Universidade do Minho
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STATEMENT OF INTEGRITY

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

Avaliação dos efeitos neuroprotetores de extratos e compostos de *Alhagi*

Resumo

O stresse oxidativo e neuroinflamação foram identificados como fatores importantes no desenvolvimento e progressão da maioria das doenças neurodegenerativas. Assim, o potencial antioxidante das plantas bem como os seus compostos bioativos têm recebido muita atenção como forma de prevenir/tratar essas condições.

Alhagi spp. são um grupo de plantas, ainda pouco exploradas, que têm demonstrado propriedades antioxidantes, anti-inflamatórias e neuroprotetoras promissoras. O objetivo deste trabalho foi avaliar o potencial biológico da espécie de *Alhagi graecorum*, através da realização de um extrato metanólico, quanto às suas propriedades antioxidantes, anti-inflamatórias e neuroprotetoras, bem como realizar a caracterização química do extrato.

Primeiramente, foi caracterizado o perfil fitoquímico do extrato metanólico de folhas de *Alhagi graecorum*. Em seguida, para avaliar sua atividade antioxidante *in vitro*, a capacidade de reduzir o radical DPPH, o superóxido e o óxido nítrico foi avaliada, juntamente com a capacidade do extrato de quelar o ferro (ICA). A atividade anti-inflamatória *in vitro* também foi avaliada, usando o ensaio de peroxidase para a medição da atividade da COX-2. Por fim, a citotoxicidade do extrato nas linhas celulares BV2 e HepG2 foi testada, bem como o efeito neuroprotetor do extrato, submetendo-as ao hidroperóxido de terc-butila (t-BHP), um composto capaz de induzir stresse oxidativo e danos nas células.

A análise fitoquímica do extrato mostrou que esta planta é composta principalmente por flavonoides, nomeadamente derivados de quercetina e apigenina foram os principais compostos encontrados. O extrato apresentou resultados positivos para todas as atividades antioxidantes, principalmente nos ensaios relativos à capacidade de redução do radical DPPH e redução do anião superóxido. O extrato também apresentou propriedades anti-inflamatórias, inibindo a atividade da COX-2. O extrato apresentou citotoxicidade, em ambas as linhagens celulares, nas concentrações de 250 e 100 µg/mL, respetivamente. Nas concentrações de 50 e 25 µg/mL o extrato não apresentou toxicidade, quer em incubação de 4 horas ou de 24 horas. Além disso, o extrato provou ser capaz de proteger ambas as linhagens celulares contra o t-BHP, com melhores resultados quando pré-incubado por 20 horas. Estes resultados sugerem a potencialidade do extrato de *Alhagi graecorum* na proteção contra neuroinflamação e stresse oxidativo.

Evaluation of neuroprotective effects of *Alhagi* extracts and compounds

Abstract

Oxidative stress and neuroinflammation have been identified as a major trigger factor in the development and progression of most neurodegenerative diseases. Thus, the antioxidant potential of plants and their bioactive compounds have been receiving a lot of attention to prevent/treat these conditions.

Alhagi spp. are a group of plants not much explored that have demonstrated antioxidant, anti-inflammatory, and promising neuroprotective properties. The goal of this work was to evaluate the biological potential of *Alhagi graecorum* metanolic extract, regarding its antioxidant, anti-inflammatory, and neuroprotective properties, as well characterize the phytochemical profile of the extract.

First, the phytochemical profile of a methanolic extract of the leaves of *Alhagi graecorum* was characterized. Next, to evaluate its *in vitro* antioxidant activity, the DPPH, superoxide, and nitric oxide scavenging activities were assessed along with the extract's capacity to chelate iron (ICA). Also, the *in vitro* anti-inflammatory activity was evaluated using the peroxidase assay for the measurement of COX-2 activity. Finally, a screen of the extract cytotoxicity was performed in BV2 and HepG2 cell lines, as well as the extract's neuroprotective effect on both cultures, subjecting them to tert-Butyl hydroperoxide (t-BHP), a compound capable of inducing oxidative stress and damage in cells.

The phytochemical analysis of the extract showed that this plant is mainly composed of flavonoids. Quercetin and apigenin derivatives were the main compounds found.

Overall, the extract showed positive results for antioxidant activities, above all in the capacity of reducing DPPH radical and Superoxide anion. The extract also showed anti-inflammatory properties, inhibiting COX-2 activity.

The extract exhibited cytotoxicity in both cell lines, at concentrations of 250 and 100 µg/mL. At concentrations of 50 and 25 µg/mL, the extract did not exhibit toxicity. This happened equally in the 4h incubation assay and the 24 hours incubation assay. In addition, the extract proved to be capable of protecting both cell lines against t-BHP insult, with better outcomes when pre-incubated for 20 hours.

Overall, these results suggest the potentiality of *Alhagi graecorum* extract in protecting against neuroinflammation and oxidative stress.

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Abbreviations

AB_{25-35} peptide: Amyloid beta-peptide (25-35)

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

AD: Alzheimer's Disease

ALS: Amyotrophic Lateral Sclerosis

ATP: Adenosine triphosphate

BV2 and N9: Type of microglial cells

cAMP: Cyclic adenosine monophosphate

CAT: Catalase

CDA: Cytidine deaminase activity

CNS: Central Nervous System

COX: Cyclooxygenase

COX-2: Cyclooxygenase-2

DAMPS: Damage-Associated Molecular Patterns

DMEM: Dulbecco´s modified Eagle´s medium

DNA: Deoxyribonucleic Acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DPPHr: Reduced 2,2-diphenyl-1-picrylhydrazyl

EC50: Half maximal effective concentration

ERK1/2: Extracellular Signal-Regulated Kinase

FBS: Fetal Bovine Serum

Fe^{2+}: Ferrous ions

FRAP: Ferric Reducing Antioxidant Power

GABA: Gamma-AminoButyric Acid

GPX: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Reduced Glutathione

GSSG: Oxidized Glutathione

H2DCF-DA- 2′,7′: Dichlorodihydrofluorescein diacetate

H_2O_2: Hydrogen peroxide

HD: Huntington´s disease

HDL: High-density lipoprotein

HepG2: Human liver cancer cell line

HO·: Hydroxyl radicals

HPLC: High-performance liquid chromatography

HPLC-DAD: High-performance liquid chromatography-diode array detector

HPLC-DAD-MSn: High-performance liquid chromatography coupled with diode-array detection tandem mass spectrometry

