction

To induce glucotoxicity following the protocol by Schlotterer *et al*. (2009), gravid adult nematodes were transferred for a duration of 2 hours to NGM plates supplemented with glucose at a concentration of 100 mmol/L. This was performed to facilitate the production of eggs and generate a synchronized population born under hyperglycemic conditions. The control groups, including uninduced worms seeded with the OP50 strain of *Escherichia coli* grown in LB (i), uninduced worms seeded with the OP50 strain of *Escherichia coli* grown in LB supplemented with *G. tridentata* extract (ii), glucotoxicity-induced worms seeded with the OP50 strain of *Escherichia coli* grown in LB (iii), and glucotoxicity-induced worms seeded with the OP50 strain of *Escherichia coli* grown in LB supplemented with *G. tridentata* extract (iv), were maintained in triplicates as were the experimental groups.

4.3.2.3.4 *C. elegans* lifespan assays

To assess the lifespan of *C. elegans* following the protocol by Vilasboas-Campos *et al*. (2021), animals were cultured on NGM solid plates or NGM solid plates supplemented with glucose (100mM). These plates were seeded with *G. tridentata* extracts (1 mg/mL) resuspended in inactivated OP50. The L4 stage was designated as day 0. During the initial two weeks, animals were transferred daily to freshly seeded plates containing inactivated OP50 and *G. tridentata* extracts (1 mg/mL). Subsequently, transfers occurred every 2-3 days for the remaining duration of the experiment.

Mortality was recorded daily, considering an animal dead if there was no mechanical response after ten consecutive touches with a platinum wire. In cases where internal hatching, extrusion, or desiccation at the border of the plate had occurred, censoring was applied to account for these factors in the analysis of lifespan data. The drug vehicle utilized was the control DMSO (1%) and daf-2 and daf-16 mutants were utilized as positive and negative controls, respectively.

4.3.2.4. Exploring *G. tridentata* effects in rats with experimental T2DM

4.3.2.4.1. Animals, ethical considerations and handling

The animals in this study were provided by the Life and Health Sciences Research Institute (ICVS) bioterium. All experimental protocols carried out was approved by the Institutional and National Ethical Commission (DGAV 23875/2019) and followed the European Community Council Directive

2010/63/EU, specific for the treatment of animals in experimental procedures. All efforts were made to reduce the number of animals to produce reliable scientific data and their suffering.

The housing conditions involved placing two animals in each polycarbonate cage (45.4x 25.5x 20 cm) within a temperature-controlled environment (22 °C, 55% relative humidity), a 12-hour light/dark cycle, and constant access to water and food (F0021; BioServ, New Jersey, US). Regular evaluations by the resident veterinarian occurred weekly throughout the experimental period to ensure animal welfare. Additionally, general health parameters and bodyweight were assessed weekly by the researcher.

During an initial phase lasting 10 to 15 days, the animals were acclimated to the researchers and the apparatus used for the tests. All experiments were conducted during the light cycle (8:00 a.m. to 8:00 p.m.). Following the tests, the animals were returned to their home cage and the animal house. Trained researchers executed all experiments, and efforts were made to ensure that the experimenters were blinded to the treatment conditions.

4.3.2.4.2. Induction of experimental type 2 diabetes (T2DM)

To induce T2DM, a dose of 180 mg/kg of nicotinamide (NA) dissolved in saline (NaCl 0.9%, Unither, Amiens, France) was administered intraperitoneally (i.p.) using a needle coupled with a syringe (0.6x25mm, Terumo, Neolus, Belgium). This procedure was followed 15 minutes later by i.p. administration of 65 mg/kg streptozotocin (STZ, Merck, Algés, Portugal) dissolved in citrate buffer (citrate buffer 0.1M, pH 4.5, Sigma Aldrich, Sintra, Portugal). Animals assigned to the control group (SHAM) received only the vehicle solutions: NaCl 0.9% (0.9% NaCl pH 7.2, Unither, Amiens, France) and citrate buffer 15 minutes later (citrate buffer 0.1M, pH 4.5, Sigma Aldrich, Sintra, Portugal) (Masiello *et al.*, 1998).

For monitoring the progression of T2DM, the glycemic index was measured after 24 and 72 hours by a blood glucose test (described in the section 4.3.2.4.4). Only animals with a glycemic index exceeding 300 mg/dl were included in the diabetic group, following the criteria established by Arulmozhi *et al.* (2004).

Considering the severity of experimental T2DM, humane endpoints were predefined before the experiment started. These included a 20% weight loss per week, blindness, and urinary tract infections, and were supervised by both researchers and the resident veterinarian. Additionally, daily monitoring was conducted to observe the development of polyuria, polydipsia, polyphagia, and cataracts in the animals.

4.3.2.4.3. Extract preparation and administration

The powdered extract of *G. tridentata* was dissolved in PBS 10 mM (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 1% Tween 20 from Applichem, Panreac, ITW companies, Barcelona, Spain) with a pH of 7.4. The solution was then subjected to ultrasound treatment using a Sonicator Branson 2510 (Emerson Corporate, London, UK) for a duration of 30 minutes and subsequently stored in the dark at room temperature.

The concentration of the administered extract was adjusted weekly based on the animals' body weight, resulting in final concentrations of 100 mg/kg and 300 mg/kg of *G. tridentata* leaves extract, respectively. Prior to administration, the extract solution underwent a 15-minute sonication process.

Treatment administration occurred daily for a four-week period through gavage. Control animals were administered the vehicle solution.

4.3.2.4.4. Glycemia

Blood glucose levels (glycemia) were quantified through a weekly blood glucose test. This procedure involved puncturing the tip of the tail using a 26G needle (0.45 x 13 mm) to obtain a drop of blood, approximately 32 µl in volume. The obtained blood drop was then transferred to a test strip until the strip's deposit was filled. The test strip had previously been inserted into the blood glucose measuring device (Contour Next XT, Ascensia, Switzerland).

Upon insertion, the device provided a reading of the blood glycemic value in mg/ml, which was displayed on the device monitor. Subsequently, this value was recorded as part of the monitoring process (Okyar *et al.*, 2001).

4.3.2.4.5. Nociceptive behavioural analyses

4.3.2.4.5.1. Tactile allodynia – Von Frey

To evaluate mechanical allodynia, characterized by an exaggerated response to a non-painful stimulus, the Von Frey test was conducted weekly, following the method described by Fonseca-Rodrigues *et al.* (2021). A calibrated Von Frey monofilament (North Coast Medical Inc, Morgan Hill, CA), was applied to the plantar surface of the rat for 1-2 seconds and allodynia determined using the up-down method (Bonin *et al.*, 2014). Briefly, the animals were positioned on an elevated grid inside an inverted transparent box, and each plantar surface of the posterior paw was probed with Von Frey monofilaments exerting forces of 0.4g, 0.6g, 1.0g, 2g, 4g, 6g, 8g, and 15g. The test started with the use of a monofilament

corresponding to 2g of force. If the animal exhibited a reaction to the stimuli, a weaker monofilament stimulus was applied; conversely, if the animal did not react, a stronger monofilament was used.

The up-down method was repeated four times following the first positive response, and the 50% threshold for paw withdrawal was determined by considering each animal's specific response pattern and the value of the final monofilament used (Trierweiler *et al.*, 2011).

4.3.2.4.5.2. Cold allodynia – Acetone test

The acetone test was conducted weekly to evaluate cold allodynia, characterized by exaggerated responses to thermal stimulation, as described by Pawar *et al.* (2021). As described by Amorim *et al.* (2017) each animal was placed above an elevated grid surface and covered by a transparent container.

During the test, approximately 100 µl of acetone was carefully placed with a syringe under the surface of each animal's posterior hind paw. This process was repeated three times on each paw at 1-minute intervals. The possible responses of the animal were scored according to the following criteria: 0 - if the animal showed no reaction; 1 - if the animal withdrew the paw quickly, tapped, or tapped the paw; 2 - if the animal displayed prolonged withdrawal of the paw or repeated movements; 3 - if the animal exhibited repeated movements with the paw while simultaneously licking the paw (Poulson *et al.*, 2020). The average of these scores was calculated for subsequent data analysis.

4.3.2.4.5.3. Mechanical hyperalgesia – Randall – Selitto test

To evaluate mechanical hyperalgesia, characterized by enhanced sensitivity to pain from a painful stimulus, the Randall-Selitto test was conducted weekly, following the method described by Vieira *et al.* (2020). This test involved placing the animal in the Randall-Selitto apparatus (Ugo Basile, Italy), and applying upward pressure on each of the hind paws until the animal either removed or moved its paw (Pawar *et al.*, 2021). The response was quantified by the mechanical force, measured in grams, that induced hind paw withdrawal.

The paw withdrawal threshold (PWT) for each animal was calculated by averaging three consecutive measurements, providing a quantitative assessment of mechanical hyperalgesia over the course of the study.

4.3.2.4.5.4. Thermal hyperalgesia – Tail flick test

To assess thermal hyperalgesia, the tail-flick test was employed weekly, following the protocol described by Pinto-Ribeiro *et al.* (2013). This test involved placing the midpoint of the awake animals' tails on the tail-flick device (The Plantar Test Instrument Model 37370, Ugo Basile, Varese, Italy) positioned above

the radiant heat point, programmed at an intensity of 30, with a cut-off set at 15 seconds. The duration between the moment of heat application and the removal of the tail was recorded as the tail withdrawal latency (TWL), providing a quantitative measure of thermal hyperalgesia.

4.3.2.4.6. Histological processing and analysis of the internal organs

Upon completion of experimental procedures, animals received a lethal dose of sodium pentobarbital (200mg/ml, Ceva, Oeiras, Portugal), and internal organs (spleen, liver, kidneys, and pancreas) were sampled. Histopathological samples were stained with haematoxylin and eosin (Oliveira *et al.*, 2010). Samples underwent dehydration using a series of ethanol/water solutions (ranging from 70% to 100%) in an automatic sample processor (Leica AutoStainer XL, Nussloch, Germany) for 19 hours, followed by three washes with xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group, France). Subsequently, the specimens were immersed in paraffin (Thermo Scientific, Cheshire, UK) at 62°C and solidified at -5°C. Sections of 4 µm thickness were then cut and mounted on Superfrost glass slides (25 mm × 75 mm × 1 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK). Deparaffinization of slides occurred in an automated slide processing apparatus (Leica AutoStainer XL, Nussloch, Germany) before staining with hematoxylin Harris (Millipore Corporation, Massachusetts, MA, USA) for 1 minute, followed by a 2-minute tap water bath. Slides were subsequently washed with 0.5% ammonia solution (Sigma Aldrich, Madrid, Spain) for 10 seconds, tap water for 1.5 minutes, and 96% ethanol solution for 45 seconds. Eosin Y solution (Thermo Scientific, Cheshire, UK) was applied for 30 seconds, followed by dehydration with 96% ethanol (1 minute), absolute ethanol (2 minutes each), and xylene (1 minute for the first wash, 2 minutes for the second wash). Slides were mounted using a resinous medium (Entellan, Merck, Darmstadt, Germany) and covered with lamellae (24 × 60 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK). Finally, the slides were air-dried for seven days. After drying, slides were examined under a light microscope (Olympus BX61, Olympus Co. Ltd, Tokyo, Japan) and photomicrograph.

4.3.2.5. Experimental design

The experimental design, outlined in Figure 4.3.1., involved a 2-week phase of adaptation and habituation of animals to the researchers. Subsequently, baseline assessments were conducted to evaluate well-being, as well as mechanical and thermal nociceptive responses, and blood glucose levels for all animals. Following these assessments, animals were randomly assigned to either the Diab (n=38) or Sham (n=14) groups.

In the second week, experimental T2DM was induced, and weekly testing was conducted throughout the subsequent 4 weeks to monitor disease progression. Four weeks post-T2DM induction, the Diab group

was randomly subdivided into three groups: GtL_100 (administered 100 mg/kg *G. tridentata* leaves extract, n= 11), GtL_300 (administered 300 mg/kg *G. tridentata* leaves extract, n= 13), and Diab (received vehicle solution as a control, n= 14). Sham group animals were also administered vehicle solutions.

Nociceptive behavior was assessed weekly using the Von Frey and acetone tests for mechanical and thermal allodynia, respectively, as well as the Randal-Sellito and tail-flick tests for mechanical and thermal hyperalgesia. *G. tridentata* leaves extract administration started four weeks after T2DM induction and continued daily via gavage for an additional 3 weeks. Additionally, hyperglycemia was monitored weekly.

Upon completion of the behavioral assessments, animals were sacrificed via transcardiac perfusion, and internal organs were sampled for further processing and analysis. This comprehensive experimental design aimed to systematically evaluate the effects of *G. tridentata* leaves extracts on T2DM-induced nociceptive behaviors, along with monitoring hyperglycemia throughout the experimental period. The subdivision of the Diab group based on *G. tridentata* leaves extract concentrations allowed for a nuanced exploration of dose-dependent effects on the observed parameters. The inclusion of the Sham group as a control facilitated a comparative analysis, enhancing the reliability and significance of the study outcomes.

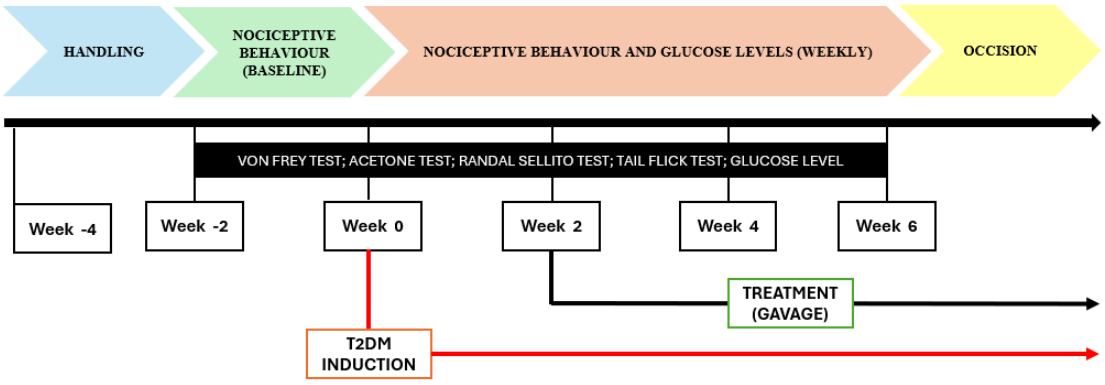


Figure 4.3.1. Schematic representation of the experimental time points. Animals were divided into 4 groups: one control group (SHAM, n=14) and the other 3 groups with experimental T2DM: one group without treatment (Diab, n=14); one group with treatment of 100mg/Kg (Diab_GtL100, n=11) of *G. tridentata* leaves extract and one group with treatment of 300mg/Kg (Diab_GtL300, n=13) of *G. tridentata* leaves extract. *G. tridentata* leaves extract administration started four weeks post-T2DM induction, continuing for three weeks. Weekly assessments included the Von Frey and acetone tests for allodynia, the Randal-Sellito, and tail-flick tests for hyperalgesia. At the end of the experimental period, animals were euthanized, and the internal organs were excised for histopathological analysis.

4.3.2.6 Statistical analysis

The statistical analysis was conducted using GraphPad Prism 8 software (GraphPad Software Inc, La Jolla, CA, USA). Initial assessments involved testing data for normality through the Shapiro-Wilks test and parametric tests were employed for all analyses. Weekly behavioural data underwent analysis of variance for repeated measures (ANOVArm), followed by a Bonferroni post hoc test. Results are presented as mean ± standard error of the mean (SEM), unless otherwise stated, with statistical significance set at $P < 0.05$.

4.3.3. Results

4.3.3.1. Toxicity of *G. tridentata* extracts

Recognizing the liver's key role in xenobiotic detoxification, we utilized HepG2 cells (human hepatocytes) to investigate any potential cytotoxicity of the *G. tridentata* extracts. HepG2 cells underwent incubation with methanolic extracts from *G. tridentata* leaves for either 4 h or 24 h, across various concentrations (up to 500 µg/ml), simulating acute and chronic cell exposure, respectively. Overall, exposure of HepG2 cells to the extracts at concentrations up to 500 µg/ml for 4 h did not induce any toxicity (Figure 4.3.2A: ANOVA $_{one\text{-}way}$: $F_{(5,51)} = 106.2$, $P < 0.0001$). However, after 24 h of exposure, cell viability declined, indicating dose-dependent cytotoxicity (Figure 4.3.2B: ANOVA $_{one\text{-}way}$: $F_{(5,54)} = 301.0$, $P < 0.0001$).

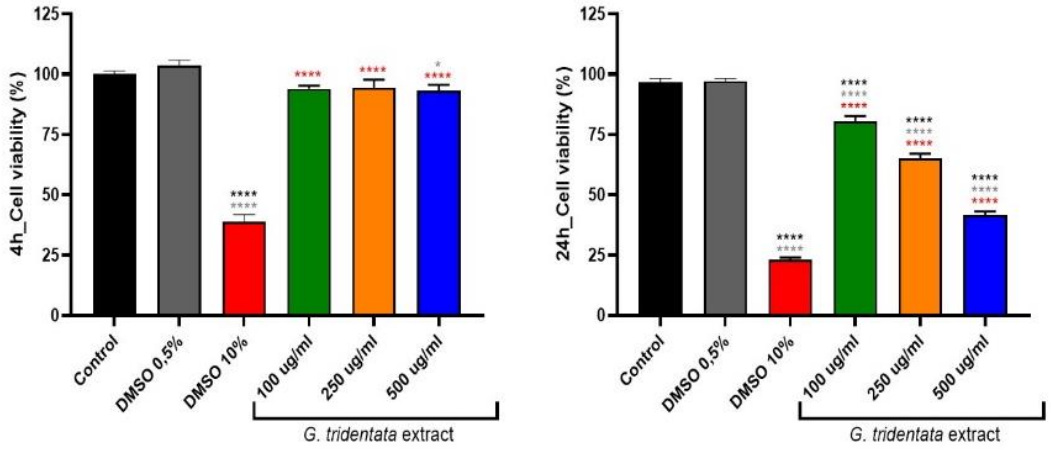


Figure 4.3.2. Cytotoxic effect of methanolic extracts from *G. tridentata* leaves on HepG2 cells. HepG2 cells were treated with *G. tridentata* methanolic extracts at concentrations of 100, 250, and 500 µg/ml for 4 h and 24 h (A and B, respectively). Results are expressed as mean ± SEM. * $P < 0.05$; **** $P < 0.0001$.

The safety profile of various concentrations of *G. tridentata* was assessed in wild-type *C. elegans* (N2) using a food clearance assay. This method measured the rate at which the worm's consumed food, providing an indirect measure of their overall growth, survival, and reproductive capacity by monitoring the optical density (OD595nm) of *E. coli* bacteria (OP50) over time. We considered a specific

concentration of the extract as safe if the treated animals exhibited a food consumption rate comparable to that of control animals treated with 1% DMSO, which served as the safe condition. Conversely, if the OD decrease in animals treated with a particular concentration of *G. tridentata* differed significantly from the 1% DMSO control condition (non-toxic vehicle) or approached the 5% DMSO control condition (considered toxic), we categorized it as toxic. The results are represented in Figure 4.3.3. Statistical analysis of the toxicity assay revealed that all tested concentrations were deemed safe for the animals (Interaction: ANOVA $_{\text{Two-way}}$: $F_{(70,176)} = 2.249$, $P < 0.0001$ Treatment: ANOVA $_{\text{Two-way}}$: $F_{(7,176)} = 9.961$, $P < 0.0001$; Time: ANOVA $_{\text{Two-way}}$: $F_{(7,176)} = 211.9$, $P < 0.0001$).

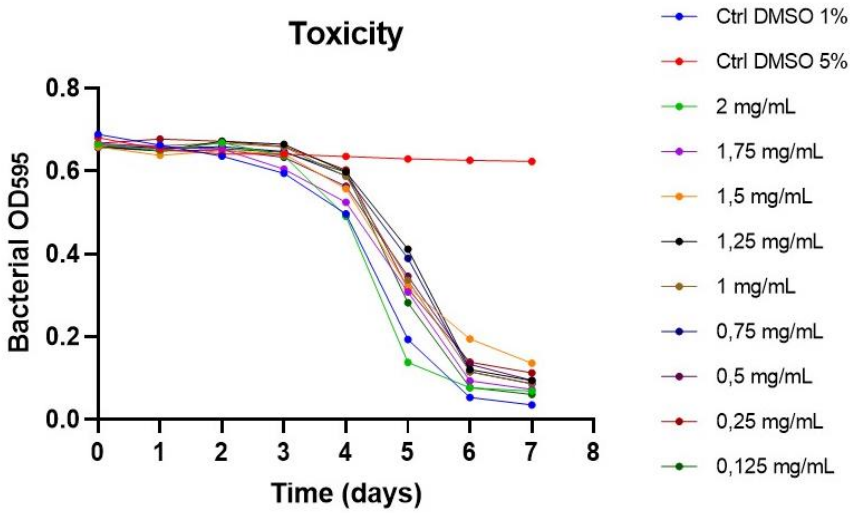


Figure 4.3.3. Toxicity of *G. tridentata* extract in the food clearance assay. Optical density readings of the OP50 suspension treated with *G. tridentata* extract in N2 animals were monitored daily across different concentrations. Mean optical density (OD) values were calculated from triplicate samples and plotted over time. The drug vehicle utilized was the control DMSO (1%), while DMSO at 5% served as the positive control for toxicity. The data were obtained from three independent experiments.

4.3.3.2. *G. tridentata* effects increase *C. elegans* lifespan under glucotoxicity conditions

Given the known influence of glucose on lifespan, our study aimed to explore how cultivating worms in media supplemented with glucose and different concentrations of *G. tridentata* extracts affected the lifespan of *C. elegans*. We observed a decrease in lifespan among *C. elegans* exposed to glucotoxic conditions. Importantly, we found that the lifespan of *C. elegans* treated with *G. tridentata* extracts showed an increase compared to the control group (Figure 4.3.4. and table 4.3.1.).

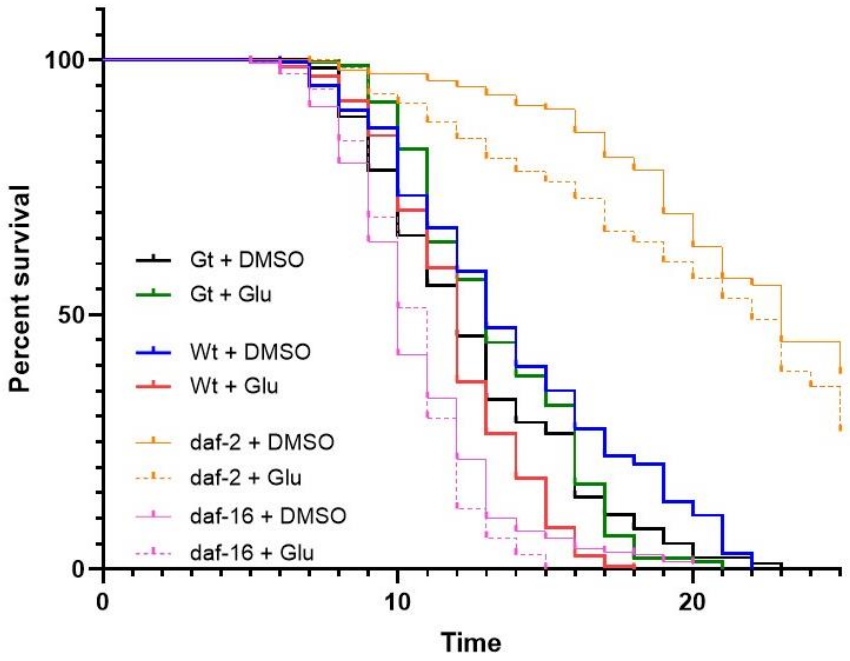


Figure 4.3.4. Kaplan-Meier survival curve illustrating the response of *C. elegans* to glucotoxicity and treatment with 1 mg/ml of *G. tridentata* extract. The results reveal increased survival rates post-treatment, while not affecting WT animals. As positive and negative controls, daf-2 and daf-16 mutants were utilized, respectively. The experiment included 300 individuals per condition, conducted across three independent assays.

Table 4.3.1. Detailed data and statistical analysis of the *C. elegans* longevity assays after treatment with PTL plant extract.

Strain (n[a])	Median lifespan (days)± SEM	Mean lifespan (days)±SEM	Control strain (n)	Median lifespan (days)± SEM	Mean lifespan (days)±SEM	*P*-value[b]
daf-2+DMSO (159)	20±0.5	21.0±0.4	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **
daf-2+DMSO (159)	20±0.5	21.0±0.4	**daf-2+DMSO+GLU (112)**	19±0.9	18.7±0.6	***p*=0.001 ** **
daf-16+DMSO (224)	10±0.1	9.9±0.1	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **
daf-16+DMSO (224)	10±0.1	9.9±0.1	**daf-16+DMSO+GLU (221)**	11±0.2	10.5±0.1	***p*=0.001 ** **
WT+GLU (187)	12±0.2	11.9±0.2	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **
WT+PTL (180)	12±0.4	12.5±0.3	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*<0.0001*** **

WT+GLU+PTL (139)	12±0.4	13.4±0.3	**WT+DMSO (191)**	13±0.4	13.9±0.3	***p*=0.018***
WT+GLU (187)	12±0.2	11.9±0.2	**WT+PTL (180)**	12±0.4	12.5±0.3	***p*=0.006****
WT+GLU (187)	12±0.2	11.9±0.2	**WT+GLU+PTL (139)**	12±0.4	13.4±0.3	***p*<0.0001*****

[a]Number of worms after censoring.
[b]The log-rank test (Mantel-Cox) was used to compare the groups.
p ≥ 0.05 (NS), p ≤ 0.05 (∗), p ≤ 0.01 (∗∗), p ≤ 0.001 (∗∗∗).

4.3.3.3 *G. tridentata* extract alleviates neuropathic pain in STZ-NA-Induced Diabetic Rats

4.3.3.3.1 Animal welfare

Maintaining control over the weight and blood glucose levels of the animals was crucial for ensuring their welfare throughout the course of the study.

Blood glucose levels were assessed over the experimental period, and the data is depicted in Figure 4.3.5A. The analysis revealed blood glucose levels varied among experimental groups over time (ANOVAtwo-way: Interaction: $F_{(21,384)}$=5.737, P<0.0001; Time: $F_{(7,384)}$=43.49, P<0.0001; Group: $F_{(3,384)}$=173.7, P<0.0001). Post hoc tests indicated that before the induction of T2DM (week -1), there were no differences in blood glucose levels between groups. However, from week 0 onwards, there was an exponential increase in blood glucose levels in T2DM groups, regardless of *G. tridentata* treatment in comparison with Sham animals.

Statistical analysis revealed animal weight varied among experimental groups over time (Figure 4.3.5B: ANOVAtwo-way: Interaction: $F_{(21,384)}$=1.959, P=0.0074; Time: $F_{(7,384)}$=27.33, P<0.0001; Group: $F_{(3,384)}$=60.13, P<0.0001). Notably, all groups exhibited a marked increase in body weight until week 0. Following the induction of T2DM by STZ-NA (week 0), post hoc tests indicated that T2DM animals gained less body weight compared to SHAM animals, regardless of *G. tridentata* extract treatment.

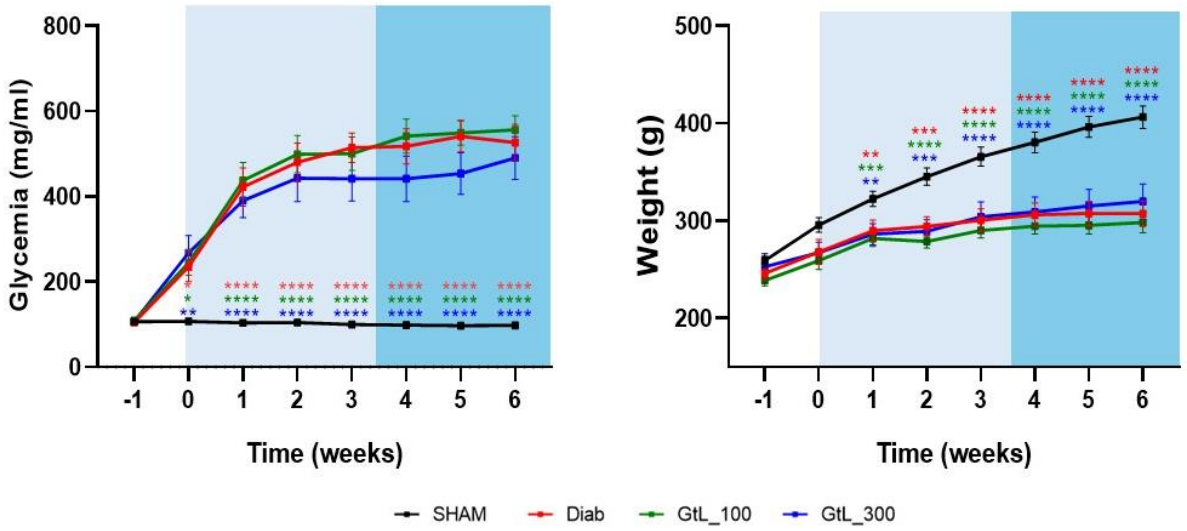


Figure 4.3.5. Assessment blood glucose levels (A) and body weight (B) throughout the experimental period. Results are expressed as mean ± SEM. ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. (Sham - control animals injected with vehicle solution (NaCl and Citrate Buffer) and administered PBS via gavage, n=14; Diab – animals injected with STZ and NA and administered PBS via gavage, n=14; GtL_100 - animals injected with STZ and NA and administered 100mg/kg of *G. tridentata* extract via gavage, n=11*;* GtL_300 - animals injected with STZ and NA and administered 300mg/kg of *G. tridentata* extract via gavage, n=13). The light blue box corresponds to the period after induction and before treatment, while the dark blue box corresponds to the period during which the treatment was administered.

4.3.3.3.2. Nociceptive behaviour

Mechanical and thermal allodynia were assessed weekly using the Von Frey and Acetone tests, respectively. Additionally, mechanical, and thermal hyperalgesia were evaluated through the Randall-Selitto and tail-flick tests, respectively.

4.3.3.3.2.1 Mechanical and thermal allodynia

The results from the Von Frey test, evaluating mechanical allodynia, are presented in Figure 4.3.6A. Statistical analysis indicates mechanical allodynia varied between experimental groups (ANOVAtwo-way: Interaction: $F_{(21,384)}=4.752$, $P<0.0001$; Time: $F_{(7,384)}=5.525$, $P<0.0001$; Group: $F_{(3,384)}=46.30$, $P<0.0001$). Post hoc analysis revealed T2DM animals developed mechanical allodynia after week 4, and this effect was effectively prevented by both *G. tridentata* treatments.

To evaluate sensitivity to a cold stimulus (cold allodynia), the acetone test was conducted (Figure 4.3.6B). Data analysis demonstrates cold allodynia varied between experimental groups over time (ANOVAtwo-way: Interaction: $F_{(21,384)}=7.896$, $P<0.0001$; Time: $F_{(7,384)}=20.01$, $P<0.0001$; Group: $F_{(3,384)}=60.80$, $P<0.0001$). Post hoc tests indicate that T2DM animals developed thermal allodynia after the second post-

induction week, and *G. tridentata* treatments effectively reduced thermal allodynia from the first week of treatment onwards.

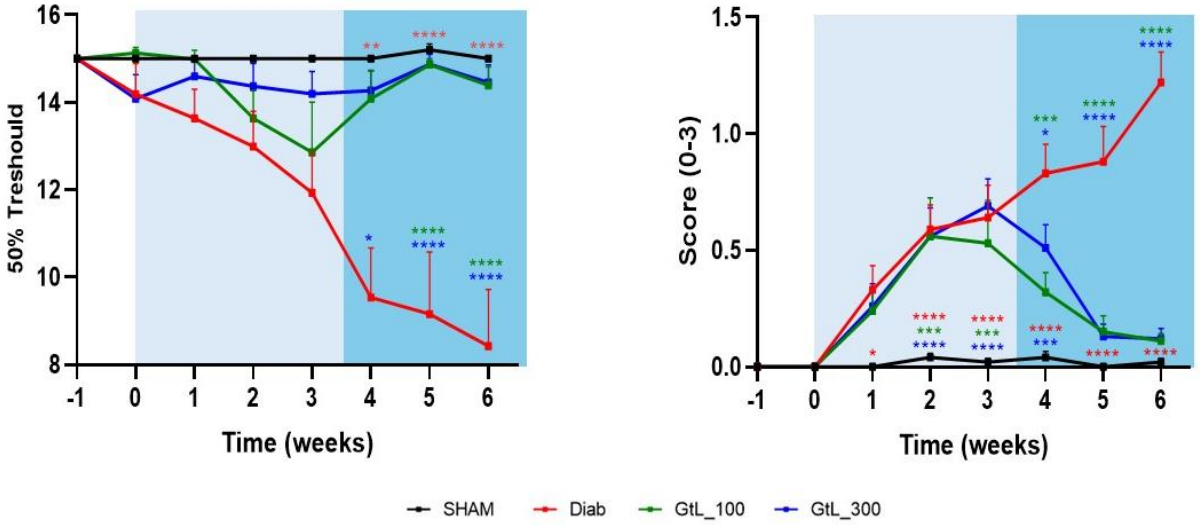


Figure 4.3.6. Evolution of mechanical (A) and thermal (B) allodynia of animals throughout the experimental period. Results are expressed as mean ± SEM. ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. (Sham - control animals injected with vehicle solution (NaCl and Citrate Buffer) and administered PBS via gavage, n=14; Diab – animals injected with STZ and NA and administered PBS via gavage, n=14; GtL_100 - animals injected with STZ and NA and administered 100mg/kg of *G. tridentata* extract via gavage, n=11; GtL_300 - animals injected with STZ and NA and administered 300mg/kg of *G. tridentata* extract via gavage, n=13). The light blue box corresponds to the period after induction and before treatment, while the dark blue box corresponds to the period during which the treatment was administered.