IC50: Half maximal inhibitory concentration

ICA: Iron chelating activity

IL: Interleukin

IL-6: Interleukin 6

IR: Infrared spectroscopy

JNK1/2: c-junNH2-terminal kinase

LPS/IFN-γ: Lipopolysaccharide/interferon-gamma

MAPK: Mitogen-activated protein kinase

MCA: Metal chelating activity

MS: Mass spectrometry

MTT: Methyl-thiazol-tetrazolium

NADPH: Nicotinamide adenine dinucleotide phosphate

NBT: Nitroblue tetrazolium

NF-kB: Transcription nuclear factor kappa B

NLRP3: NLR Family Pyrin Domain Containing 3

NMR: Nuclear magnetic resonance

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

O_2: Oxygen

$O_2^{\cdot -}$: Superoxide radical anion

PAMPS: Pathogen-Associated Molecular Patterns

PCR: Polymerase chain reaction

PD: Parkinson's disease

PLGA: Poly (lactic-co-glycolic acid)

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

QR: Quinone reductase

RNS: Reactive Nitrogen Species

ROS: Reactive oxygen species

SEM: Standard Error of the Mean

SNP: Sodium Nitroprusside

SOD: Superoxide dismutase

SRB: Sulforhodamine B

TAC: Phosphomolybdenum total antioxidant capacity

TBA: 2-Thiobarbituric Acid

TBARS: Thiobarbituric Acid Reactive Substances

T-BHP: Tert-Butyl hydroperoxide

Th2: Type 2 Helper T Cell

TLRs: Toll-Like Receptors

TNF: Tumor necrosis factor

Chapter 1: General context

1.1- Medicinal plants in health

Plants are a valuable source of bioactive natural compounds. Despite the availability of different therapy approaches, natural products remain one of the best reservoirs of new pharmacophore structural types. Standardized plant extracts provide unlimited opportunities for the new drug due to the unmatched availability of chemical diversity (Newman and Cragg, 2020).

Medicinal plants are defined as those used for maintaining health and/or treating specific illnesses. They are used in a variety of ways in both allopathic and traditional practices of medicine in many countries across the world. Even people using only allopathic medicine throughout their lives are likely to be somewhat medicinal plant reliant, since 20% to 25% of drugs prescribed are plant-derived (Smith-Hall et al., 2012). According to the World Health Organisation (WHO), 252 drugs are considered basic and essential for human life and 11% of which are exclusive of plant origin. Furthermore, a significant number are synthetic drugs obtained from natural pioneers (SO et al., 2018).

WHO has estimated that 80% of the world's population relies solely or largely on traditional remedies for health care and there is speculation that more than two billion people may be heavily reliant on medicinal plants (Smith-Hall et al., 2012). As a result, natural products have provided treatment for a large range of disorders including inflammatory, parasitic, neurological, cardiovascular, metabolic, oncological, and pain-related diseases (Soares-Bezerra et al., 2013).

These properties are associated with active compounds present in plants, such as alkaloids, phenols, tannins, cyanogenic glycosides, and terpenoids (SO et al., 2018). These compounds have been used and found effective as sweeteners and anti-bacterial, hence medicinal plants play a great role in the sustainability of human health (SO et al., 2018).

These bioactive compounds are mostly plant secondary metabolites, and many naturally occurring pure compounds have become medicines, dietary supplements, and other useful commercial products. Active lead compounds can also be further modified to enhance the biological profiles and developed as clinical trial candidates (Itokawa et al., 2008).

Therefore, several plants have been studied for their pharmacological activities and compounds of interest and *Alhagi* spp. are some of them. They have been showing promising features, although there is still little information regarding this genus.

1.2- *Alhagi* spp.

Alhagi spp. are plants from the *Fabaceae* family, commonly known as camelthorn, camelthorn bush, Caspian manna, and Persian manna plant. It comprises several species, such as *Alhagi pseudalhagi*, *A. graecorum*, *A. sparsifolia*, *A. kirgisorum*, *A. maurorum*, *A. camelorum,* and *A. persarum* (Saleem et al., 2020). These plants are widely distributed in Asia, Middle East, Europe, Russia, and Africa (Laghari et al., 2011) and are characterized by being undershrub herbs, having 60–100 cm tall, glabrous or pubescent. *Alhagi* are well known in Pakistan, Kashmir, Iran, Afghanistan, Russia, Turkey, Iraq, Syria, Palestine, Cyprus, and Africa, where they have been used in folk medicine by populations over the years (Ahmad et al., 2010; Laghari et al., 2011).

Alhagi spp. antioxidant potential and nutritional value have been explored through time along with various other medicinal properties (Muhammad et al., 2015). Studies report antioxidant property (Olas et al., 2015), anti-cancer activity (Loizzo et al., 2014), immunomodulatory capability (Wusiman et al., 2019), influence in the enlargement of the ureter (Marashdah and Farraj, 2010), benefits for nephrotoxicity (Khalifa et al., 2020), anti-inflammatory and antinociceptive power (Awaad et al., 2011) and neuroprotective potential (Marashdah and AL-Hazimi, 2010), among others.

The main *Alhagi* constituents from the aerial parts, according to the literature, are phenolic compounds, more specifically tannins and flavonoids (Laghari et al., 2012). There were also found triterpenes and unsaturated sterols (Laghari et al., 2012). To a less extent, there are studies confirming the presence of carbohydrates, glycosides, and lactones (Atta and El-Sooud, 2004; Atta and Mouneir, 2004). In their roots, *Alhagi* plants showed a strong presence of flavonoids, saponins, phenolic, and terpenoid derivatives (Saleem et al., 2020).

Overall, this genus is better known for its antioxidant capacity. To date, there are only a few studies concerning the pharmacological and phytochemical properties.

1.3- Neuroinflammation and neurodegenerative diseases

The neuroinflammatory response, excessive oxidation along with mitochondrial dysfunction and DNA damage are risk factors for several degenerative diseases (Hou et al., 2019; López-Otín et al., 2013). For instance, chronic inflammation, persistent activation of microglia, sustained elevation of pro-inflammatory mediators, and increased oxidative stress were seen in brain tissues of patients with Alzheimer´s disease (AD) (Sarlus and Heneka, 2017), Parkinson´s disease (PD) (Hirsch and Hunot, 2009), Huntington´s disease (HD) (Sapp et al., 2001) and spinal cord tissue from patients with Amyotrophic Lateral Sclerosis (ALS) (Henkel et al., 2004). Moreover, aggregated amyloid beta (Aβ) fibrils in AD, mutant α- synuclein (α-syn) in PD, mutant huntingtin in HD, and the superoxide dismutase 1 (SOD1) mutant protein SOD1 in familial ALS are capable of inducing secretion of interleukin (IL)-1β via NLRP3 inflammasome activation (Codolo et al., 2013; Halle et al., 2008; Johann et al., 2015; Ona et al., 1999). Inflammation also exacerbates Aβ deposition in AD, and both α- syn truncation and aggregation in PD, and might induce protein aggregation in HD and ALS (Wang et al., 2016, 2015).