4.3.3.3.2.2. Mechanical and thermal hyperalgesia

To evaluate mechanical hyperalgesia, the Randall-Selitto test was performed weekly (Figure 4.3.7A) throughout the experimental period. Data analysis shows this parameter varied significantly between groups (ANOVAtwo-way: Time: Interaction: $F_{(21,384)}=3.355$, $P<0.0001$; $F_{(7,384)}=7.648$, $P<0.0001$; Group: $F_{(3,384)}=108.1$, $P<0.0001$). Heat hyperalgesia was assessed by the tail-flick test, and results are presented in Figure 4.3.7B. Data analysis shows this parameter varied significantly between groups (ANOVAtwo-way: Interaction: $F_{(21,384)}=5.50$, $P<0.0001$; Time: $F_{(7,384)}=5.603$, $P<0.0001$; Group: $F_{(3,384)}=23.13$, $P<0.0001$). Post hoc tests reveal diabetic animals developed mechanical and thermal hyperalgesia 3 weeks post-induction, and both treatments (GtL_100 and GtL_300) ameliorated sensitivity to a painful stimulus after 2 weeks of treatment.

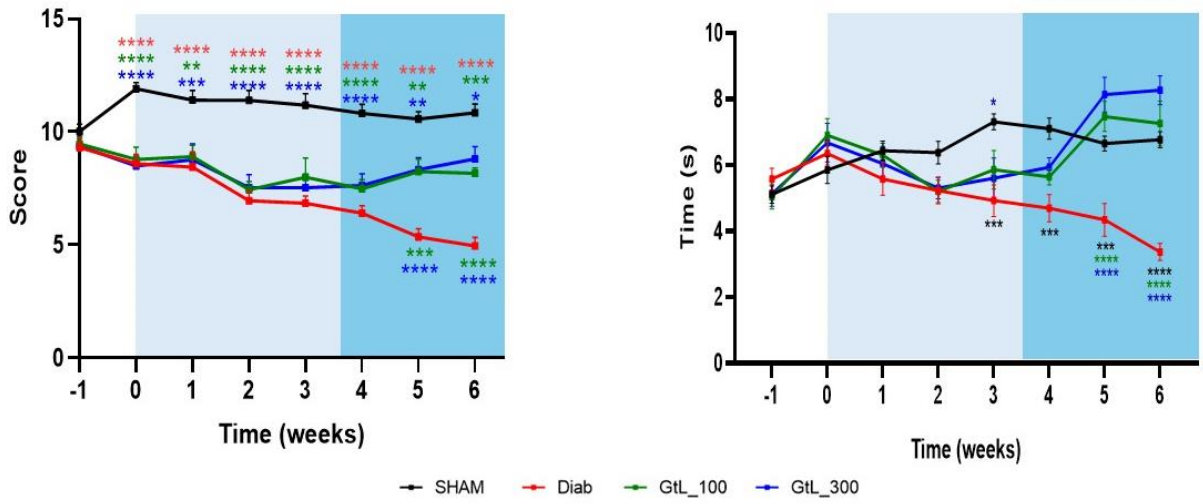


Figure 4.3.7. Evaluation of mechanical (A) and thermal (B) hyperalgesia throughout the experimental period. Results are expressed as mean ± SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. (Sham - control animals injected with vehicle solution (NaCl and Citrate Buffer) and administered PBS via gavage, n=14; Diab – animals injected with STZ and NA and administered PBS via gavage, n=14; GtL_100 - animals injected with STZ and NA and administered 100mg/kg of *G. tridentata* extract via gavage, n=11; GtL_300 - animals injected with STZ and NA and administered 300mg/kg of *G. tridentata* extract via gavage, n=13). The light blue box corresponds to the period after induction and before treatment, while the dark blue box corresponds to the period during which the treatment was administered.

4.3.3.4. Histopathological analysis of internal organs

The animals' internal organs were weighed immediately after occision. Subsequently, they were subjected to microscopic analysis using hematoxylin and eosin staining.

4.3.3.4.1. Weight of organs

The weights of the liver, kidneys, and spleen exhibited significant variations among experimental groups. ANOVA one-way analysis revealed significant differences for the liver (Figure 4.3.8A: ANOVAone-way: $F_{(3,22)}=5.402$, $P=0.0061$), kidneys (Figure 4.3.8C: ANOVAone-way: $F_{(3,22)}=11.44$, $P<0.0001$), and spleen (Figure 4.3.8E: ANOVAone-way: $F_{(3,22)}=6.013$, $P=0.0038$). Post-hoc tests revealed increased weights of the liver, kidneys, and spleen in the Diab group, with additional weight increase in the kidneys observed in the GtL_100 and GtL_300 groups.

However, the weights of the thymus (Figure 4.3.8B: ANOVAone-way: $F_{(3,22)}=0.8759$, $P=0.4710$), adrenal glands (Figure 4.3.8D: ANOVAone-way: $F_{(3,22)}=0.1353$, $P=0.9378$), lungs (Figure 4.3.8F: ANOVAone-way: $F_{(3,22)}=0.9777$, $P=0.4212$), and pancreas (Figure 4.3.8G: ANOVAone-way: $F_{(3,22)}=1.350$, $P=0.2841$) did not show significant differences among experimental groups.

Overall, *G. tridentata* treatments exhibited a protective effect, particularly in the liver and spleen, especially when administered at the higher extract dose.

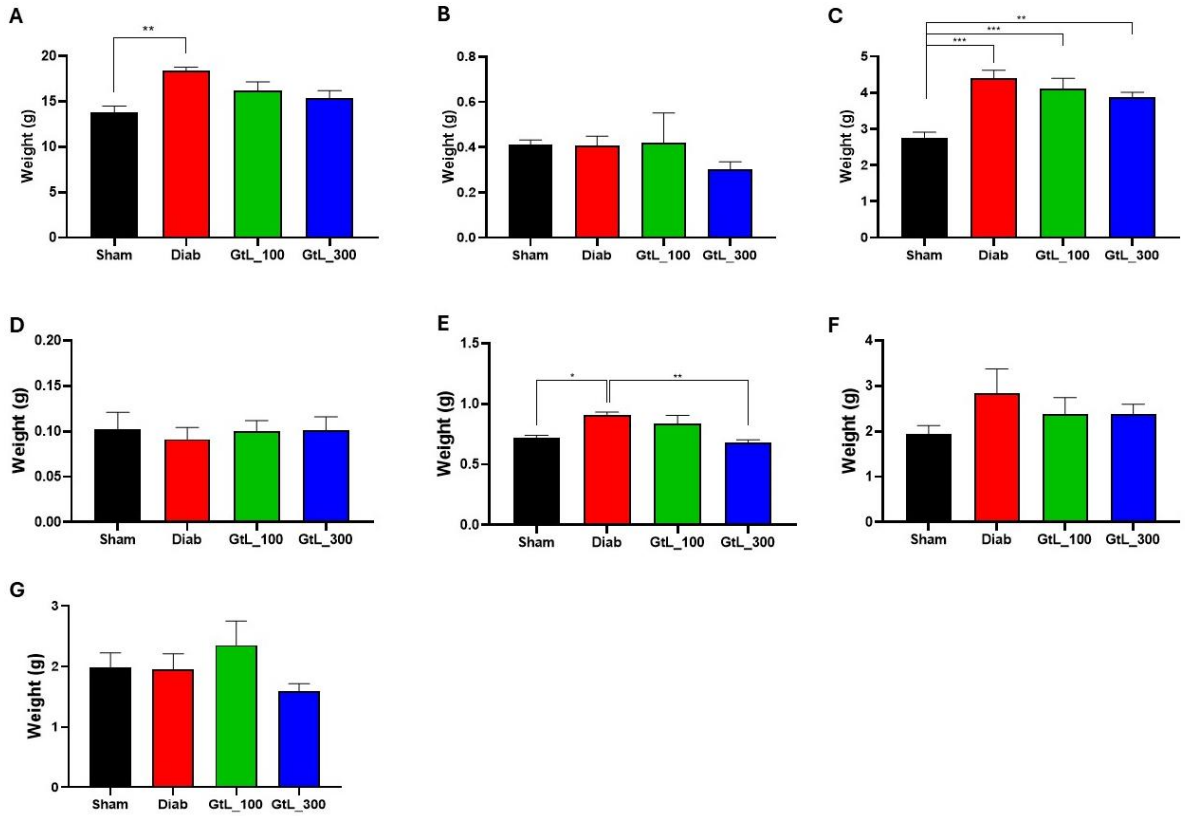


Figure 4.3.8. Assessment of internal organ weights (adjusted to the animal´s body weight). [A] Liver; [B] Thymus; [C] Kidneys; [D] Adrenal Glands; [E] Spleen; [F] Lungs; [G] Pancreas. Results are expressed as mean ± SEM. ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. (Sham - control animals injected with vehicle solution (NaCl and Citrate Buffer) and administered PBS via gavage, n=6; Diab – animals injected with STZ and NA and administered PBS via gavage, n=6; GtL_100 - animals injected with STZ and NA and administered 100mg/kg of *G. tridentata* extract via gavage, n=7*;* GtL_300 - animals injected with STZ and NA and administered 300mg/kg of *G. tridentata* extract via gavage, n=7).

4.3.3.4.2 Histopathological analysis of internal organs

Samples of internal organs underwent Hematoxylin and eosin staining for histopathological analysis (Figure 4.3.9). Photomicrographs from the Sham group were used as controls for normal histological architecture of the liver (Figure 4.3.9A), thymus (Figure 4.3.9E), kidney (Figure 4.3.9I), adrenals (Figure 4.3.9M), spleen (Figure 4.3.9Q), and pancreas (Figure 4.3.9U) tissues.

The histopathological examination of liver sections from STZ-NA T2DM induced animals (Figure 4.3.9B-D) revealed various morphological changes, including alterations in hepatic structure, sinusoid capillary hypertrophy, apoptotic hepatocytes, and cytoplasmic vacuolization.

Regarding the thymus and adrenals, no histopathological changes were observed in the general tissue structure in T2DM animals (Figure 4.3.9F, G, H, N, O, P).

Kidney histopathological analysis showed glomerular atrophy and podocyte loss in the Diab group (Figure 4.3.9J). Photomicrographs indicated an expansion of mesangial extracellular matrix and damage to proximal tubules in the Diab group.

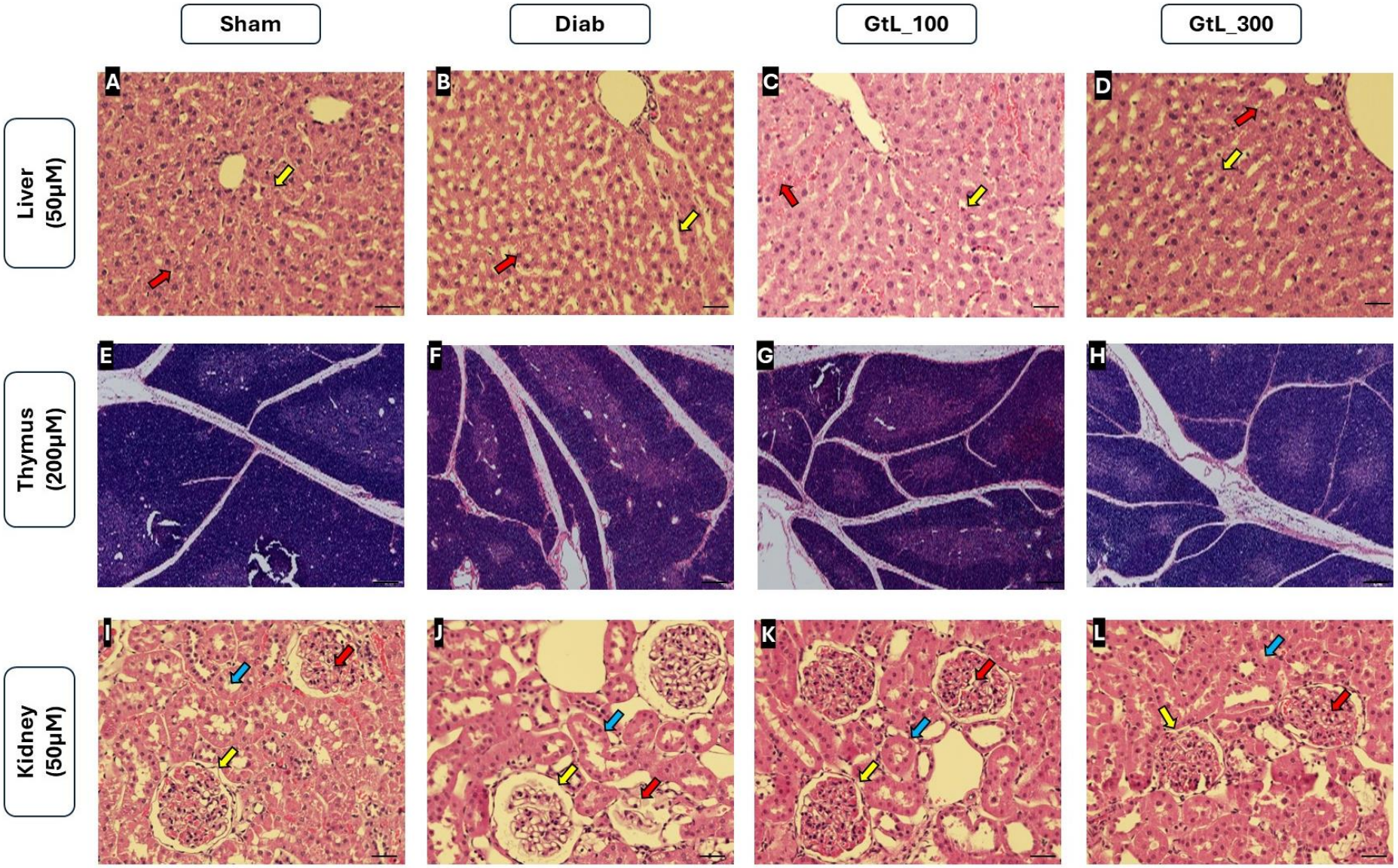
The spleen of T2DM animals exhibiting a reduction in the white pulp (Figure 4.3.9R, S, T).

The pancreas of the Diab group demonstrated a decrease in the number of Langerhans Islets, accompanied by severe structural deterioration: an increase in dispersion in the β-cells (hypertrophy) and loss of the enveloping mantle (Figure 4.3.9V).

G. tridentata three-week oral treatment (both) showed a hepato-protective effect against liver damage caused by experimental T2DM in rats, preventing morphological alterations, including partial reversion of sinusoid capillary hypertrophy, and reduced cytoplasmic vacuolization (Figure 4.3.9C and Figure 4.3.9D).

Additionally, photomicrographs of the kidney revealed that *G. tridentata* treatments (both) led to normalization of glomerular and mesangial extracellular matrix size, along with a decrease in podocyte loss in the glomerulus. Furthermore, a reduction in proximal tubule damage was observed (Figure 4.3.9K and Figure 4.3.9L).

Additionally, both treatments reversed pathological abnormalities observed in T2DM groups concerning the state of white and red pulp in the spleen (Figure 4.3.9S and Figure 4.3.9T). Furthermore, *G. tridentata* treatments improved the number and architecture of Langerhans islets, decreasing dispersion in the β-cells. However, recovery of the capsule was not observed (Figure 4.3.9X and Figure 4.3.9Y).


Sham
Diab
GtL_100
GtL_300
Liver (50µM)
Thymus (200µM)
Kidney (50µM)
A
B
C
D
E
F
G
H
I
J
K
L

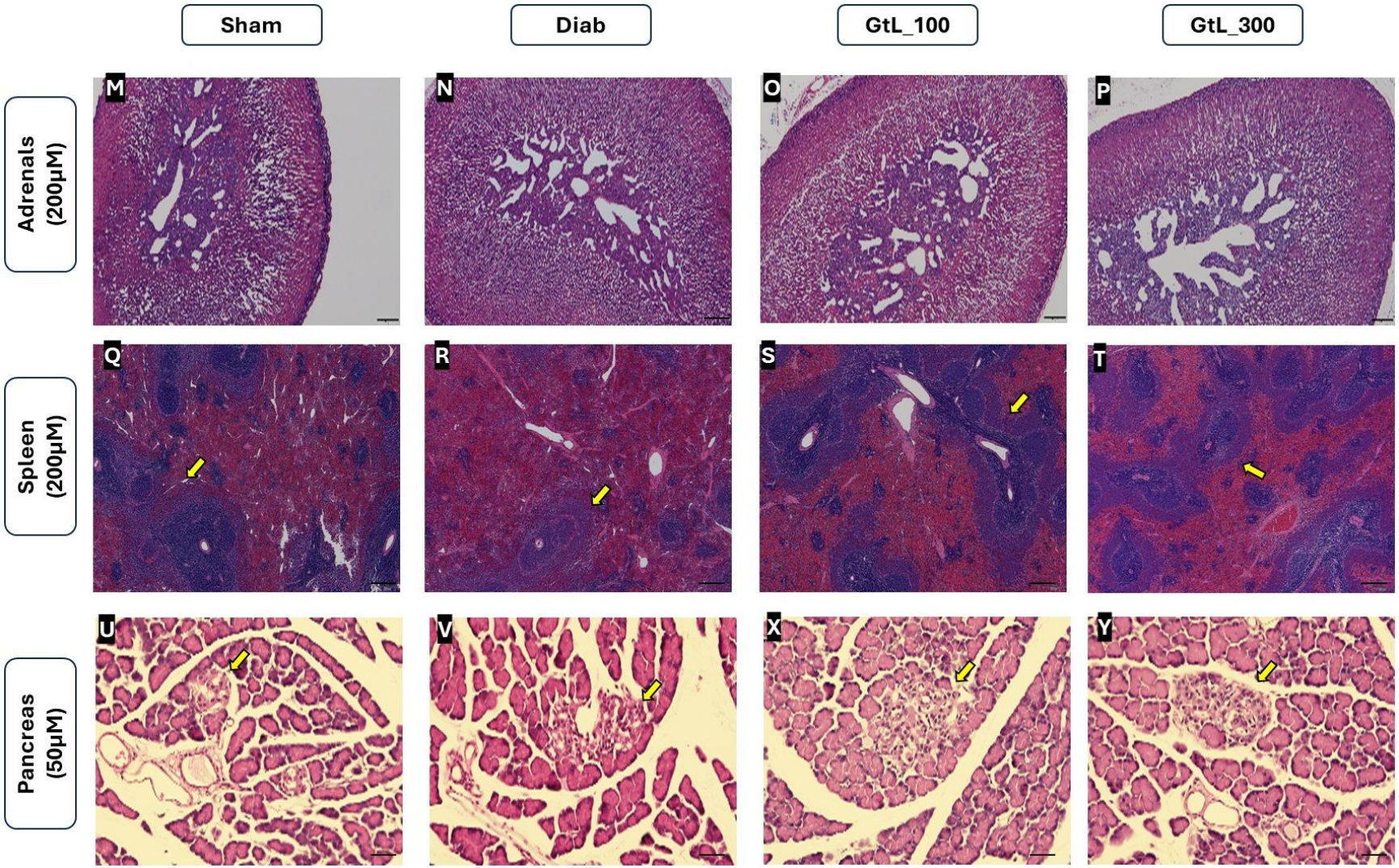


Figure 4.3.9 – Photomicrographs of hematoxylin and eosin stained sections of the Liver (A-D) magnification 40x, scale bar 50µm, Thymus (E-H) magnification 4x, scale bar 200µm, Kidney (I-L) magnification 40x, scale bar 50µm, Adrenals (M-P) magnification 4x, scale bar 200µm, Spleen (Q-T), magnification 4x, scale bar 200µm and Pancreas (U-Y) magnification 40x, scale bar 50µm.

4.3.4. Discussion

Our investigation into *G. tridentata* extract yielded several significant findings. Firstly, Our investigation revealed cytotoxic effects of the extract in HepG2 cells at doses exceeding 250 μg/mL, while no toxic effects were observed in *C.elegans*, indicating a favorable safety profile. Additionally, oral administration of the extract extended the lifespan of *C.elegans* under glucotoxic conditions, suggesting potential anti-glucotoxicity effects. Furthermore, *G. tridentata* extract effectively mitigated diabetic neuropathic pain in rat models, indicating its potential as an analgesic agent. Finally, the extract demonstrated protective effects on organ morphology, including the liver, kidneys, and pancreas, in diabetic rat models, suggesting a broader organ-protective role. These results provide valuable insights into the therapeutic properties of *G. tridentata* extract, warranting further investigation for potential clinical applications.

4.3.4.1. *G. tridentata* toxicity

The results of the analysis showed that the *G. tridentata* extract exhibited no cytotoxicity in HepG2 cells at lower doses, or *C.elegans*. These findings are supported by a series of previous studies that investigated the extract's toxic effects at various levels, from human cells to rat liver mitochondria (Ferreira *et al.*, 2012; Falé *et al.,* 2013). For instance, studies conducted by Falé *et al.* (2013) and Ferreira *et al.* (2012) examined cytotoxicity in human cell lines such as Caco-2, HeLa, and HePG2, without finding any evidence of toxicity. Additionally, Ferreira *et al.* (2012) explored the toxicological effects on rat liver mitochondria, also finding no toxicity.

In support of these findings, more recent studies by Caleja *et al.* (2019) and Garcia-Oliveira *et al.* (2022) assessed cytotoxicity in tumor cell lines and non-tumor cell cultures, finding 50% growth inhibition in some tumor cell lines but no toxicity in non-tumor cells. These results align with the observed growth inhibition in the HepG2 cell line, as reported in our study.

Furthermore, in a study with laboratory animals conducted by Laranjeira et al. (2023), no signs of behavioral or histological toxicity were observed at significant doses of the methanolic extract. These findings suggest a low toxicity profile for the *G. tridentata* extract, which is encouraging for its potential beneficial use. However, it's important to note the need for caution when handling organic extracts due to the possibility of containing potentially toxic components. In summary, the experimental results indicate that *G. tridentata* extracts, particularly the methanolic extract, exhibit good safety profiles, providing insights into potential doses for future efficacy studies.

4.3.4.2. *G. tridentata's* influence on lifespan during glucotoxic conditions in *C. elegans*

Emerging evidence suggests that excessive glucose intake negatively affects the lifespan of *C. elegans* (Wang *et al.*, 2020; Franco-Juárez *et al.*, 2021), leading to diminished functional abilities (Choi *et al.*, 2011; Wang *et al.*, 2020; Franco-Juárez *et al.*, 2021). However, the potential impact of *G. tridentata* on *C. elegans* lifespan under conditions of glucotoxicity remains poorly understood. Our findings suggest that *G. tridentata*, which is rich in phenolic compounds like Isoquercitrin, Genistein, and Quercetin derivatives, partially increases the shortened lifespan and mitigates health span deterioration induced by a high glucose diet.

While it is widely recognized that high dietary sugar intake contributes to conditions such as obesity, diabetes, and cardiovascular diseases (Aston, 2006; Jenkins *et al.*, 2021), limited information exists regarding its direct effects on aging and longevity. Our results demonstrate that glucotoxic conditions decrease the lifespan of *C. elegans*. Notably, only one scientific report (Paulo *et al.*, 2008) addresses the antihyperglycemic properties of *G. tridentata in vivo*, where the aqueous extract showed significant antihyperglycemic effects in normoglycemic rats during the initial 30 minutes after a glucose challenge. However, later, a paradoxical hyperglycemic effect was observed, suggesting the presence of compounds with varying effects on glucose tolerance. Additionally, sissotrin and isoquercitrin isolated from *G. tridentata* showed opposite effects on glucose tolerance in normoglycemic rats, with the former decreasing blood glucose levels, and the latter increasing, suggesting potential in regulating glucose metabolism (Paulo *et al.*, 2008).

Excessive glucose intake can increase oxidative stress by increasing cellular reactive oxygen species (ROS), accelerating the aging process (Pitocco *et al.*, 2013; Mikrut *et al.*, 2016). Sustained hyperglycemia induces ROS formation by enhancing advanced glycation end products, activating protein kinase C, and impacting the mitochondrial electron transport chain, leading to macromolecular damage (Luo *et al.*, 2016; Zhang *et al.*, 2016). Thus, removing excess ROS induced by hyperglycemia is considered an effective strategy to alleviate diabetes complications (Lim & Park, 2023).

Our study strongly suggests that the methanolic extract of *G. tridentata* leaves offers protection against glucose toxicity, positioning *G. tridentata* as a promising candidate for the development of anti-diabetic drugs.

4.3.4.3. Antinociceptive potential of *G. tridentata* methanolic extract

According to Cheng *et al.* (2021), T2DM animals are expected to exhibit an increasing sensitivity to pain. Several authors have reported the development of mechanical and thermal allodynia, as well as

mechanical and thermal hyperalgesia, in rat diabetes models, which can persist for up to 12 weeks post-induction (Obrosova *et al.*, 2008; Xu *et al.*, 2012), including STZ-NA-induced T2DM (Shekunova *et al.*, 2020; Cheng *et al.*, 2021). Our results regarding mechanical and thermal allodynia and thermal and mechanical hyperalgesia in this study align with the literature, demonstrating a progressive increase in nociceptive behavior in STZ-NA T2DM animals.

G. tridentata treatments exhibited a reduction in mechanical and thermal allodynia after 2 weeks of treatment, suggesting a potential analgesic effect of *G. tridentata* extract. The heightened pain sensitivity observed in diabetes may be linked to increased oxidative stress and inflammatory responses resulting from hyperglycemia and insulin resistance (Cheng *et al.*, 2021).

In a prior study (Laranjeira *et al.*, 2023), our research team examined the phytochemical composition of *G. tridentata* leaf methanolic extract, pinpointing genistein, isoquercitrin, and quercetin derivatives as prominent compounds within it. Notably, these same bioactive constituents have been the focus of several studies exploring their potential antinociceptive effects in rat models of diabetes. For instance, Resham *et al.* (2019) observed neuropathy development in Type 1 diabetic rats, manifested by reduced thresholds for thermal and mechanical hyperalgesia. Additionally, their study revealed that treatment with isoquercetin significantly mitigated heat hyperalgesia, cold hyperalgesia, and mechanical hyperalgesia compared to untreated diabetic animals.

Moreover, our findings align with those of Valsecchi *et al.* (2011), who demonstrated the reversal of mechanical allodynia in T1DM mice following genistein administration. Furthermore, the presence of rutin in our *G. tridentata* extract is of particular interest, given its observed analgesic effects in diabetic rats as reported by Al-Enazi *et al.* (2014). Similarly, Tian *et al.* (2016) also reported that rutin administration alleviated cold allodynia, mechanical hyperalgesia, and thermal hyperalgesia in diabetic rats.

Also, the analgesic potential of quercetin, another constituent identified in our extract, is supported by the findings of Anjaneyulu and Chopra (2004), who demonstrated its efficacy in reducing thermal allodynia and hyperalgesia in rats with STZ-induced T1DM.

These collective findings suggest a promising avenue for the use of *G. tridentata* extract in relieving pain associated with diabetic neuropathy, with its bioactive compounds potentially acting synergistically to modulate oxidative stress and inflammatory pathways. Further investigations are warranted to elucidate the precise mechanisms underlying these effects and to evaluate the extract's therapeutic potential.

4.3.4.4. Internal organs

T2DM predominantly affects the pancreas, liver, and kidneys (Chatterjee *et al.*, 2020), as they perform essential functions such as blood filtration, detoxification, excretion of unwanted substances (Brzoska *et al.*, 2003), and insulin production, which regulates carbohydrate and fat metabolism (Brzoska *et al.*, 2003).

Recent research has highlighted histopathological changes in liver tissue following both STZ-NA-induced T2DM (Asokan *et al.*, 2019; El-Marasy *et al.*, 2020) and STZ-induced T1DM (Al-Ani *et al.*, 2017; Rodríguez *et al.*, 2018; Samadi-Noshahr *et al.,* 2021). It's noteworthy that our diabetic rats also exhibited similar morphological alterations in the kidneys, liver, and pancreas. However, our latest findings (Figure 5.3.9A-D) reveal that oral administration of *G. tridentata* (100 and 300 mg/kg) for three weeks not only protects the liver but also mitigates these morphological alterations induced by STZ-NA-induced diabetic pathology in rats. Furthermore, we observed similar protective effects on kidney, liver, and pancreas morphology in diabetic animals, suggesting a broader organ-protective role of *G. tridentata* in diabetes-induced damage.

Research has reported that rutin treatment (50 and 100 mg/kg) could enhance the antioxidant status of the liver by increasing levels of glutathione peroxidase, catalase, and superoxide dismutase (Kamalakkannan and Prince, 2006; Ahmed *et al.*, 2010; Niture *et al.*, 2014). Additionally, rutin has been shown to reduce levels of liver enzymes in the serum and improve histological injury of hepatocytes (Kamalakkannan and Prince, 2006; Fernandes *et al.*, 2010). In fact, a study by Lai and colleagues (2021) demonstrated that quercetin improves T2DM-induced autophagy in the liver and reduces liver fatty accumulation. Also, Kim *et al.* (2023) showed that isoquercetin inhibits lipid accumulation induced by free fatty acids in HepG2 cells by properly regulating lipid metabolism. Isoquercetin (30 mg/kg i.p) has hepatoprotective activity against the loss of hepatocyte architecture, hepatic necrosis associated with vacuolar or balloon degeneration of hepatocytes in T2DM rats (Huang *et al.*, 2017). Additionally, Zhang *et al.* (2022) showed that genistein prevents insulin resistance and hyperglycemia by improving hepatic function in mice with pre-diabetes.

The primary function of the kidney is to eliminate waste products and excess fluid from the body through urine (Tanner, 2009). Notably, several authors have confirmed renal lesions in T2DM, characterized by microvascular injury and loss of podocytes in the glomerulus (Sakai *et al.*, 2019; Lai *et al.*, 2021; Najafian *et al.*, 2008; Peres and Michelacci, 2015; Qiao *et al.*, 2017). Additionally, histopathological alterations include expansion of the mesangial extracellular matrix, thickening of the glomerulus, interstitial fibrosis, and tubular atrophy (Kanwar *et al.*, 2011; Kumari *et al.*, 2021).

As anticipated, our study demonstrated, through histopathological analysis of the kidneys, the loss of podocytes in the glomerulus, expansion of the mesangial extracellular matrix, and tubular atrophy in STZ-NA-induced diabetic rats. Furthermore, we showed that both doses of *G. tridentata* extract treatment could effectively delay the progression of these morphological changes (Figure 4.3.9I-L).

Additionally, Lai and colleagues (2021) reported improvements in kidney morphological changes following Quercetin treatment in a model of a high-fat diet and STZ-induced T2DM. Jia *et al.* (2019) found that genistein exhibited renal protective effects by inhibiting oxidative stress through activation of the Nrf2 HO 1/NQO1 pathway.

The thymus and spleen are critical immune organs in the body. The thymus, a primary lymphoid organ, plays a vital role in regulating immune stability and maintaining autoimmune equilibrium (Liu *et al.*, 2016). The spleen, a secondary lymphoid organ, is closely linked to cellular protection and immunity (Liu *et al.*, 2016). In our study, no abnormalities were found in the histopathological analyses of the thymus (Figure 4.3.9E-H).

However, some authors have noted thymic atrophy accompanied by the loss of architecture between cortical and medullary regions in DM (Barreto *et al.*, 2005; Nagib *et al.*, 2010). It is possible that in STZ-NA-induced T2DM, the loss of thymic architecture is undetectable after 7 weeks or may be related to the diabetes model used, as these studies were conducted using an alloxan-induced diabetes model, whereas our model involves STZ-NA-induced diabetes.

The spleen's structure comprises two distinct sections: the white pulp and the red pulp. The white pulp, consisting of lymphoid tissue, contains lymphocytes and macrophages, while the red pulp contains parenchymal cells and vascular sinuses (Vondenhoff *et al.*, 2008; Ghosh *et al.*, 2018). Several studies have demonstrated a decrease in the white pulp and activation of the red pulp as key characteristics of both T1 and T2DM (Ebaid *et al.*, 2015; Ghosh *et al.*, 2018; Said *et al.*, 2020).

Histological examination of spleen sections has revealed that *G. tridentata* treatment following diabetes induction reverses the reduced white pulp state (Figure 4.3.9Q-T), indicating its ability to restore spleen immunological functions.

The pancreas plays a pivotal role in maintaining metabolic balance by regulating the synthesis and release of insulin and glucagon (Campbell and Newgard, 2021). Insulin, produced and secreted by pancreatic β-cells, along with glucagon from α-cells, are crucial for managing blood glucose levels. Dysregulation of these systems leads to diabetes mellitus (DM), characterized by impaired insulin action and abnormal insulin secretion in response to glucose (Ouni *et al.*, 2020; Campbell and Newgard, 2021).

The pancreatic islets of Langerhans are essential for maintaining metabolic homeostasis, and their dysfunction contributes to DM (Ouni *et al.*, 2020). The architecture of these islets varies among species. Immunohistochemical staining of normal adult human islets shows a diffuse mass of β-cells with other islet cell types dispersed among them, whereas rodent islets typically feature a larger β-cell center with other cells forming an enveloping mantle (Da Silva Xavier, 2018; Wieland *et al.*, 2021).

STZ acts as a β-cell toxin, causing islet atrophy due to β-cell death and a reduction in the central core of islet tissue. Additionally, STZ administration is associated with an increase in the thickness of the peripheral layer. However, the impact of the STZ-NA model on β-cells is mixed, with some cells being severely damaged and apoptotic while others remain functional (Nugent *et al.*, 2008; Szkudelski, 2012).

Metabolic changes resulting from loss of glucose control and insulin sensitivity, characteristic symptoms of T2DM, are reflected in changes in islet architecture. Islet hypertrophy is stimulated by an increased need for insulin, which may be associated with hyperphagia, insulin resistance, or a combination of both factors. Therefore, islet structure affects glucose responsiveness (Nugent *et al.*, 2008; Wieland *et al.*, 2021).

As expected, our results demonstrated a decrease in the number of Langerhans islets and alterations in their structure, including increased dispersion of β-cells (hypertrophy) and loss of the enveloping mantle (Figure 5.3.9U-Y). Treatment with *G. tridentata* showed glucose levels in the blood similar to those in the untreated diabetic group. However, the damage to Langerhans islets observed in *G. tridentata*-treated animals was milder than that in the untreated T2DM group. *G. tridentata* treatments improved the architecture of the islets, although the recovery of the capsule was not observed.