In summary, neuroinflammation together with oxidative stress are major pathophysiological features of neurodegenerative disorders. Anti-inflammatory interventions have, however, been largely ineffective (Hou et al., 2019). Nonetheless, natural bioactive and antioxidant compounds including vitamin E, quercetin, N-acetyl-l-cysteine, curcumin, carotenoids, flavonoids, isothiocyanates, terpenoids, proanthocyanidins, and omega-3 fatty are thought to be neuroprotective and are becoming increasingly relevant as a therapeutic approach for neuroinflammation (Hou et al., 2019).

1.4- Neuroinflammation

The two main categories of cells populating the Central Nervous System (CNS) are the neurons and the glial cells (Kwon and Koh, 2020). Glial cells do not produce electrical impulses and can regulate neuronal activity. These include microglia, which are the inherent resident immune cells in the CNS, and other glial support cells, such as astrocytes and oligodendrocytes (Kwon and Koh, 2020).

In response to injurious stimuli, microglia cells act as the macrophages of the brain and are able to initiate an inflammatory response (Kwon and Koh, 2020). Microglia activation mechanisms begin with the recognition of injurious stimuli, including pathogen-associated and danger-associated molecular patterns

(PAMPs and DAMPs) through a limited number of pattern-recognition receptors, including Toll-like receptor (TLRs), highly expressed in the membrane of these cells (Piovan et al., 2021).

In a similar context to infection, proteins such as Aβ or α-syn in AD and PD, respectively, can bind and activate TLRs, particularly TLR2 (Shabab et al., 2017).

The activated microglia produce proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-16, and chemokines, including the C-C motif chemokine ligand 2 (CCL2) and IL-18. These cytokines help to up-regulate genes encoding for more cytokines, chemokines, and other mediators, through the activation of transcription factors such as nuclear factor kB (NF-kB), signal transducer and activator of transcription-1 (STAT1), and NLRP3 inflammasomes (Kwon and Koh, 2020; Valacchi et al., 2018). The activation of these pathways subsequently induces NADPH oxidase (NOX2), and inducible Nitric Oxide synthase (iNOS), thus contributing to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Cyclooxygenase-2 (COX-2) expression is also induced, which is involved in the generation of prostaglandins (Kwon and Koh, 2020; Shabab et al., 2017; Valacchi et al., 2018). The activation of NF-kB also induces proinflammatory astrocytes that likewise play a role in the inflammatory response (Kwon and Koh, 2020).

These varied mechanisms work in a coordinated way to consent the proper inflammatory resolution by producing bactericidal chemicals (including ROS and RNS), increasing the local blood flow, and activating phagocytic microglia to eliminate dead cells and tissue debris (Kwon and Koh, 2020; Shabab et al., 2017; Valacchi et al., 2018).

In general, the acute inflammatory episodes are terminated by the removal of pathogens and exogenous agents and the inflammatory episode is cleared. Microglia can then switch to a neuroprotective state associated with tissue healing and homeostasis (Kwon and Koh, 2020; Shabab et al., 2017; Valacchi et al., 2018). Anti-inflammatory cytokines (IL-4, IL-10, IL-13, and transforming growth factor-β (TGF-β)) are also involved in the feedback mechanism and activate neuroprotective microglia, leading to the release of diverse factors (FIZZ1, Chitinase-3-Like-3 (Chi3l3), Arginase 1, Ym1, CD206, insulin-like growth factor 1 (IGF-1), and Frizzled class receptor 1) that switch off the inflammatory event (Kwon and Koh, 2020).

In summary, inflammation should be a rapid, intense response aimed at tissue protection, and recovery promptly controlled to avoid detrimental consequences (Valacchi et al., 2018).

1.5- Oxidative and nitrosative stress

Living in an aerobic environment, the generation of potentially noxious oxidants is an inevitable phenomenon (Valacchi et al., 2018). ROS and RNS are involved in an extremely delicate and easily corruptible balance between biological damage and redox signaling (Valacchi et al., 2018). At low concentrations, these are signaling molecules that regulate cell proliferation. On the other hand, at high concentrations, they are key cytotoxic molecules (Shabab et al., 2017).

ROS are multi-potent, diffusible species of chemicals atom or molecules that possess an unpaired electron, so they are capable of carrying out signal transduction processes in response to extracellular stimuli (Shabab et al., 2017). In addition to ROS direct toxic impact on biological macromolecules, they can trigger the inflammatory response by stimulating several genes which are regulating the inflammatory-signaling cascades (Shabab et al., 2017). Neurons have high energy demands to perform neuronal activities and are particularly sensitive to changes in mitochondria function. Mitochondria are an important source of ROS leaked from the electron transport chain while they are susceptible to oxidative damage, leading to mitochondrial dysfunction and tissue injury (Shabab et al., 2017). During its endogenous metabolic reactions, superoxide anion (O_2-.), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and organic peroxides are produced as normal products.

Nitric Oxide (NO) itself is produced by NO synthases, which have three isoforms in the CNS, endothelial (eNOS), neuronal (nNOS) and iNOS, being the last one highly expressed in glial cells under inflammatory conditions (Shabab et al., 2017). Under hypoxic conditions, the mitochondria also produce NO which generates RNS (as NO· and nonradical species as peroxynitrite, ONOO-), that can further generate other reactive species such as malondialdehyde (MDA) through lipid peroxidation (Biswas, 2016; Morgan and Liu, 2011).

Mitochondrial dysfunction is commonly observed in many types of neurodegenerative diseases such as AD, PD, HD, alcohol-related dementia, and brain ischemia reperfusion-related injury (Shabab et al., 2017). Moreover, increased NO levels can stimulate the nitration of many proteins which is reported in the neuronal tissues of patients suffering from the diseases mentioned above (Shabab et al., 2017). Under pathological conditions and after exposure to neurotoxic agents, increased amounts of superoxide anion and NO can be produced, resulting in oxidative and nitrosative stress in the brain (Shabab et al., 2017).

The human body has natural antioxidant defense mechanisms to face oxidative stress composed of biological antioxidants that may be non-enzymatic such as radical scavengers and quenchers (ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, and flavonoids), or enzymatic (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT)) (Assunção et al., 2017).

Although the antioxidant mechanisms of the human body are quite effective, in some disease scenarios they are not enough (Assunção et al., 2017).