Several studies have shown damage to the antioxidant balance in STZ-NA-induced diabetic rats. STZ administration generates reactive oxygen species in β-cells, which can exacerbate β-cell damage due to their vulnerability to antioxidant defense (Nukatsuka *et al.*, 1988; Szkudelski, 2012; Kilari *et al.*, 2021). The decline in antioxidant levels and reduced capability to eliminate free radicals may contribute to increased oxidative stress in DM, impacting β-cell function and viability (Drews *et al.*, 2010; Szkudelski, 2012).

It has been confirmed in various studies that bioactive compounds found in *G. tridentata* methanolic extracts exhibit protective activity against pancreatic β-cell damage, thereby improving DM (Vessal *et al.*, 2003; Adewole *et al.*, 2007; Oza and Kulkarni, 2018). For instance, in rats with STZ-induced T1DM, the administration of Quercetin two weeks after induction resulted in the regeneration of pancreatic β-cells, preserving their structure and integrity (Vessal *et al.*, 2003; Adewole *et al.*, 2007). Additionally, Quercetin

treatment improved glucose uptake in insulin-resistant tissues and increased insulin secretion (Lee *et al.*, 2012). Similarly, Biochanin A, a Genistein derivative, has been reported to protect pancreatic islet β-cells and improve glucose tolerance in T2DM rat models (Oza and Kulkarni, 2018). Hence, the antioxidant bioactive compounds present in *G. tridentata* extract may play a role in the observed changes in Langerhans islets in T2DM rats undergoing *G. tridentata* treatment.

Overall, these findings underscore the importance of exploring natural treatments like *G. tridentata* and its bioactive compounds in mitigating the multi-organ complications of T2DM.

4.3.5. Conclusion

Our study provides comprehensive insights into the potential therapeutic effects of *G. tridentata* extracts in mitigating the complications associated with T2DM and associated comorbidities.

Firstly, our investigation into the toxicity of *G. tridentata* extracts revealed a favorable safety profile, with no observed toxicity. Secondly, our study explored the influence of *G. tridentata* on lifespan under glucotoxic conditions using a *C. elegans* model. We found that *G. tridentata* extracts partially attenuated the shortened lifespan induced by high glucose diets, which suggests a promising role for *G. tridentata* in mitigating glucotoxicity and its associated health span deterioration. Thirdly, our investigation into the antinociceptive potential of *G. tridentata* methanolic extracts revealed a reduction in mechanical and thermal allodynia, suggesting a potential analgesic effect in diabetic neuropathic pain. Lastly, our study highlighted the beneficial effects of *G. tridentata* extracts on internal organs affected by T2DM. *G. tridentata* treatments demonstrated hepatoprotective effects, delayed progression of renal morphological changes, and improved the architecture of pancreatic islets, potentially through the modulation of oxidative stress and inflammation.

Overall, our findings underscore the potential of *G. tridentata* and its bioactive compounds in ameliorating the complications of T2DM. Further research is warranted to elucidate the underlying mechanisms and optimize the therapeutic potential of *G. tridentata* as a natural component to improve medicines for T2DM and its associated conditions.

4.3.6. References

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4.4. Topical application of *Genista tridentata* leaf extract: A promising strategy for diabetic wound management

4.4.1. Introduction

The prevalence of Diabetes mellitus (DM) has reached epidemic proportions on a global scale (IDF, 2021). According to the International Diabetes Federation (IDF) report from 2021, approximately 537 million adults (20-79 years) were diagnosed with DM. Moreover, this number is projected to rise significantly to 783 million by the year 2045. These alarming data demonstrate that DM constitutes one of the most rapidly growing global health emergencies of the 21st century (IDF, 2021).

The increasing epidemic of DM can be attributed to several factors, as highlighted by Zheng *et al.* (2018). These include population aging, economic development, urbanization, unhealthy eating habits, and sedentary lifestyles. Collectively, these multiple factors contribute to the growing prevalence of DM, making it a significant public health concern worldwide (Zheng *et al.,* 2018).

Based on IDF's data, the economic burden of DM has exhibited substantial growth over the years. In 2007, global spending on diabetes was recorded at USD 232 billion, which increased significantly to USD 966 billion in 2017 for adults (20–79 years old). Projections indicate that this economic impact will continue to rise, reaching a staggering USD 1.05 trillion by 2045. However, it's essential to note that these predictions assume constant mean expenses per person and DM prevalence while just considering demographic changes (IDF, 2021).

Furthermore, DM has been linked to an increased risk of long-term complications, including foot ulceration and amputation (Hunt, 2011). Disturbingly, around 30% of diabetic patients may develop foot ulcers (Chang & Nguyen, 2021). These foot ulcers are chronic wounds that often resist healing and, in severe cases, can result in the need for amputations (Hunt, 2011; Chang & Nguyen, 2021).

Extensive research has been conducted on the role of reactive oxygen species (ROS) in the wound-healing process. Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot}$) are crucial intracellular secondary messengers that regulate various stages of wound healing, including cell recruitment, cytokine production, cell migration, proliferation, and angiogenesis (Cano Sanchez *et al.*, 2018; Wang *et al.*, 2023).

Importantly, in diabetic patients, prolonged hyperglycemia leads to an overproduction of ROS due to increased oxygen consumption by mitochondria (Yaribeygi *et al.*, 2020; Zhang *et al.*, 2020) and induces oxidative stress through the polyol pathway and protein kinase C (PKC) signaling, as well as the accumulation of Advanced Glycation End Products (AGEs) (Yaribeygi *et al.*, 2020; Zhang *et al.*, 2020).

The involvement of ROS in the impaired wound-healing process in diabetic patients is multifaceted. Studies have aimed to highlight the role of ROS in delayed wound healing, as increased ROS production disrupts redox homeostasis, causing cellular oxidative damage that inhibits proper wound healing (Deng *et al.*, 2021; Wang *et al.*, 2023). Thus, oxidative stress, characterized by an increase in ROS levels and/or a decrease in antioxidant defenses, is a key factor in the healing impairment (Wang *et al.*, 2023).

Several plants and herbs have been recognized for their ability to promote wound healing, and they contain a variety of phytochemical constituents, namely phenolic compounds (Budovsky *et al.*, 2015; Shedoeva *et al.*, 2019; Sharma *et al.*, 2021). The use of medicinal plant is preferable due to their lower toxicity and reduced adverse effects compared to conventional therapeutic drugs (Budovsky *et al.*, 2015). Consequently, there has been a renewed interest in utilizing medicinal plants for wound healing, both in diabetic and non-diabetic cases (Shedoeva *et al.*, 2019; Sharma *et al.*, 2021), especially in resource-limited areas (Shedoeva *et al.*, 2019).

Genista tridentata (L.) Willk. (also known as *Pterospartum tridentatum* L. Willk) is a European endemic Leguminosae (Fabaceae) species, subfamily Papilionoideae, autochthonous in the Iberian Peninsula, Morocco, Tunisia, and Algeria (Laranjeira *et al.*, 2023). The utilization of freshly picked or shade-dried flowers from *G. tridentata* is common in folk medicine, administered through infusions, decoctions, and tonics (Pinela *et al.*, 2011). These floral preparations are recognized for their anti-inflammatory activity (Gião *et al.*, 2007; Bremner *et al.*, 2009; Laranjeira *et al.*, 2023) and their effectiveness in the reduction of hyperglycemia (Grosso *et al.*, 2007; Coelho *et al.*, 2011; Luis *et al.*, 2011; Ferreira *et al.*, 2012; Gonçalves *et al.*, 2013; Aires *et al.*, 2016; Balanč *et al.*, 2016; Gonçalves *et al.*, 2018).

Taking into account *G. tridentata* anti-inflammatory and antioxidant activity, the main aim of the present study was to further investigate the potential wound healing effect of the *G. tridentata* methanolic leaves extract. We evaluated the antioxidant activity of the *G. tridentata* leaves methanolic extract using *in vitro* radicals scavenging assays and *in vivo* fibroblasts cell culture. Additionally, an animal excision wound healing model in Type 2 DM STZ-NA induced rats was also used to evaluate the wound healing effect of the extract through a detailed analysis of tissue regeneration.

4.4.2. Materials and methods

4.4.2.1. Preparation of *G. tridentata* extracts

Leaves of *G. tridentata* were collected in Esposende, Portugal (41°34′30″ N; 8°46′0″ W), in the spring of 2019, during the flowering period (May). The plant material was identified at the Biology Department of the University of Minho (Biology Department Herbarium, University of Minho: voucher reference

12/2019). Biomass was triturated and macerated with methanol 70% (Sigma-Aldrich, Barcelona, Spain), in the dark, for 7 days. Afterward, the mixture was filtered and concentrated with a rotary evaporator (Buchi® RII, Buchegg, Switzerland) (under reduced pressure, in the dark, at 50 °C). The extract was then lyophilized (Christ® Alpha 2-4, B. Braun, Gottingen, Germany) for two days to obtain a powder and stored in a closed container (dark at room temperature) until further use (Dias *et al.*, 2007).

4.4.2.2. *In vitro* antioxidant activity

4.4.2.2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The assessment of radical scavenging activity was conducted using a modified method based on the stable DPPH free radical, as described by Oliveira *et al.* (2018). In a 96-well plate (Sigma-Aldrich, Barcelona, Spain), 140 µl of a 400 µM DPPH solution (dissolved in 100% ethanol, Sigma-Aldrich, Barcelona, Spain) was mixed with 10 µl of *G. tridentata* extracts at various concentrations (46.875 µg/ml, 93.75 µg/ml, 187.5 µg/ml, 375 µg/ml, 750 µg/ml, and 1000 µg/ml) to achieve a final concentration of 1000 µg/ml. After incubating for 1 hour in darkness at room temperature, absorbance was measured at 517 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices, USA). Graph Pad Prism software (GraphPad Software Inc., version 5, LaJolla, CA, USA) was utilized to determine the extract concentration required to scavenge 50% of the initial DPPH (EC50), with Quercetin (Sigma-Aldrich, Barcelona, Spain) employed as the reference standard. The measurements were performed in triplicate across three independent sets, and the results are presented as the mean ± standard deviation (SD).

4.4.2.2.2. Nitric oxide (NO) scavenging activity

The evaluation of NO scavenging activity was conducted following a protocol with slight modifications by Silva *et al.* (2008). In brief, a reaction mixture (200 µl) containing Sodium Nitroprusside (SNP, Sigma-Aldrich, Barcelona, Spain, 10 mM, in PBS, pH 7.4) and *G. tridentata* extract up to a final concentration of 1000 µg/ml, was incubated under light at room temperature for 2 hours. A control group, substituting the extract with PBS, pH 7.4, was included. Afterwards, 50 µl of Griess reagent (1% (w/v) sulfanilamide and 0.1% (w/v) NED (N-(1-naphthyl) ethylenediamine) in 2% phosphoric acid) (Sigma-Aldrich, Barcelona, Spain) was added and incubated for 10 min in the dark. Two blanks, the sample blank and the control blank, with a composition mirroring their respective counterparts but containing 2% phosphoric acid (Sigma-Aldrich, Barcelona, Spain) instead of the Griess reagent, were integrated. The absorbance of the resulting chromophore (purple azo dye) was measured at 543 nm. Gallic acid (Sigma-Aldrich, Barcelona, Spain) was used as the reference standard, and Graph Pad Prism software (GraphPad Software Inc., version 5, LaJolla, CA, USA) was utilized to determine the extract EC50 values. The measurements were

performed in three independent sets, each set consisting of three replicates. The results are presented as the mean ± SD.

4.4.2.2.3. Super Oxide (SO) scavenging activity

Superoxide (SO) production induced by the extracts was assessed following the protocol outlined by Valentão *et al.* (2002). The reaction mixture comprised 50µl of NADH stock solution (1.97mM in phosphate buffer, pH 7.4) (Sigma-Aldrich, Barcelona, Spain), 150 µl of NBT stock solution (Nitro blue tetrazolium, 81.5 µM in phosphate buffer, pH 7.4) (Roche, Basel, Switzerland), 50 µl of PMS solution (3.26 mM in phosphate buffer, pH 7.4) (Acros Organics; Thermo Scientific Chemicals; Antwerpen, Belgium), and 50µl of *G. tridentata* extract up to a concentration of 1000 µg/ml, and was incubated for 2 minutes at room temperature. Two blanks, one for the sample and one for the control, were prepared to match their respective counterparts, with the sample blank containing 50 µl of the appropriate solutions and 50 µl of NADH stock, and the negative control comprising phosphate buffer (pH 7.4), NADH stock, NBT stock, and PMS solution. The absorbance at 560 nm was measured using a SpectraMax Plus 384 microplate reader (Molecular Devices, USA), with the color change from blue to yellow indicating SO production. Graphs were generated using GraphPad Prism (GraphPad Software Inc., version 5, LaJolla, CA, USA) to determine the extract concentration required for 50% SO scavenging (EC50), with Quercetin (Sigma-Aldrich, Barcelona, Spain) serving as the reference standard. The results, obtained from three sets of three replicates, are presented as mean ± SD.

4.4.2.2.4. Iron Chelating activity (ICA)

The chelation of ferrous ions by *G. tridentata* extracts was assessed using a method previously outlined by Oliveira *et al.* (2018), with minor modifications. In a 96-well plate (Sigma-Aldrich, Barcelona, Spain), 50 µl of 0.12 mM FeSO4 solution (Sigma-Aldrich, Barcelona, Spain) was mixed with 50 µl of varying concentrations of the extract to achieve a final concentration of 1000 µg/ml. The reaction was initiated by adding 50 µl of 0.6 mM ferrozine solution (Sigma-Aldrich, Barcelona, Spain), followed by a 15-minute incubation at room temperature in the dark. Subsequently, the absorbance of the solution was measured at 562 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, USA). EDTA (Sigma-Aldrich, Barcelona, Spain) was used as the reference standard, and the extract concentration required to chelate 50% of the ferrous ions (EC50) was determined. The measurements were performed in three sets, each consisting of three replicates, and the results are presented as the mean ± SD.

4.4.2.3. Cell culture

The L929 cell line (Mouse Fibroblast cells) was sourced from the European Collection of Cell Cultures (ECACC). These cells were cultivated in 25 cm^2 polystyrene flasks (Nunc Cell Culture, Thermofisher Scientific, Antwerpen, Belgium) using complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, 1% antibiotic–antimycotic solution, and 1.5 g/L sodium bicarbonate; Sigma-Aldrich, Barcelona, Spain) at 37 °C in a humidified incubator with 5% CO2 (Binder CB150, Labstuff.eu e. K., Malente, Germany). Sub-culturing was performed three times a week using 0.05% trypsin (Sigma-Aldrich, Barcelona, Spain), and cells were seeded at a density of 5×10^5 cells per ml. For all subsequent assays, DMEM without FBS was employed.

4.4.2.3.1. Cell viability/cytotoxicity assay

To evaluate the potential cytotoxicity of *G. tridentata* extracts, L929 cells were plated in 96-well culture plates (Sigma-Aldrich, Barcelona, Spain) at a density of $1.5x10^5$ cells/ml (200 µl/well) in complete DMEM. Following a 24-hour incubation period, the medium was aspirated, and the cells were exposed to varying concentrations of *G. tridentata* extracts (100, 250 and 500 µg/ml) for durations of 4 hours and 24 hours. Cells cultured in DMEM without extracts and cells treated with 0.5% and 10 % DMSO in DMEM served as controls for normal growth and cell death, respectively. Cell viability was determined using the MTT assay as previously outlined by Oliveira *et al.* (2018), with viability expressed as a percentage relative to the control (cells cultured solely in DMEM).

4.4.2.3.2. Cytoprotective effects of *G. tridentata* against t-BHP (*tert*-Butyl hydroperoxide-induced oxidative damage

To examine the potential cytoprotective effects of *G. tridentata* extract against T-BHP-induced insult, L929 cells were seeded in 96-well culture plates (Sigma-Aldrich, Barcelona, Spain) at a density of $1.5x10^5$ cells/ml (200 µl/well) in complete DMEM (Sigma-Aldrich, Barcelona, Spain). After a 24-hour incubation period, the medium was replaced, and the cells were exposed to various concentrations of *G. tridentata* extracts (100, 250, and 500 µg/ml) along with T-BHP (0.1mM; Sigma, Darmstadt, Germany) for 4 hours.

For the pre-incubation assay, L929 cells were similarly seeded in 96-well culture plates and allowed to incubate for 24 hours. Afterward, the cells were pre-incubated with different concentrations of *G. tridentata* extracts (100, 250, and 500 µg/ml) for 20 hours before being co-incubated with T-BHP (0.1 mM) for an additional 4 hours. Cellular viability was evaluated using the MTT assay described by Oliveira *et al.*, (2018).

As controls, L929 cells cultured in DMEM without extracts and cells treated with 0.5% and 10 % DMSO in DMEM served as references for normal growth and cell death, respectively. Additionally, L929 cells cultured in DMEM with T-BHP (0.1 mM) were included as controls for the cytoprotective assays.