1.6- Oxinflammation

As mentioned above, neuroinflammation is switched-off when the feedback mechanism triggers the expression of anti-inflammatory molecules (Valacchi et al., 2018). In some cases, when the trigger that activated the acute inflammatory response persists or, for different causes, the resolution phase is not efficient, a chronic inflammatory state may occur (Valacchi et al., 2018). Exogenous stressors (pollution, smoking, fat-based diet, etc.) or endogen factors (diseases and inflammation itself) are potential able disruptors of this finely tuned homeostasis. They induce the formation of oxidants since one of the most evident features of the inflammatory response is the generation of a pro-oxidative environment due to the production of high fluxes of pro-oxidant species (Shabab et al., 2017). However, not only inflammatory mediators such as the release of cytokines can implement the production of ROS via the activation of specific enzymes present in the cells (NOX, XO, etc.), but also ROS can themselves modulate inflammation (Shabab et al., 2017; Valacchi et al., 2018). For instance, ROS can activate transcription factors involved in the inflammatory process such as NF-kB, and act as second messengers and induce a cascade of events by MAPK activation (p38, JNK, and ERK1/2) which leads to migration of inflammatory cells and therefore augment the inflammatory response (Shabab et al., 2017; Valacchi et al., 2018).

The above-mentioned mutual activation between inflammatory and oxidative stress mediators is named “oxinflammation” (Valacchi et al., 2018). “Oxinflammation” refers to a permanent pro-oxidative feature that interacts, in a positive feedback manner, with a not yet clinically detectable inflammatory process. In a long term, this leads to derangement from the adaptive homeostatic capacities of the organism and eventually inducing or aggravating pathological metabolic dysfunctions such as neurodegenerative diseases (Valacchi et al., 2018).

1.7- Objectives

Initially, the goal of this work was to evaluate the pharmacological potential of *Alhagi graecorum* and *Alhagi maurorum*, regarding their antioxidant, anti-inflammatory, and neuroprotective properties. It was decided to proceed only with *A. graecorum* since it proved to be more promising compared to *A. maurorum*, taking into account the results of preliminary tests carried out with direct methanolic extracts from the leaves of both species. Subsequently, the main tasks of this work were characterizing the phytochemical profile of methanolic extract of the leaves of *A. graecorum*, investigating its *in vitro* antioxidant and anti-inflammatory activity, evaluating its cytotoxicity in BV2 and HepG2 cell lines and assessing the extract neuroprotective effect against incubation with t-BHP in the same cell lines.

Chapter 2: Evaluation of neuroprotective effects of *Alhagi* extracts and compounds: a Systematic Review

Abstract

Alhagi spp. are plants from the Fabaceae family, commonly known as camelthorn. This genus is widely distributed in Asia, Middle East, Europe, Russia, and Africa, where is used in folk medicine by various cultures.

This study aims to do a systematically review of the *Alhagi* bioactivities, as well as their phytochemistry composition. In the search process, PubMed and ScienceDirect databases were utilized, and the keywords "Alhagi" and "Camelthorn" were used. All the studies published until 12th April 2022 were covered, resulting in a total of 834 records. After screening, only 55 articles were accepted for the full read, resulting in 17 articles regarding phytochemistry and 42 regarding bioactivities of *Alhagi*.

This genus has shown a big variety of properties. The percentage of articles regarding each bioactivity found was antioxidant (11%), anti-inflammatory and antipyretic (11%), immunomodulatory (9%), anti-cancer (9%), anti-nephrotoxicity and enlargement of the ureter (9%), neuroprotective and antinociceptive (7%), antimicrobial (5%), cardioprotective (5%), anti-ulcer (5%) and anti-diabetic (4%). The remaining articles approached bioactivities that are less explored. *Alhagi maurorum* is the most studied species, and possibly because of that, is the one that shown to have more bioactivities.

The phytochemistry studies showed a big presence of flavonoids, phenolics and terpenoid derivatives. Some articles confirm the presence of unsaturated sterols, tannins, carbohydrates, saponins, and glycosides, among others.

However, there are still very few articles regarding this genus, so more research is required to confirm and understand better these properties.

2.1- Introduction

Alhagi is a plant genus that belongs to the Fabaceae family, commonly known as camelthorn, camelthorn bush, Caspian manna, and Persian manna plant (Saleem et al., 2020). There are several species of *Alhagi* such as *Alhagi pseudalhagi*, *A. graecorum*, *A. sparsifolia*, *A. kirgisorum*, *A. maurorum*, *A. camelorum,* and *A. persarum*. They have been explored over the years for their antioxidant potential and nutritive value along with various medicinal properties (Muhammad et al., 2015).

These plants are characterized by being undershrub herbs, having 60–100 cm tall, glabrous or pubescent, and are widely distributed in Asia, Middle East, Europe, Russia, and Africa (Laghari et al., 2011). *Alhagi* are

well known in Pakistan, Kashmir, Iran, Afghanistan, Russia, Turkey, Iraq, Syria, Palestine, Cyprus, and Africa, where have been used in folk medicine by populations, (Ahmad et al., 2010; Laghari et al., 2011).

Due to their very deep-reaching root system, plants of this genus can successfully grow in desert and saline areas, and due to this, they prefer dry steppes, gravelly and clayey semi-deserts, and deserts (Nishanbaev et al., 2019).

A large number of bioactivities had been assigned to *Alhagi* plants. There are reports of these plants having antioxidant properties (Olas et al., 2015), anti-cancer activity (Loizzo et al., 2014), immunomodulatory properties (Wusiman et al., 2019), influence in enlargement of the ureter (Marashdah and Farraj, 2010), benefits in nephrotoxicity (Khalifa et al., 2020), anti-inflammatory and antinociceptive capabilities (Awaad et al., 2011), neuroprotective qualities (Marashdah and AL-Hazimi, 2010), among others that are summarized in this systematic review.

These plants are also described to have various compounds of interest, such as tannins, flavonoids, unsaturated sterols/triterpenes, carbohydrates, lactones, and proteins/amino acids (Atta and Mouneir, 2004). *Alhagi* plants were also proved to be a very good source of lupeol (Laghari et al., 2011).

The present systematic review intends to describe and elucidate scientific studies related to the main bioactivities and the phytochemistry of the *Alhagi* genus.

2.2- Methods

2.2.1- Search strategy and data sources

A systematic review of published studies concerning the bioactivities and phytochemistry of *Alhagi* plants was developed following the PRISMA statement guidelines (Liberati et al., 2009; Moher et al., 2009). A literature search was conducted using the PubMed and ScienceDirect databases, covering all studies published before 12th April 2022. The keywords used for a first screening were “Alhagi” and “Camel Thorn”. The search process was carried out using the software EPPI reviewer (https://eppi.ioe.ac.uk/cms/Default.aspx?alias=eppi.ioe.ac.uk/cms/er4) where the total references obtained were pooled together.

2.2.2- Inclusion and exclusion criteria

In this systematic review, it was included only English-language articles addressing the phytochemistry or biological properties of *Alhagi*. Studies involving other plants or investigations with other research directions than those intended, such as ecology, agriculture, plant biology, among others, were excluded. Book chapters, review articles, conference documentation, and articles without abstract were also discarded.