4.4.2.4. *G. tridentata* effects in wound-healing in rats with experimental T2DM

4.4.2.4.1. Animals, ethical considerations and handling

Animals from the Life and Health Sciences Research Institute (ICVS) vivarium were used in this study. All experimental procedures were approved by the Institutional and National Ethical Commission (DGAV 23875/2019) and adhered to the European Community Council Directive 2010/63/EU for animal treatment in experiments. Steps were taken to minimize animal usage and distress for reliable scientific outcomes. Two animals were housed per polycarbonate cage (45.4x 25.5x 20 cm) in a controlled environment (22 °C, 55% relative humidity), with a 12-hour light/dark cycle, and provided constant water and food access (F0021; BioServ, New Jersey, US). Regular veterinarian checks and weekly health assessments by researchers ensured animal welfare. Trained researchers, blinded to treatment conditions, executed all procedures.

4.4.2.4.2. Anesthesia and euthanasia

To induce the wound excision model, animals were intraperitoneally anesthetized with ketamine (0.75 mg/kg, Merial, Lyon, France) and medetomidine (0.5 mg/kg, Esteve Veterinaria, Léon, Spain) (Laranjeira *et al.*, 2024). The depth of anesthesia was assessed by monitoring pupil size, general muscle tone, and absence of nociceptive withdrawal reflexes. Body temperature was maintained using a warming blanket to ensure physiological range. Anesthesia reversal was achieved with atipamezole hydrochloride (1 mg/kg, Pfizer, Oeiras, Portugal). Euthanasia was performed with a lethal dose of sodium pentobarbital (200 mg/mL, Ceva, Oeiras, Portugal).

4.4.2.4.3. Induction of type 2 diabetes

To induce T2DM, rats were intraperitoneally injected with 180 mg/kg of nicotinamide (NA) dissolved in saline (NaCl 0.9%, Unither, Amiens, France), followed by a subsequent injection of 65 mg/kg streptozotocin (STZ, Merck, Algés, Portugal) dissolved in citrate buffer (citrate buffer 0.1M, pH 4.5, Sigma Aldrich, Sintra, Portugal) 15 minutes later. Animals in the control group (SHAM) received only vehicle solutions: NaCl 0.9% (0.9% NaCl pH 7.2, Unither, Amiens, France) and citrate buffer 15 minutes later (citrate buffer 0.1M, pH 4.5, Sigma Aldrich, Sintra, Portugal) (Masiello *et al.*, 1998). The glycemic index was monitored at intervals of 24 and 72 hours initially, followed by weekly checks, achieved by puncturing

the tail tip with a 26G needle (0.45 x 13 mm) to collect approximately 32 µl of blood, which was then transferred onto a test strip inserted into the Contour Next XT glucose meter (Ascensia, Switzerland). Animals exceeding 300 mg/dl included in the diabetic group according to criteria by Arulmozhi *et al.* (2004). Predefined humane endpoints for diabetes severity included weekly weight loss of 20%, blindness, and urinary tract infections, and were overseen by the researchers and the resident veterinarian. Daily observations tracked polyuria, polydipsia, polyphagia, and cataract development.

4.4.2.4.4. Excision wound model

After administering anesthesia to immobilize the rats (n=7 each group), their flanks in the dorsum were shaved bilaterally and disinfected 3 times with chlorhexidine interspersed with 70% alcohol, using centrifugal movements. Utilizing a punch-biopsy tool (Stiefel Laboratories Inc., Research Triangle Park, North Carolina, USA), four circular wounds of consistent size (1.0 cm diameter each) were created on the shaved region—two on each side, and these wounds were deliberately left open. A minimum separation of 2 cm was maintained between all wounds to minimize potential effects from adjacent wounds. The day of wounding was labeled as day zero, following the protocol described by Nasir *et al.* (2016).

4.4.2.4.5. Extract-cream preparation and application

A cream containing 0.25 % *G. tridentata* extract was prepared. The formulation of the cream was made by using 3 different phases (table 4.4.1.). Firstly, phase A and B were separately heated to 70 °C using a hot water bath. Once both faces reached the desired temperature, phase A was added to phase B and emulsified at 3000 rpm using a turbo Silverson mixer. The *G. tridentata* extract was present in phase C of the cream formulation and added after cooling around 35 Celsius. Phase C was gradually added, under continuous agitation, and the mixture was left to cool to lab temperature (*circa* 25 Celsius).

Table 4.4.1. Cream formulation containing *G. tridentata* extract.

Phase	Ingredient	Percentage
A	Distilled water	94.75
	Xanthan	0.5
B	Montanov™ 68	3.5
	Cetyl alcohol	0.5
C	Preservative	0.5
	G. tridentata extract	0.25

The animal was properly restrained by the researcher, and a layer of cream (±5 mg) was applied to each wound accordingly to the animal's group (once a day, for 10 days), gently and uniformly with a sterilized spatula, covering the entire wound surface (Nasir *et al.*, 2016). After the application, the animal was held by researcher for 2 minutes to prevent immediate removal of the cream and subsequently placed in a clean box to prevent contaminations. The animals were observed daily to ensure there were no adverse reactions to the treatment, such as rubor or swelling. The treatment application was performed daily, during 10 days.

4.4.2.4.6. Histological processing

Following completion of experimental procedures, animals were euthanized with a lethal dose (0.5ml) of sodium pentobarbital (200mg/ml, Ceva, Oeiras, Portugal). Photographs of the area were taken positioning the camera (smartphone SM-A307FN/DS - Samsung A30S, camera version 11.0.16.16; Fnac, Portugal) at a distance of 15 cm perpendicular to the wound area. The skin samples were collected (Wound area±0.5 cm^2) and preserved in 4% paraformaldehyde (PFA) (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain) until further processing. Histopathological samples were stained with haematoxylin and eosin (Oliveira *et al.*, 2010). The samples were dehydrated using a series of ascending ethanol/water solutions, ranging from 70% to 100%, utilizing an automated sample processor (Leica AutoStainer XL, Nussloch, Germany) for a duration of 19 hours. Following dehydration, the specimens underwent triple rinsing with xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group, France). Subsequently, they were immersed in paraffin (Thermo Scientific, Cheshire, UK) at a temperature of 62°C, allowing them to solidify at -5°C. Sections measuring 4 µm in thickness were then cut in a vibrotome (Leica RM2155, Nussloch, Germany) and fixed onto Superfrost glass slides (25 mm × 75 mm × 1 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK). The slides were subjected to deparaffinization in an automated slide processing apparatus (Leica AutoStainer XL, Nussloch, Germany) prior to staining. Hematoxylin Harris (Millipore Corporation, Massachusetts, MA, USA) staining was conducted for 1 minute, followed by immersion in tap water for 2 minutes. Subsequent steps involved washing the slides with a 0.5% ammonia solution (Sigma Aldrich, Madrid, Spain) for 10 seconds, followed by rinsing in tap water for 1.5 minutes, and then in a 96% ethanol solution for 45 seconds. Eosin Y solution (Thermo Scientific, Cheshire, UK) was then applied for 35 seconds, followed by dehydration with 96% ethanol (1 minute), absolute ethanol (2 minutes each), and xylene (1 minute for the first wash and 2 minutes for the second). Finally, the slides were mounted using a resinous medium (Entellan, Merck, Darmstadt, Germany) and covered with lamellae (24 × 60 mm, Superfrost Plus, Thermo Scientific, Cheshire, UK),

after which they were allowed to air dry for a period of seven days. After drying, slides were examined under a light microscope (Olympus BX61, Olympus Co. Ltd, Tokyo, Japan) and photomicrographed.

4.4.2.5. Wound healing assessment

The three parameters evaluated were wound opening, granulocytic tissue area, and neo-epidermis thickness. Measurements were conducted using ImageJ software (Schneider *et al.*, 2012). Wound opening percentage was derived by subtracting the length of the exposed wound from the total wound length. The resulting value, measured in megapixels, was converted into centimeters by referencing the microscope scale marker (known in both centimeters and pixels), using the corresponding megapixel count to calculate length in cm. Subsequently, granulocytic tissue area was determined by outlining its contour alongside the microscope scale marker, with the corresponding megapixel count used to compute the area in cm^2. Neo-epidermis thickness was assessed by tracing three lines at different points, measuring them in megapixels, and converting the measurements into centimeters as described. These parameters provided crucial insights into wound condition and the healing process progression.

4.4.2.6. Statistical analysis

Biochemical experiments were independently repeated three times, with each repetition consisting of three replicates. EC50 values were calculated through non-linear regression analysis. Data analysis was performed using GraphPad Prism v. 8.0 Software (GraphPad Software Inc, La Jolla, CA, USA). The results are presented as mean ± SD. T-tests were used to evaluate the antioxidant capacity of the *G. tridentata* extract. Statistical analyses included one-way ANOVA followed by Bonferroni pos hoc tests for multiple comparisons to evaluate the cytotoxicity and the antioxidant activity of the extracts in cellular assays, as well as, to evaluate the wound-healing parameters. For these assays, the results are presented as mean ± Standard Error of the Mean (SEM). Significance was determined at $p \leq 0.05$.

4.4.3. Results

4.4.3.1. Antioxidant activities (*in vitro* biochemical assays) of *G. tridentata* extracts

An initial assessment to evaluate the antioxidant activity of *G. tridentata* extracts was conducted using various *in vitro* methods, assessing several pertinent activities indicative of the overall antioxidant potential of the extracts. The findings are summarized in Table 4.4.2. Overall, *G. tridentata* extracts exhibited potent antioxidant effects, characterized by low EC50 values in scavenging methods (DPPH, NO and SO) and ICA. Additionally, they demonstrated strong superoxide scavenging capability.

Table 4.4.2. *In vitro* antioxidant capability of the *G. tridentata* extracts (GtL). Results are expressed as mean ± SD. *** **p** < 0.001; **** **p** < 0.0001. (DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay; NO: Nitric Oxide scavenging assay;SO: Super Oxide Scavenging assay; ICA: Iron Chelating Activity assay)

EC50 (µg/ml) ± SD			
Assay	**GtL extract**	**Positive control**	**Statistical analysis**
DPPH	101,37 ± 23,02	6,59 ± 1.2	Ttest: $T_{(16)}$=12.33,****
SO	42,5 ± 8,42	13,19 ± 6,4	Ttest: $T_{(16)}$=7.673, ****
NO	153,52 ± 71,96	48,85 ± 21,71	Ttest: $T_{(16)}$=4.178, ***
ICA	199,25 ± 40,04	40,44 ± 12,72	Ttest: $T_{(16)}$=11.3, ****

4.4.3.2. Cytotoxicity of *G. tridentata* extracts

Recognizing the pivotal role of fibroblasts in tissue homeostasis, we employed fibroblast cells to explore any potential cytotoxic effects of *G. tridentata* extracts. Fibroblast cells were subjected to incubation with methanolic extracts of *G. tridentata* leaves for durations of either 4 h or 24 h, across a range of concentrations (100, 250 and 500 µg/ml), to mimic acute and chronic cellular exposure, respectively.

Notably, exposure of fibroblast cells to the extracts at concentrations up to 500 µg/ml for 4 h and 24h did not elicit any signs of toxicity (Figure 4.4.1A: $ANOVA_{one\text{-}way}$: $F_{(5,71)}$=52.77, P<0.0001 and Figure 4.4.1B: $ANOVA_{one\text{-}way}$: $F_{(5,53)}$=1846, P<0.0001, respectively).

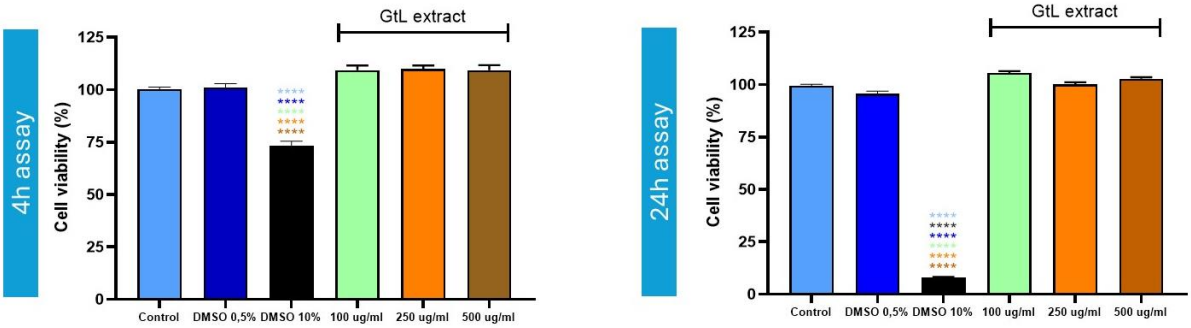


Figure 4.4.1. The cytotoxic effects of methanolic extracts from *G. tridentata* leaves on L929 cells. L929 cells were treated with *G. tridentata* methanolic extracts at concentrations of 100, 250, and 500 µg/ml for 4 h and 24 h (A and B, respectively). Results are expressed as mean ± SEM. **** P< 0.0001.

4.4.3.3. Cytoprotective effect of *G. tridentata* extracts

Since *G. tridentata* extract proved to display *in vitro* antioxidant activities, we investigated the cytoprotective potential of these extracts against t-BHP induced oxidative stress in L929 cells. The extracts were evaluated for their putative L929 cytoprotection against oxidative insult (t-BHP 0.1 mM, 4 h), in two types of incubation regimes: (i) Co-incubation: addition of the extract and t-BHP during 4h; (ii) Pre-Incubation: addiction of extracts 20 h, following 4 h with the insult. A dose dependent cytoprotective effect against t-BHP insult was found in both assays (Figure 4.4.2A: $ANOVA_{one\text{-}way}$: $F_{(6,68)}=746.9$, $P<0.0001$ and Figure 4.4.2B: $ANOVA_{one\text{-}way}$: $F_{(6,71)}=448.6$, $P<0.0001$, respectively).

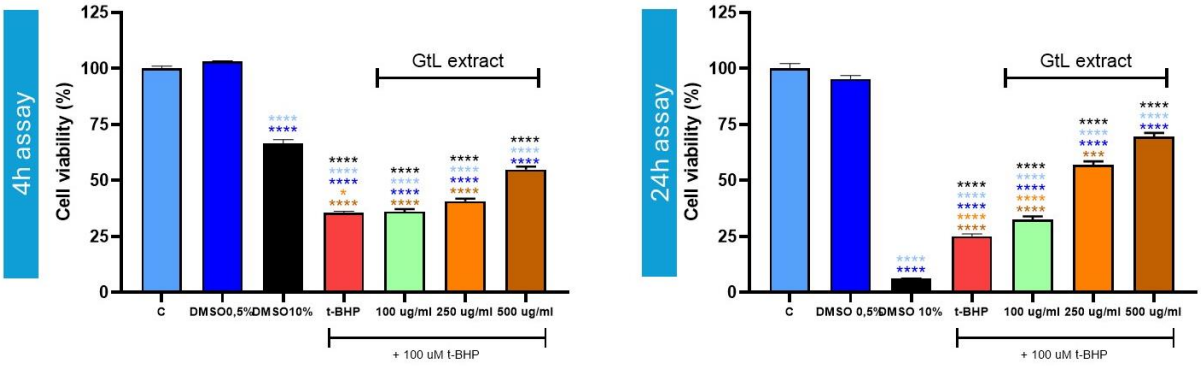


Figure 4.4.2. The cytoprotective effects of methanolic extracts from *G. tridentata* leaves on L929 cells against t-BHP 0.1 mM. L929 cells were treated with *G. tridentata* methanolic extracts at concentrations of 100, 250, and 500 µg/ml and co-incubated with t-BHP 0.1 mM for 4 h (A) and L929 cells were treated with *G. tridentata* methanolic extracts at concentrations of 100, 250, and 500 µg/ml for 20 h and co-incubated with t-BHP 0.1 mM for 4h (B). Results are expressed as mean ± SEM. * $P \leq 0.05$; *** $P \leq 0.001$; **** $P \leq 0.0001$. (C: cells with no treatment; DMSO 0.5%: cells treated with DMSO 0.5%; DMSO 10%: cells treated with DMSO 10%; t-BHP: cells treated with t-BHP 100mM; 100, 250, and 500 µg/ml: cells treated with 100, 250, and 500 µg/ml of *G. tridentata* methanolic leaf extract, respectively).

4.4.3.4. Blood glucose levels

Blood glucose levels were assessed over the experimental period, and the data is depicted in Figure 4.4.3. The analysis revealed no significant variations in blood glucose levels among experimental groups over time (ANOVAtwo-way: Time: $F_{(6,126)}=63.30$, $P<0.0001$; Group: $F_{(2,126)}=1.122$, $P=0.3289$; Interaction: $F_{(12,126)}=0.3297$, $P=0.9825$).

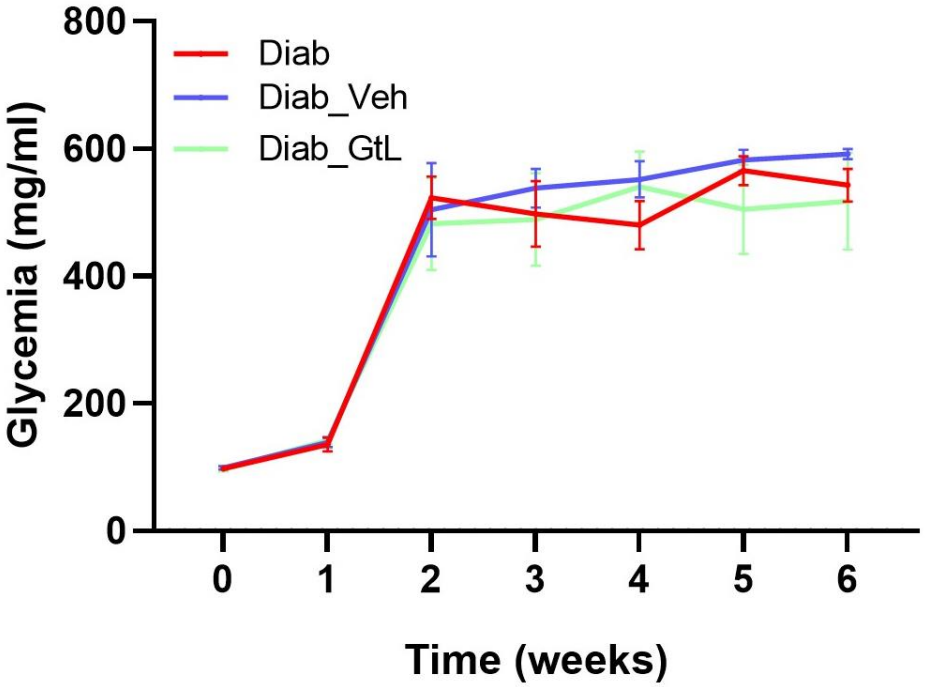


Figure 4.4.3. Blood glucose levels were assessed throughout the experimental period. Results are presented as mean ± SEM. Statistical significance was denoted as ** for P< 0.005, *** for P< 0.001, and **** for P< 0.0001. Animals were categorized as follows: Diab – animals injected with STZ and NA and with no topical treatment after the induction of wound excision model (n=7); Diab_Veh – animals injected with STZ and NA and with topically treatment with a base-cream after the induction of wound excision model (n=7); Diab_GtL – animals injected with STZ and NA and with topically treatment with *G. tridentata* incorporated base-cream after the induction of wound excision model (n=7).

4.4.3.5. Animal welfare

Throughout the experimental period, diabetic animals exhibited polydipsia and polyuria. However, none experienced a body weight decrease exceeding 20% weekly, thus no animals were sacrificed due to diabetes-related complications. Additionally, daily wound observations revealed no adverse effects from the applied treatments.

4.4.3.6. Wound-healing evaluation

The histological examination of wound tissues, supported by macroscopic photographs and histological photomicrographs (Figure 4.4.4.), revealed significant improvements in wound healing following the topical application of the vehicle and the methanolic leaf extract from *G. tridentata*. A significantly decrease in wound opening was observed (Figure 4.4.5A: ANOVA $_{one\text{-}way}$: $F_{(2,39)}$ =12.63, P<0.0001). Furthermore, a decrease in granulocytic tissue was noted (Figure 4.4.5B: ANOVA $_{one\text{-}way}$: $F_{(2,39)}$ =18.16, P<0.0001, respectively), indicating a reduction in the inflammatory response associated with the wound.

Additionally, wounds treated with the extract exhibited an increased thickness of the neoepidermis (Figure 4.4.5C: $ANOVA_{one\text{-}way}$: $F_{(2,39)}$=26.55, P<0.0001), suggesting enhanced skin regeneration.

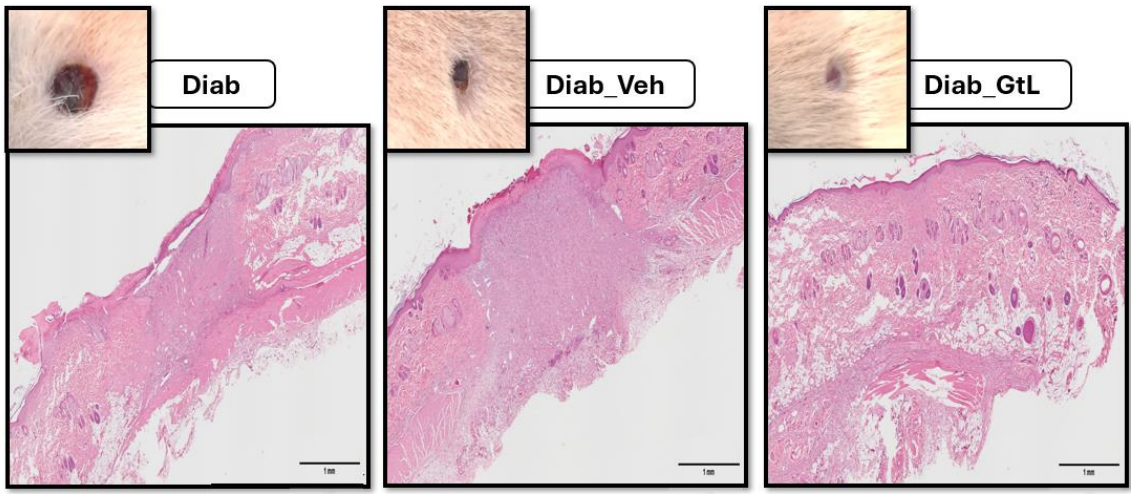


Figure 4.4.4. Macroscopic and microscopic examination of wound tissues: Representative images stained with hematoxylin and eosin of untreated wound tissues (Diab); after daily treatment with a base cream (Diab_Veh); and following daily treatment with a cream incorporating methanolic leaf extract of *G. tridentata* (Diab_GtL). Magnification: 250x.

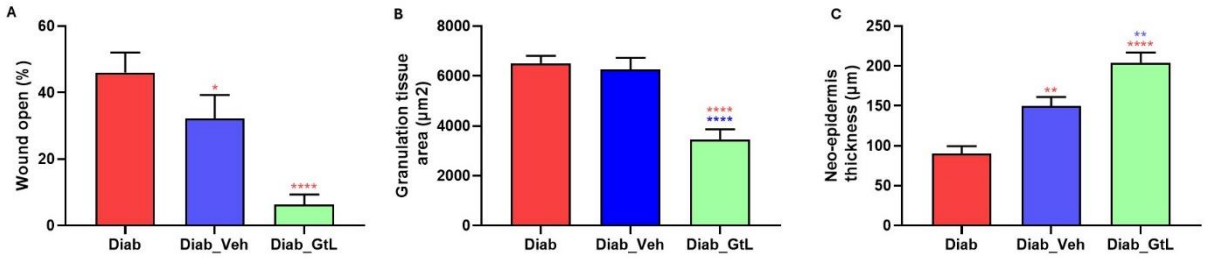


Figure 4.4.5. Analysis of wound parameters, depicting the effects of methanolic leaf extract of *G. tridentata* on skin healing and inflammatory response after 10-day treatment. (A) Wound opening; (B) Granulocytic tissue area; and (C) Neoepidermis thickness. Results are presented as mean ± SEM. Statistical significance was denoted as * for P< 0.05, ** for P< 0.005, and **** for P< 0.0001. Animals were categorized as follows: Diab – animals injected with STZ and NA and with no topical treatment after the induction of wound excision model (n=7); Diab_Veh – animals injected with STZ and NA and with topically treatment with a base-cream after the induction of wound excision model (n=7); Diab_GtL – animals injected with STZ and NA and with topically treatment with *G. tridentata* incorporated base-cream after the induction of wound excision model (n=7).

4.4.4. Discussion

Wound healing is a complex process involving a series of coordinated events, including inflammation, cell proliferation, extracellular matrix deposition, and tissue remodeling (Wallace *et al.*, 2017; Aitcheson *et al.*, 2021). In conditions such as diabetic wounds, this process can be compromised due to factors such as oxidative stress, chronic inflammation, and cellular dysfunction (Burgess *et al.*, 2021). Therefore, identifying therapeutic agents that can modulate these processes and promote effective wound healing is of significant clinical importance.

The results of this study demonstrated that the tested extract possesses several beneficial properties that may contribute to the improvement of wound parameters. Firstly, biochemical assays revealed significant antioxidant activity of the extract, as evidenced by its ability to chelate iron, and scavenge ROS. This antioxidant activity is crucial, as oxidative stress plays a crucial role in the pathogenesis of chronic wounds (Yaribeygi *et al.*, 2020; Zhang *et al.*, 2020), interfering with cell proliferation, extracellular matrix deposition, and fibroblast function (Deng *et al.*, 2021; Wang *et al.*, 2023).

Additionally, cellular assays with TBHP showed that the extract was able to protect cells against induced oxidative stress, suggesting its potential in preventing cellular damage associated with inflammation and oxidative stress in the wound (Wedel *et al.*, 2020). This cellular protection capacity is particularly relevant in diabetic wounds (Cho *et al.*, 2019), where cellular dysfunction is a common feature due to prolonged exposure to high levels of blood glucose (Yaribeygi *et al.*, 2020; Zhang *et al.*, 2020).

The antioxidant and anti-inflammatory properties exhibited by the methanolic extract of *G. tridentata* leaves may be linked to the presence of some phytochemical compounds, such as isoquercetin, genistein, and derivatives of quercetin in the extracts reported in our earlier study (Laranjeira *et al.*, 2023). These bioactive compounds may be one of the main contributors to the observed effects. These compounds are known for their antioxidant and anti-inflammatory properties (Sobhani *et al.*, 2021), which can help protect cells against oxidative damage, modulate the inflammatory response, and promote wound healing (Bhatia *et al.*, 2016; Polerà *et al.*, 2019; Čoma *et al.*, 2021).

However, it is important to note that the biological activity of the extract may be attributed not only to its individual components but also to their possible interaction and synergistic effect (Mitra *et al.*, 2023), potentiate their antioxidant and anti-inflammatory effects (Zhang *et al.*, 2019; Mitra *et al.*, 2023) and contributing to the overall effectiveness of the extract in promoting wound healing.

In this study, we induced T2DM in rats using a combination of STZ and NA. Within 72 hours of induction, all diabetic rats exhibited elevated blood glucose levels throughout the experimental period, consistent with findings from previous studies (Masiello *et al.*, 1998; Pauzi *et al.*, 2013; Undale *et al.*, 2020). We opted for topical application of a methanolic extract of *G. tridentata* leaves due to its advantages, including the ability to maintain high and sustained concentrations of agents at the injury site, with minimal risk of systemic absorption and toxicity (Lipsky and Hoey, 2009). Research has shown that topically administered drugs facilitate faster wound contraction, closure, and overall healing by exerting a localized effect directly at the wound site (Panchatcharam *et al.*, 2006).

The wound healing process aims to restore damaged tissue as closely as possible to its original state. Wound contraction, a crucial aspect of this process, involves the closure of wounds to the external environment (Hsu and Mustoe, 2010). Successful healing relies on tissue repair capability, the extent and type of damage, and the overall health status of the tissue (Teoh *et al.*, 2009).

In our investigation, we observed significant improvements in wound healing at the histological level with the topical application of methanolic leaf extract from *G. tridentata*. We noted a decrease of granulocytic tissue, suggesting a reduction in the inflammatory response associated with the wound. Non-resolved inflammation tends to favor wound matrix degradation, amplifying the presence of pre-existing granulation tissue productive cells (Acosta *et al.*, 2008; Aitcheson *et al.*, 2021). This modulation of the inflammatory response can be attributed to the antioxidant/anti-inflammatory activity of the extract (Merecz-Sadowska *et al.*, 2021), which may help reduce the production of pro-inflammatory mediators and resolve inflammation more effectively (Laranjeira *et al.*, 2023).

Furthermore, an increased thickness of the neo epidermis was observed in wounds treated with the extract, indicating more complete skin regeneration (Yang *et al.*, 2021). This is crucial for restoring skin barrier function and protecting the underlying tissue (Li *et al.*, 2017). The ability of the extract to promote the formation of a thicker neo epidermis may be attributed to its ability to promote cell proliferation and tissue repair (Li *et al.*, 2017; Merecz-Sadowska *et al.*, 2021), possibly through the modulation of cell signaling pathways involved in skin regeneration (Pastore *et al.*, 2012; Merecz-Sadowska *et al.*, 2021), further studies are needed to uncover the mechanisms.

Despite the promising findings presented in this study, it is important to acknowledge several limitations. Firstly, the research was conducted using animal and cellular models. While these models provide valuable insights into potential therapeutic effects, they may not fully replicate the complexity of human physiology and pathology (Grada *et al.*, 2018). Therefore, additional clinical studies involving human subjects are essential to confirm and extrapolate the observed effects to real-world scenarios (Wilhelm *et al.*, 2017).

Furthermore, the translation of findings from preclinical models to clinical practice necessitates careful consideration of factors such as dosing, formulation, and safety profiles (McGonigle and Ruggeri, 2014). Additionally, clinical studies allow for the evaluation of secondary outcomes, such as patient-reported outcomes and long-term effects, which are critical for informing clinical decision-making and healthcare policy (Möller, 2011; Wilhelm *et al.*, 2017).

4.4.5. Conclusion

The observed antioxidant activity of the extract, coupled with its ability to protect cells against oxidative stress, suggests a promising avenue for mitigating cellular damage associated with inflammation and oxidative stress in wounds, especially in the context of diabetic wounds where cellular dysfunction is prevalent. Moreover, the histological improvements observed, including decreased granulocytic tissue and increased neo-epidermal thickness, underscore the extract's potential in modulating the inflammatory response and promoting skin regeneration, crucial aspects of successful wound healing.

Overall, our data shows the topical application of a methanolic extract from *Genista tridentata* leaves improves wound healing in diabetic rats, highlighting its potential use in the cosmeceutical industry.

4.4.6. References

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Chapter 5: Discussion and final considerations

5.1. Discussion

Medicinal plants have been used for centuries in various folk medicine systems around the world (Petrovska, 2012 Süntar, 2020), owing to their diverse bioactive compounds and therapeutic properties (Süntar, 2020). In recent years, scientific research has increasingly focused on uncovering the pharmacological potential of these plants (Salmerón-Manzano *et al.*, 2020). In this thesis, we focused particularly on three edible, aromatic and medicinal plants (*C. tinctoria*, *B. dracunculifolia* and *G. tridentata*) and their potential effects in the areas of antioxidant, anti-inflammatory, antinociceptive, and antidiabetic properties. This discussion will delve into the existing literature on medicinal plants, highlighting their potential implications in managing oxidative stress, inflammation, pain, and diabetes and highlighting their possible applications and economic impact.

One of the most well-studied aspects of medicinal plants is their antioxidant activity (Munteanu and Apetrei, 2021). Oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms (Preiser, 2012; Sies *et al.*, 2017; Liguori *et al.*, 2018) plays a central role in the pathogenesis of various diseases, including cardiovascular diseases, neurodegenerative disorders, and cancer (Spector, 2000; Liguori *et al.*, 2018). Medicinal plants rich in antioxidants, such as flavonoids, phenolic compounds, and vitamins, have emerged as promising candidates for combating oxidative stress and its associated complications (Rajkapoor *et al.*, 2010; Hassan *et al.*, 2017).

When comparing the antioxidant activity of *C. tinctoria, B. dracunculifolia*, and *G. tridentata*, their distinct compositions contribute to variations in efficacy. *C. tinctoria,* with its rich in quercetin derivates, caffeic acid derivatives, kaempherol and catechins, *B. dracunculifolia*, abundant in caffeic acid derivatives, kaempherol and kaempheride, and *G. tridentata*, containing significant levels of genistein and isoquercetin, all show promising antioxidant effects, especially in mitigating oxidative stress-induced damage. These plants, with their diverse antioxidant profiles, can be integrated into various traditional medicine systems, herbal remedies, pharmaceuticals, dietary supplements, and skincare products, offering accessible and effective means to harness their antioxidant potential for promoting overall health and well-being.

Incorporating plants with well-established antioxidant properties into various applications can provide insight into the potential utilization of *C. tinctoria, B. dracunculifolia*, and *G. tridentata*. For instance, plants such as green tea (*Camellia sinensis*), turmeric (*Curcuma longa*), and grape seed (*Vitis vinifera*) extract are widely utilized in dietary supplements and skincare products for their antioxidant benefits (Gramza-

Michalowska and Regula, 2007; Vaughn *et al.,* 2016; Koch *et al.,* 2019; Sharafan *et al.*, 2023). These plants contain bioactive compounds such as catechins, curcuminoids, and proanthocyanidins, respectively, which contribute to their antioxidant activity and protective effects against oxidative stress-related damage (Gramza-Michalowska and Regula, 2007; Vaughn *et al.,* 2016; Koch *et al.,* 2019; Sharafan *et al.*, 2023). By drawing parallels with these established examples, the potential applications of *C. tinctoria, B. dracunculifolia*, and *G. tridentata* in various health and wellness products can be further explored and developed.

In addition to their antioxidant properties, several medicinal plants possess significant anti-inflammatory effects (Maione *et al.*, 2017; Tasneem *et al.*, 2019), which are closely linked with their antioxidant mechanisms (Furman *et al.*, 2019). Chronic inflammation is a key driver of various pathological conditions, including arthritis (Falconer *et al.*, 2018), inflammatory bowel disease (Padoan *et al.*, 2023), and cardiovascular diseases (Libby, 2006; Alfaddagh *et al.*, 2020). By modulating inflammatory pathways and cytokine production, various bioactive compounds can attenuate inflammatory responses and alleviate associated symptoms (Maione *et al.*, 2017; Tasneem *et al.*, 2019).