2.3- Results

2.3.1- Literature search

The literature search using the mentioned keywords identified the following number of articles in the respective databases: PubMed (n = 137) and ScienceDirect (n = 697), obtaining a total of 834 articles. After removing duplicates (n = 54), 780 articles remained definitive. The screening was conducted considering the title and the abstract, and through this process, publications that did not satisfy the inclusion criteria were removed. The article search and selection process are summarized in Figure 1. A total of 55 articles remained for complete reading, of which full text was not available in 7, so these were not considered in this study. This process resulted in 17 articles regarding phytochemistry (29%) and 42 articles regarding the pharmacological properties (71%) of the *Alhagi* genus, some of which talked about both topics.

According to the articles extracted from the two mentioned databases, it was found that most of the studies focused on ecology, environmental, causes, and the use of *Alhagi* in agriculture, but only a few studies investigated bioactivities and phytochemistry, as is showed in Figure 2.

Environmental studies focusing mostly on plant communities of protected areas, *Alhagi*'s halophytic properties, monitoring the responses to different stresses of certain ecosystems, species diversity, and adaptation to limitations in growth. Articles regarding agriculture were mainly centred on saline agriculture, on the *Alhagi*'s capacity to alleviate saline stress of the crops, and the properties of soils where these plants grow. In addition, many articles involving ethnobotanical and ethnopharmacological studies were found, in which questionnaires were made to the local population to collect information concerning not only the use of medicinal plants in different regions but also which plants were most commonly used. Was also observed a considerable number of articles concerning plants/microorganisms' associations and identification of microorganisms, as well as the impact on plant growth and soil quality. These excluded research directions are summarized in Figure 2 (B).

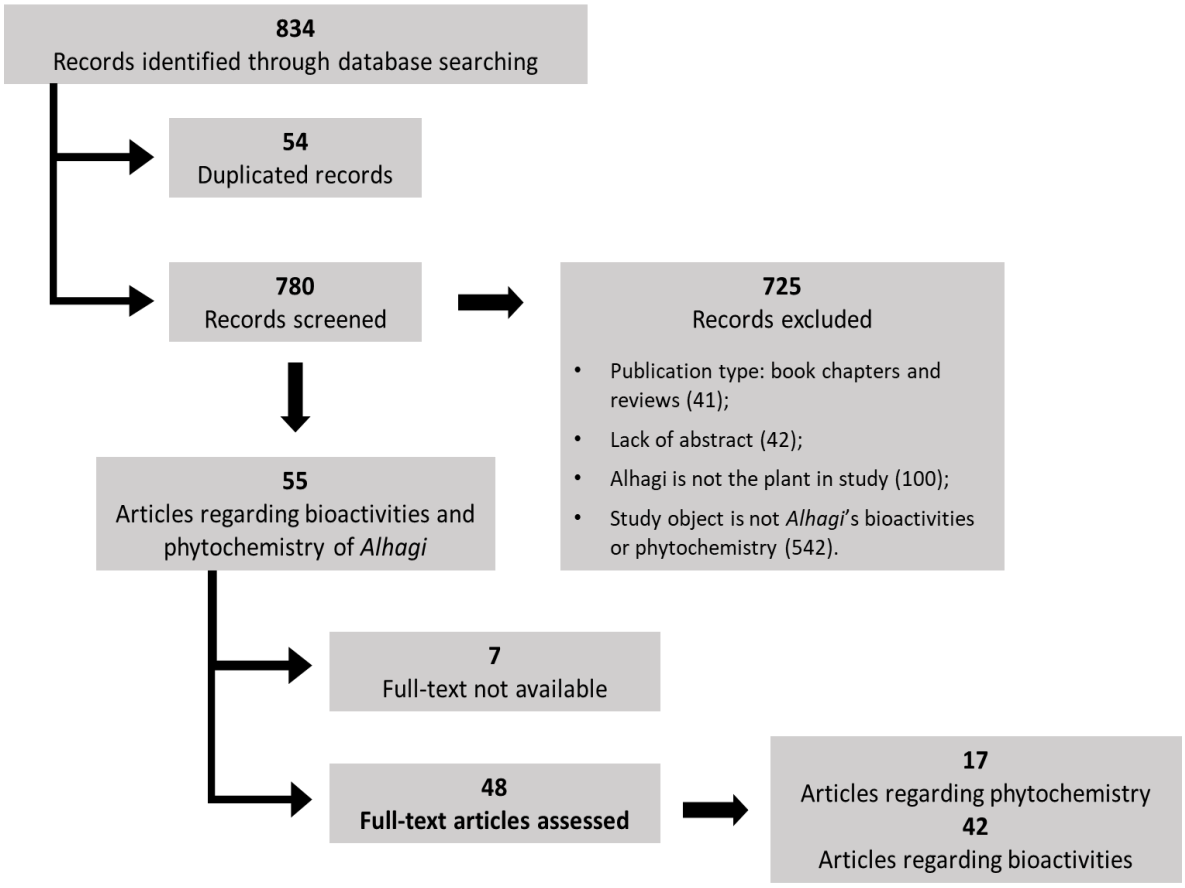

Figure 1. Flowchart of the search process.

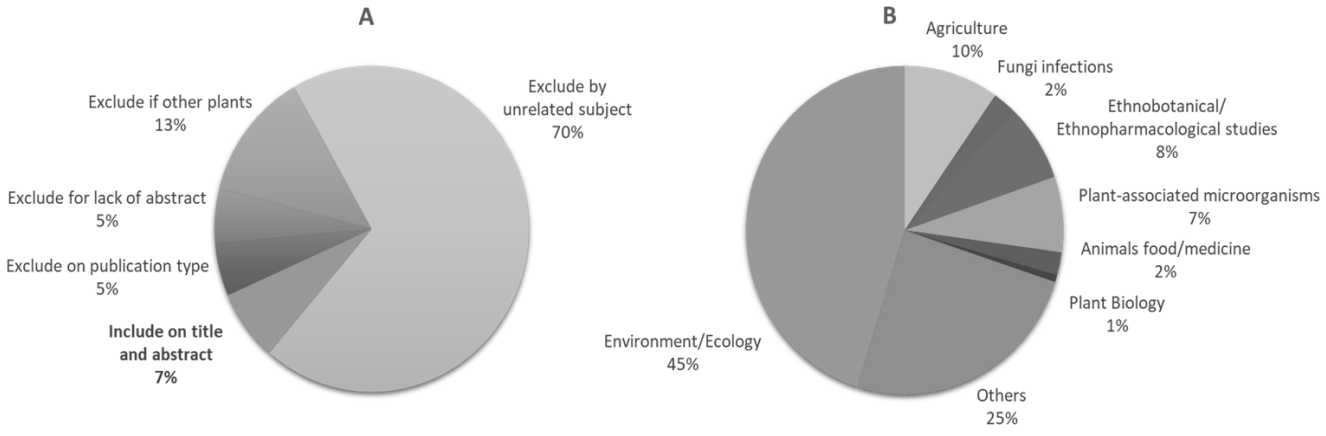

Figure 2. Excluded and included articles during the screening by title and abstract. **A**- Excluded and included percentage of articles, considering exclusion and inclusion criteria. **B**- Percentage of different research directions excluded, within the exclusion criteria "Exclude by unrelated subject".