For instance, flavonoids such as quercetin, genistein, and rutin exert anti-inflammatory effects by inhibiting the expression of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) (O'Leary *et al.*, 2004; Min *et al.*, 2007; Shukla *et al.*, 2019; Mahmoud *et al.*, 2022). Similarly, other phenolic compounds like isoquercitrin and kaempherol have been shown to suppress inflammatory signaling pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Frattaruolo *et al.*, 2019; Yu *et al.*, 2022).

Moreover, aqueous extracts of *Salvia miltiorrhiza* and *Artimisia absinthium* demonstrate comparable anti-inflammatory effects, modulating diverse inflammatory pathways like NF-κB and MAPK to mitigate inflammatory responses (Gonfa *et al.*, 2023). These botanical compounds, alongside flavonoids and phenolic compounds, underscore the vast therapeutic promise of plant-based interventions against inflammation and related ailments.

Additionally, pain management is a major healthcare challenge (Brennan *et al.*, 2007; Maixner *et al.*, 2016), with millions of people worldwide suffering from chronic pain conditions such as neuropathic pain (Colloca *et al.*, 2017), fibromyalgia (Queiroz, 2013), and osteoarthritis (Hawker, 2019). Medicinal plants offer a promising opportunity for pain relief due to their antinociceptive properties (Akram *et al.*, 2013; Forouzanfar and Hosseinzadeh, 2018), which involve modulation of pain signaling pathways and neurotransmitter release (Zakaria *et al.*, 2018; Silva-Correa *et al.*, 2021).

Medicinal plants have also emerged as promising adjunctive therapies for diabetes management due to their ability to regulate blood glucose levels, enhance insulin sensitivity, and mitigate diabetic complications (Arumugam *et al.*, 2013; Nazarian-Samani *et al.*, 2018). Plant extracts containing bioactive compounds like flavonoids, alkaloids, and polyphenols have been shown to exert hypoglycemic effects by various mechanisms, including inhibition of carbohydrate digestion and absorption (Ansari *et al.*, 2020; Srinuanchai *et al.*, 2021; Ogbe *et al.*, 2023), stimulation of insulin secretion (Kaur *et al.*, 2011; Keller *et al.*, 2011; Ríos *et al.*, 2019) and enhancement of glucose uptake by peripheral tissues (Lahrita *et al.*, 2015; Harley *et al.*, 2020; Akdad and Eddouks, 2024). Additionally, these plants possess antioxidant and anti-inflammatory properties, which play a crucial role in mitigating oxidative stress and chronic inflammation associated with diabetes (Hassan *et al.*, 2017; Unuofin and Lebelo, 2020).

This work contributes to a deeper understanding of the pharmacological properties of different plant extracts, which can lead to the identification of new bioactive compounds with therapeutic potential. Additionally, it provides solid scientific evidence for the traditional use of the studied medicinal plants in some health conditions, such as *Diabetes mellitus* and osteoarthritis, helping to validate their efficacy and safety. Lastly, by exploring the therapeutic potential of these plants, enabling the development of new medicines and therapies based on natural products, offering more accessible, sustainable, and culturally relevant treatment options for diverse communities. Moreover, the plants studied are consumed as food, adding an extra dimension to their pharmacological investigation. Primarily, this may indicate a potential source of nutraceuticals. Furthermore, the safety of these plants as food may suggest a wider margin of safety for their therapeutic use, increasing the acceptance and accessibility of these natural treatments. However, it is also important to consider the potential interactions between these plants and people's diet, as well as the long-term effects of consuming these extracts at higher concentrations than typically found in the diet.

The research demonstrates the biological activities of *C. tinctoria*, *B. dracunculifolia* and *G. tridentata*, such as their anti-inflammatory, antioxidant, and potential anti-diabetic properties. So, there is ample potential for the incorporation of these plants into products with anti-inflammatory, antioxidant, and anti-diabetic properties. Some of these products include phytopharmaceuticals, nutraceuticals, cosmetics, and personal care products.

Some phytopharmaceutical drugs may be developed based on extracts from the studied plants. In the future, these drugs may be prescribed by healthcare professionals as a complement to conventional treatment (Antonio *et al.*, 2014; Motti *et al*, 2018) for a variety of conditions, including inflammation,

chronic pain, and metabolic issues. Capsules, tablets, or powders containing extracts of *G. tridentata*, *B. dracunculifolia*, or *C. tinctoria*, can be produced and marketed as nutritional supplements to promote overall health, support the immune system, or improve metabolic health, as they have demonstrated antioxidant, anti-inflammatory, and anti-nociceptive activities with low toxicity. Currently, these types of supplements are often consumed by individuals seeking a natural approach to managing specific health conditions (Tewfik and Tewfik, 2008; Teoh *et al.*, 2019). Also, shampoos, facial creams, body lotions, and other personal care products may contain extracts of medicinal plants (Shivanand *et al.*, 2010) known for their beneficial properties for the skin and hair (Shivanand *et al.*, 2010; Desam and Al-Rajab, 2021). These products are promoted as being gentler and more natural than conventional products, appealing to consumers concerned about the ingredients they apply to their bodies (Antignac *et al.*, 2011; Desam and Al-Rajab, 2021).

The specific economic impact of these products can vary depending on several factors, such as market size, consumer acceptance, production costs, and marketing, among others (Lubbe and Verpoorte, 2011). Overall, the industry of phytopharmaceuticals, nutraceuticals, and cosmetics incorporating medicinal plant extracts has grown in recent years (Maurya *et al.*, 2021; Aziz and Setapar, 2022), reflecting the consumers' increasing interest in natural and alternative products (Scott *et al.*, 2020; Song *et al.*, 2022). This trend brings a positive economic impact in terms of job creation, increased company profits, and contributions to the local and global economy (Wijijayanti *et al.*, 2020; Mofokeng *et al.*, 2022).

Moreover, beyond their intrinsic value in promoting health (Bernardini *et al.*, 2018; Süntar, 2020), medicinal plants also represent value-added products in various aspects (Lubbe and Verpoorte, 2011; Wijijayanti *et al.*, 2020; Mofokeng *et al.*, 2022). The economic impact of medicinal plants is significant and encompasses a variety of aspects, from production (Lubbe and Verpoorte, 2011) and marketing (Sharma, 2021) to tourism and local development (Wijijayanti *et al.*, 2020). Firstly, the global market for herbal medicines is constantly growing, driven by the increasing demand for natural and holistic alternatives to conventional pharmaceuticals (Lubbe and Verpoorte, 2011). For instance, in 1980, the expenditure on traditional medicine in the USA amounted to USD 800 million (Sher *et al.*, 2014). By 2000, this figure surged to USD 17 billion (Allkin, 2017), reflecting a relative increase of 20-25%. The traditional Chinese medicine industry alone was valued at USD 83 billion in 2012 (Allkin, 2017). In India, the herbal products trade was estimated at USD 120 billion in 2015, with projections indicating a leap to USD 7 trillion by 2050 (Hoareau and Da Silva, 1999). Broadly, Europe annually imports around 400,000 tonnes of medicinal plants from Africa and Asia, totaling USD 1 billion in value (Sher *et al.*, 2014; Sen and Chakraborty, 2015). Moreover, the global trade in medicinal plants and their derivatives was valued

at USD 33 billion in 2014, with growth rates of 2.4% in volume and 9.2% in value per annum (Vasisht *et al.*, 2016). Estimates suggest that the international trade of medicinal plants and their products reached USD 60 billion in 2010, with projections indicating a rise to USD 5 trillion by 2050 (Zahra *et al.*, 2020). The worldwide cosmeceutical market reached a value of USD 44 billion in 2017 and is expected to expand at an annual growth rate of 7%. Likewise, the market share of the USA was USD 2.8 billion in 2001, with an annual growth rate exceeding 7% until 2012 (Riaz *et al.*, 2021). Thus, companies dedicated to the production, processing, and distribution of extracts, essential oils, teas, and other products derived from medicinal plants are finding lucrative opportunities in an expanding market.

Additionally, the cultivation of medicinal plants can represent a stable source of income for farmers and rural communities (Sher *et al.*, 2014; Lima *et al.*, 2016). Many medicinal plants can be grown on small-scale farms or in agroforestry systems, providing an opportunity to diversify agricultural production and increase household incomes (Makunga *et al.*, 2008; Braga, 2021).

International trade in medicinal plants also plays a significant role in the global economy, with many countries exporting their medicinal plants to foreign markets (Sher *et al.*, 2014; Sen and Chakraborty, 2015; Riaz *et al.*, 2021). This not only generates profits for producers and exporters but also promotes cultural and commercial exchange between different regions of the world (Riaz *et al.*, 2021). However, it is crucial to ensure that this trade is conducted ethically and sustainably, respecting the rights of indigenous and traditional communities, and promoting fair trade practices (Braga, 2021).

Furthermore, tourism related to medicinal plants can also have a significant economic impact in areas where these plants are an important part of the local culture and heritage (Lima *et al.*, 2016). Fairs and festivals of medicinal herbs, botanical trails, and ecotourism experiences can attract visitors interested in learning about the properties and uses of medicinal plants (Hussain, 2022; Suhariyanto, 2022), generating economical returns for hotels, restaurants, tour guides, and local artisans (Figini and Patuelli, 2022).

Additionally, medicinal plants also hold profound cultural and historical value in the traditions of various communities around the world over centuries (Braga, 2021; Mishra and Kumar, 2023). They are intrinsically linked to the traditions, rituals, and healing practices of many ethnic groups and indigenous societies (Lima *et al.*, 2016; Mishra and Kumar, 2023). For instance, in traditional Chinese medicine, the use of herbs like ginseng, ginkgo biloba, and licorice dates back thousands of years and is considered an integral part of the healthcare system (Efferth *et al*, 2016; Suroowan and Mahomoodally, 2019). Similarly,

indigenous cultures in South America have a rich tradition of knowledge about the medicinal properties of native plants (Lima *et al.*, 2016; Braga, 2021).

These ancestral traditions not only employ medicinal plants to treat physical ailments but also recognize their significance in the spiritual and emotional context (Mishra and Kumar, 2023). Often, the process of harvesting, preparing, and administering medicinal plants is steeped in rituals and ceremonies aimed at not only healing the body but also strengthening the connection between individuals and nature, and promoting balance between mind, body, and spirit (Mishra and Kumar, 2023).

Preserving and promoting this traditional knowledge not only enriches the cultural identity of communities (Volenzo and Odiyo, 2020; Mishra and Kumar, 2023) but also offers opportunities for cultural tourism and sustainable economic development of rural areas (Hussain, 2022; Suhariyanto, 2022). Through festivals, workshops, and tours, communities can share their ancestral wisdom with interested visitors (Lima *et al.*, 2016), generating income and fostering greater understanding and appreciation of local traditions (Figini and Patuelli, 2022).

Furthermore, recognizing and respecting the traditional knowledge of medicinal plants is also essential for promoting social justice and the rights of indigenous and traditional communities (Kuspraningrum *et al.*, 2019). This includes ensuring that these communities are properly recognized and compensated for their role as custodians of ecological and medicinal knowledge passed down through generations (Kuspraningrum *et al.*, 2019; Volenzo and Odiyo, 2020). Ultimately, by valuing medicinal plants not only for their healing properties but also for their cultural and historical context, we can promote a more holistic and sustainable approach to human health and well-being (Kuspraningrum *et al.*, 2019).

Another important aspect is the environmental value of medicinal plants (Akerele, 2015). The environmental value of medicinal plants is multifaceted and crucial for biodiversity conservation and the preservation of natural ecosystems (Domínguez-Martín *et al.*, 2020; Linhares *et al.*, 2023). Firstly, many medicinal plants are native species that play important roles in local ecosystems, contributing to ecological stability and the health of natural habitats (Volenzo and Odiyo, 2020). Promoting the sustainable cultivation and harvesting of these plants can help protect and even restore threatened ecosystems, combating biodiversity loss and environmental degradation (Volenzo and Odiyo, 2020; Linhares *et al.*, 2023).

Furthermore, cultivating medicinal plants organically and sustainably can have significant benefits for soil health (Ait Elallem *et al.*, 2021; Wang *et al.*, 2022) and water quality (Singh *et al.*, 2022a). Agricultural methods that avoid the use of pesticides and chemical fertilizers help preserve soil fertility and microbial

biodiversity, thus promoting the health of agricultural ecosystems and reducing environmental contamination (Kumar and Kumar, 2019). This is especially important in areas where intensive cultivation of medicinal plants may have negative impacts on the environment, such as soil degradation and water pollution (Volenzo and Odiyo, 2020).

Additionally, by promoting sustainable cultivation and harvesting of medicinal plants, we can encourage responsible forest management practices and conservation of natural habitats (Volenzo and Odiyo, 2020). This is particularly relevant in regions where medicinal plants are harvested from wild habitats, such as forests and savannas, and where overexploitation can lead to biodiversity loss and ecosystem degradation (Mofokeng *et al.*, 2022). By adopting science-based and traditional knowledge-based approaches to the management of these natural resources, we can ensure their long-term availability for both present and future generations (Volenzo and Odiyo, 2020; Mofokeng *et al.*, 2022). Therefore, by recognizing and promoting the environmental value of medicinal plants, we can not only harness their therapeutic benefits but also contribute to nature conservation and sustainable development of our communities and ecosystems (Kumar and Kumar, 2019; Volenzo and Odiyo, 2020; Mofokeng *et al.*, 2022).

Thus, investing in responsible agricultural and management practices, as well as scientific research and environmental education programs, is crucial to ensuring that medicinal plants continue to play a vital role in promoting human health and protecting the environment (Singh *et al.*, 2022). Also, it is evident that industries related to healthcare, especially the pharmaceutical sector, profit from products derived from biodiversity, especially medicinal plants (Linhares *et al.*, 2023). Currently, there are several botanicals approved by the FDA available in the global market, such as Veregen® (Tea catechins) for treating external genital and perianal warts (Chen *et al.*, 2008), and Fulyzaq® (extract from the red sap of Croton lechleri) for managing HIV-related diarrhea (Georgiev, 2014). Sativex®, a titrated extract containing δ-9-tetrahydrocannabinol (psychoactive) and cannabidiol (anti-inflammatory), has been approved since 2005 in numerous countries (e.g., Canada, The United Kingdom, Germany, and New Zealand) (Barnes, 2006). This botanical prescription drug is an oromucosal spray cannabinoid medicine used to treat spasticity due to multiple sclerosis and neuropathy (Barnes, 2006). Marinol® (dronabinol) and Cesamet® (nabilone) are available in the North American market for alleviating vomiting and nausea associated with chemotherapy in cancer patients (Davis *et al.*, 2007). Biodiversity is crucial for drug discovery and serves as the primary source of inspiration for the development of treatments and interventions for current and future health threats (Linhares *et al.*, 2023). Unexpectedly, even with the mounting evidence emphasizing the risks to their business models by the decline in biodiversity, the

pharmaceutical industry largely remains unengaged in biodiversity conservation efforts (Linhares *et al.*, 2023).

Biodiversity for biomedicine strongly relies on the balance of three main factors: (i) the preservation of thousands of different species that populate the Earth, (ii) the rescue of the species faced with extinction, and (iii) the development of methods and technologies that allow extraction of biological/pharmaceutical compounds without harming existing ecosystems (Linhares *et al.*, 2023). It is dramatically clear that our society relies deeply on the Earth's biological resources, and we cannot afford to deplete them. A sustainable and circular bioeconomy considers not only the health and needs of living generations but also those of future generations (D'amato and Korhonen, 2021).

5.2. Conclusion and future perspectives

This work contributes to a deeper understanding of the pharmacological properties of different plant extracts, validating their efficacy and safety for various health conditions. Furthermore, by exploring the therapeutic potential of these plants, new medicines and therapies based on natural products can be developed, offering more accessible, sustainable, and culturally relevant treatment options for diverse communities.

Looking to the future, several paths of exploration and development emerge in the realm of medicinal plants. Further research is needed to elucidate the mechanisms of action underlying the therapeutic effects of these plants. Advanced technologies such as omics approaches and computational modeling can provide deeper insights into the molecular pathways involved, paving the way for the development of novel plant-derived therapeutics with enhanced efficacy and safety profiles.

Efforts to standardize the cultivation, processing, and formulation of medicinal plant products are crucial to ensure their quality, consistency, and regulatory compliance. Establishing rigorous quality control measures and standardized protocols will enhance the reliability of medicinal plant-based interventions, facilitating their integration into mainstream healthcare systems.

Exploring synergistic interactions between medicinal plants and conventional therapies holds promise for improving treatment outcomes and reducing side effects. By combining plant-derived compounds with existing pharmaceuticals, therapeutic efficacy can be enhanced while minimizing drug resistance and toxicity.

Integrating medicinal plants into interdisciplinary research initiatives fosters cross-disciplinary collaborations and knowledge exchange, unlocking their full potential and addressing complex health challenges from a holistic perspective.

Furthermore, prioritizing the conservation and sustainable use of medicinal plant resources is essential to ensure their long-term availability. Implementing policies and practices that promote biodiversity conservation, ethical sourcing, and fair-trade support the livelihoods of local communities while preserving traditional knowledge.

In conclusion, the future of medicinal plants lies in harnessing their therapeutic potential through interdisciplinary research, standardization, innovation, and sustainability. By embracing these principles, we can unlock new opportunities for improving human health, advancing scientific knowledge, and fostering environmental stewardship.

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