2.3.2- *Alhagi* bioactivities

Out of the 48 studies included in this revision, 42 were related to different bioactivities exhibited by the *Alhagi* genus. The most mentioned properties were antioxidant, anti-inflammatory and antipyretic, prevention of nephrotoxicity and the benefits to the ureter, anti-cancer, and immunomodulatory. To a less extent, neuroprotective, and antinociceptive properties were also indicated. In a very small number of articles, antimicrobial, cardioprotective, anti-ulcer, hepatoprotective and anti-diabetic activities were also found to be studied. The percentage of studies related to each bioactivity found during the search process of the present systematic review is exposed in Figure 3.

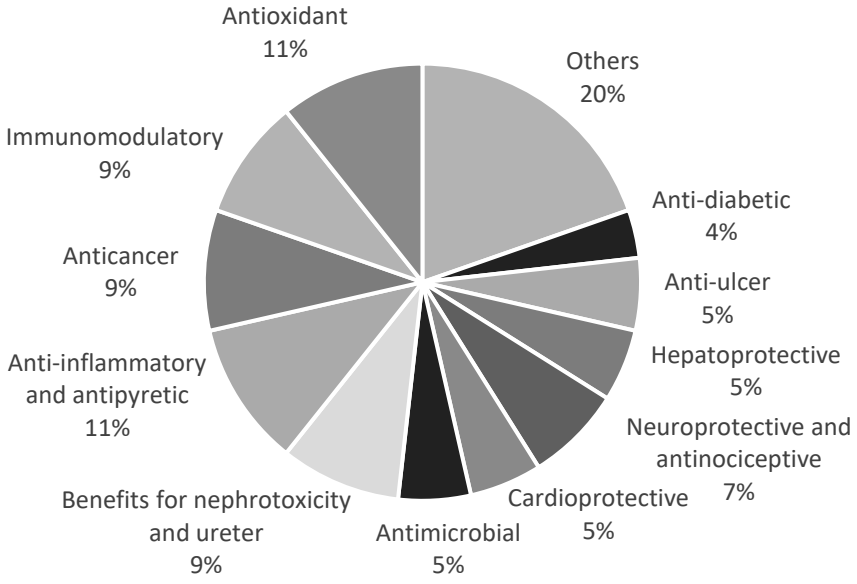

Figure 1. Percentage of *Alhagi* bioactivities studies found in the search process.

a) Antioxidant properties

A higher number of studies focused on the antioxidant properties of *Alhagi*, with a total of 6 articles found. Many other bioactivities are correlated with this property, such as anticancer and neuroprotection since oxidative stress can be a crucial problem in the development of several diseases. *Alhagi maurorum* was the species studied in all the experiments. Most studies proceeded to biochemical assays, such as DPPH assay, ABTS free radical scavenging, ferric reducing antioxidant assay (FRAP), CUPric reducing antioxidant capacity (CUPRAC), total antioxidant capacity (TAC), and metal chelating activity (MCA) of different types of extract.

The research of Olas *et al.* (2015) was the only one found that used cell cultures of human blood platelets treated with hydrogen peroxide to evaluate this feature.

Overall, the results suggest that *A. maurorum* has antioxidant properties, probably due to the presence of some antioxidant compounds, like phenolics. This plant had good outcomes in a variety of biochemical tests and experiments. The articles found, as well as their methodologies and results, are summarized in Table 1.

b) Anti-cancer activity

Five articles regarding the anti-cancer activity of *Alhagi* plants were found. Three of them studied *A. maurorum* and two of them *Alhagi pseudalhagi*. Unlike antioxidant properties, in this subject, most of the research was performed using cell cultures *in vitro*, specifically different human tumor cell lines. MTT assay and IC50 were the main parameters used to evaluate the activity of different extracts, but the induction of apoptosis by the expression levels of p53, BCL-2, Bax, and caspase-3 genes, and the quinone reductase (QR) inducing activities were also assessed by some studies.

According to the results, *Alhagi* have shown good antiproliferative and cytotoxic effects on cancer cell lines, with good IC50 values. Also, compounds of interest for the development of therapeutical approaches in cancer disease were revealed to exist in these plants. The articles found, as well as their methodologies and results are summarized in Table 3.

c) Immunomodulatory

Five articles were found that investigated the immunomodulatory capabilities of *Alhagi* plants, all of them using *Alhagi* polysaccharides for their experiments. Most of the studies used *in vivo* and *in vitro* assays, performing experiments with mice and with isolated tissues from them. One of the studies investigated the effect of these polysaccharides on the activity of RAW264.7 mouse macrophage cells (Li et al., 2018). The other studies used cationic polymer modified PLGA nanoparticles encapsulating *Alhagi* honey polysaccharides to study the effects of these polysaccharides on mice immune systems.

All the findings come into agreement that *Alhagi* polysaccharides can modulate significantly the immune system. Three of the articles found also proved that PEI-AHPP has the potential to be an effective adjuvant to induce strong and long-lasting Th1 and Th2 mixed immune responses. The articles found, as well as their methodologies and results are summarized in Table 5.

d) Nephrotoxicity and Ureter

The benefits of *Alhagi* interactions in the excretory system were also explored. There were found 5 articles and all studied in *A. maurorum*.

All the articles found proceed to *in vivo* assays. Three articles studied very similarly the effects of this plant on the ureter, using Guinea-pigs. Two of them used different extracts of the plant and one used an isolated compound. Regarding anti-nephrotoxicity properties, the two studies used rats. One used cisplatin-induced nephrotoxicity in rats, and the other used norfloxacin-induced side effects in rats.

According to all the results, *A. maurorum* can induce relaxation and enlargement of the ureter, as well can prevent, and treat different situations that cause toxicity in the kidney. The articles found, as well as their methodologies and results are summarized in Table 9.

e) Anti-inflammatory and antipyretic

Concerning the anti-inflammatory capacities of *Alhagi* plants, were found two articles that investigated this effect, both with *A. maurorum*. This property was specifically evaluated in these studies, but some other bioactivities such as anti-ulcer or neuroprotective may be also correlated to the anti-inflammatory properties of these plants, as indicated by some articles presented in other sections of this review.

One of the studies performed *in vivo* assays in adult albino rats by the carrageenan-induced paw edema method (Awaad et al., 2011), and the other one executed biochemical assays that showed the presence of a very active compound with a large anti-inflammatory capacity, named lupeol (Laghari et al., 2011).

Both these studies revealed and come into agreement that this plant has, in fact, anti-inflammatory activities, as well as an active compound, lupeol, that can be explored by the pharmaceutical industry.

Four articles related to the *Alhagi* antipyretic activities were also found, where three of them analysed *A. maurorum* and one *Alhagi hedysurum*. All these studies performed *in vivo* assays, two of them with rats, other with mice, and lastly one with rabbits.

Summing up, most assays performed showed no significant antipyretic properties, unless the study of (Marashdah and Farraj, 2010) that showed positive results. Additional research is needed to draw more

accurate conclusions on this subject. The articles found, as well as their methodologies and results are summarized in Table 7.

f) Neuroprotective and antinociceptive

Four articles were obtained in the research process that investigated the benefits of *Alhagi* in neurological conditions, as well antinociceptive effects. Three of them used *A. maurorum* and one of them used *Alhagi sparsifolia* for their experiments.

The two studies concerning antipyretic properties were performed with *in vivo* assays in mice. One of them used the writhing and the hot plate tests (Awaad et al., 2011), and the other one used writhing and tail-flick tests (Atta and El-Sooud, 2004).

The studies of (Marashdah and AL-Hazimi, 2010) and Zhou *et al.* (2017) were centred on other neurological effects of *Alhagi*, and both performed *in vitro* assays. The first one utilized frog tissue. The second one utilized LPS-induced N9 cells and was the only one using *A. sparsifolia* for the experiments.

Considering all the results, it´s possible to assume that *A. maurorum* can perform antinociceptive effects. Also, *Alhagi* plants showed to have interesting neuro-potentialities to be more explored and compounds that are very powerful in inhibiting neuronal inflammation.

The articles found, as well as their methodologies and results are summarized in Table 10.

g) Antimicrobial

There were also found three articles exploring the antimicrobial activities of *Alhagi* plants, in which two of them are concerning *A. maurorum* and one focused on *Alhagi canescens.* All the experiments were performed with *in vitro* assays, in which a screen for the antibacterial and antifungal activity of extracts was made for different bacteria and fungi species.

Considering all the data is possible to assume that *Alhagi* plants have antimicrobial activities, but very low antifungal activity. Also, this activity can be higher or lower, depending on the species of bacteria. The articles found, as well as their methodologies and results are summarized in Table 8.

h) Cardioprotective

The effects on the heart and blood circulation of *Alhagi* were also investigated by three studies obtained in this research. All of them utilized *A. maurorum* and carry out *in vivo* studies with rats. The articles found, as well as their methodologies and results are summarized in Table 2.

It seems that *A. maurorum* have benefits on the cardiovascular system, especially in reducing cardiac output and decrease peripheral vascular resistance, preventing atherosclerosis and heart failure. These capacities are very useful for many illnesses associated with the cardiovascular system.

i) Anti-ulcer

Three studies discussing the properties of *Alhagi* plants for the treatment of different types of ulcers were found, and all of them used *A. maurorum* for their experiments. Two of these studies performed *in vivo* assays, one with human patients and the other with rats. The last one performed biochemical assays to evaluate the urease-inhibition activity of one isolated flavanenol.

With the outcomes of these experiments, is feasible to believe that *A. maurorum* can treat ulcers in a way equivalent to that of drugs for that purpose, and this may be associated partially with its flavonoids with urease-inhibitory effect and with its anti-inflammatory properties. The articles found, as well as their methodologies and results are summarized in Table 4.

j) Anti-diabetic

Two different articles were found regarding the effects of *Alhagi* plants in diabetes. Both these studies performed *in vivo* assays in Streptozotocin-induced Diabetes Mellitus in rats, but one of them with *A. maurorum* and the other with *Alhagi camelorum*.

Through these two studies, both *Alhagi* species showed interesting effects on diabetes mellitus rats, suggesting its potentialities to decrease hyperglycaemia. However, is a very reduced number of studies, so more research is needed to confirm these outcomes and go even further analysing these properties. The articles found, as well as their methodologies and results are summarized in Table 6.

2.3.3- *Alhagi* phytochemistry

Out of the 48 studies included, 17 were related to the phytochemistry of *Alhagi* plants and their different extracts. Within these studies, the most used were ethanolic and methanolic extracts, and in less extend chloroform or ethyl acetate extracts. Essential oils and polysaccharide extracts were also studied. It is important to underline that some of these extracts are produced from aerial parts and others from roots. *A. maurorum* (10 studies) was the most analysed, but there were also articles concerning *A. pseudalhagi* (4 studies), *A. sparsifolia* (1 study) and *A. canescens* (1 study). Additionally, was found one article investigating Alhagi-honey.

All the studies involved the isolation of compounds including flavonoids, saponins, phenolic and terpenoid derivatives, aliphatic esters and flavanols. Some articles also confirm the presence of unsaturated sterols, triterpenes, tannins, carbohydrates, and glycosides, among others.

The articles found regarding phytochemistry analyses of *Alhagi*, as well as their methodologies and results are summarized in Table 11.

2.4- Conclusion

Overall, *Alhagi* plants have proven to have a vast number of bioactivities, as well compounds that may be interesting pharmacologically or for forthcoming studies. Despite this, it is a genus that has not been very studied, as we can see by the number of included articles for this systematic review. *A. maurorum* is, no doubt, the most investigated species of the *Alhagi* genus, and probably due to that, it´s the one that showed a greater number of properties and potentialities.

With all the data compiled in this systematic review, it is clear that this group of plants have interesting properties and a lot of potential to be explored in a substantial variety of conditions. However, these bioactivities have not yet been much explored., so it is necessary to go through more research to better understand and go beyond the information already established.

Table 1. Studies concerning the antioxidant properties of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Antioxidant**	Biochemical assays	Leaf aqueous extract green-synthesized zinc nanoparticles.	DPPH test.	Zinc nanoparticles have an effective antioxidant feature (Chinnathambi and Alahmadi, 2021).
			Methanol and dichloromethane (DCM) extracts of roots.	DPPH, ABTS, FRAP, CUPRAC, TAC and MCA.	Methanol extract exhibited the highest radical scavenging, reducing power capabilities and antioxidant capacity than the DCM extract (Saleem et al., 2020).
			Properties of leaves and flowers compared with quercetin.	DPPH assay.	The extract of leaves exhibited a higher scavenging capacity than the extract of flowers, followed by quercetin (Laghari et al., 2012).
			Diethyl ether (DE) and petroleum ether (PE) extracts.	DPPH, ABTS, carotene bleaching test, FRAP and Fe-chelating activity assay.	DE extract showed the highest radical scavenging activity against ABTS radical. It was also able to reduce the metal transition ion iron with a FRAP value of 69.6 µM Fe(II)/g that is comparable to the positive control butylated hydroxytoluene (63.2 µM Fe(II)/g) (Loizzo et al., 2014).
			Methanolic extract of air-dried whole plant material.	DPPH assay.	Isorhamnetin-3-O-[-α-L-rhamnopyranosyl-(1-3)]-β-D-glucopyranoside and 3'-O-methylorobol exhibit moderate antioxidant activity (Ahmad et al., 2010).
		In vitro assays	Phenolic fraction from roots in human blood platelets and plasma after treatment with hydrogen peroxide (H2O2).	Levels of protein thiol groups. Concentration of thiobarbituric acid reactive species.	May protect proteins/lipids against oxidative modifications, but the mechanism of action is not known, and had stronger antioxidant properties than known antioxidants (aronia and Y. schidigera extracts), but these differences were not statistically significant (Olas et al., 2015).

Table 2. Studies regarding cardioprotective properties of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Cardioprotective**	*In vivo* assays	Ethanolic extract (EE) of powdered roots.	Rats anaesthetized with urethane. ECG.	The intraperitoneal administration of EE decreased heart rate by 22.5%, and at a dose of 1 g/kg induced bradycardia only and not myocardial depressant (Marashdah and AL-Hazimi, 2010).
			2% aqueous acetic acid extract of powdered roots.		Intraperitoneal administration of the extract decreased the heart rate by 10% and depressed the force of contraction of the heart by 66.7%. The extract at a dose of 1 g/kg not only was bradycardiogenic but it also was myocardial depressant (Marashdah and Farraj, 2010).
			Ethanolic extract.	Older rats and rats treated with NO-synthase inhibitor.	The intake of extract with drinking water reduced the activity of angiotensin-converting enzyme and this effect increased with increasing the dose. In both types of rats ACE decreased to the values observed in young control rats at extract concentration of 0.2%. The extract was not inferior to taxifolin in preventing the early stages of aortic atherosclerosis (Arutyunyan et al., 2014).

Table 3. Studies regarding anti-cancer activities of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Cancer**	*In vitro* assays	Diethyl ether (DE) and petroleum ether (PE) extracts.	Sulforhodamine B (SRB) assay. MCF7, ACHN, C32, A375, LNCaP, HeLa and COR-L23 human tumor cell lines and one normal cell line, 142BR.	DE extract showed promising IC50 values against C32 and COR-L23 cell lines. PE extract showed an interesting activity against the human Caucasian lung large cell carcinoma (COR-L23). Both extracts showed moderate activity against renal cell adenocarcinoma (ACHN). DE inhibited hormone-dependent prostate carcinoma LNCaP (Chinnathambi and Alahmadi, 2021).
			Leaf aqueous extract green-synthesized zinc nanoparticles.	MTT assay. Normal (HUVEC) and bone malignancy cell lines (G-292, clone A141B1, MG-63, HOS, Hs 707(A).T, and Saos-2).	The viability of malignant bone cell line reduced dose-dependently in the presence of leaf aqueous extract green-synthesized zinc nanoparticles. The best results of cytotoxicity are in the case of HOS without any cytotoxicity against normal cell line, HUVEC (Loizzo et al., 2014).
			Methanolic extract of powdered aerial parts.	MTT assay, NMR, PCR. Breast cancer cell lines (MCF-7 and MDA-MB-231) and normal breast cell line (MCF 10A).	An active fraction was detected as lupeol. The cytotoxic activity of lupeol epoxide was significantly more than lupeol. The mRNA expression level of p53, caspase-3 and bax genes were increased in both cancer cells treated with the pure compound. However, bcl-2 gene expression decreased in treated breast cancer cells (Behbahani, 2014).
Alhagi pseudalhagi		Biochemical and *in vitro* assays	Chloroform extracts from the air-dried aerial part.	NMR, MS, IR, UV and CD. Hepa 1c1c7 wild-type hepatoma cell lines (ATCC).	A new isoflavonolignan was isolated and named pseudalhagin A. This compound exhibited moderate quinone reductase (QR) inducing activity for hepa lclc7 cells, with IR value 2.3 (Li et al., 2010).
		In vitro assays	Methanol extracts from aerial sections.	MTT assay. Human leukemia cell lines: K562, Jurkat and Raji. Solid tumor cell lines: Fen and HeLa.	Except for the K562 cell line, this extract showed no significant effects on the different cell lines. The highest percentage of inhibition was (44.2 ± 0.3%) at 200 µg/mL. The IC50 was calculated as 224 µg/mL (Esmaeilbeig et al., 2015).

Table 4. Studies regarding anti-ulcer activities of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Anti-ulcer**	*In vivo* assays	Camel thorn distillate.	Human patients with oral aphthous ulcers.	Complete resolution time ranged from 3 to 7 days in the group treated with distillate, whereas in the control group, ranged from 7 to 14 days. In the test group, the severity of pain and diameter of the lesion reached zero by the fifth day, whereas in the control group, these values did not reach zero until the fourteenth day (Pourahmad et al., 2010).
			Ethanolic extract.	Rats administrated with aspirin.	Acid output was decreased for plant extract followed by ranitidine. No ulcer patterns have been shown in the histopathological study (Shaker, Mahmoud and Mnaa, 2010).
		Biochemical assays	Flavanenol from the ethyl acetate fraction of roots.	Enzymatic colorimetric method.	The compound showed 24% greater enzyme inhibition activity as compared to that of protocatechuic acid (Laghari et al., 2010).

Table 5. Studies regarding immunomodulatory activities of *Alhagi* plants found in the search process of this review.

	Bioactivity	Models and methodologies			Main results
Alhagi honey polysaccharide (AHP)	**Immunomodulatory**	*In vitro* assays	SAP-1 (50% alcohol precipitation) and SAP-2 (80% alcohol precipitation).	RAW264.7 cells. MTT assay, Griess method, neutral red uptake assay and ELISAs.	SAP increased the proliferative activity and activated the immune function of cells and was lacking in cytotoxicity. SAP-1 exhibited a stronger overall effect in all the measure parameters than did SAP-2. SAP can enhance the immune activity of mouse macrophages (Li et al., 2018).
		In vitro and *in vivo* assays	Cationic polymer modified PLGA nanoparticles encapsulating AHP.	ICR mice and peritoneal macrophages isolated and purified from ICR mice.	AHPP significantly stimulated phagocytic activity, MHCII and CD86 expression in macrophages. AHPP/OVA significantly enhanced lymphocyte proliferation and improved the CD4+ /CD8+ T cell ratio. This treatment significantly increased IgG levels and up-regulated Th-associated cytokines with overall Th1 polarization (Wusiman, Xu, et al., 2019).
		In vitro assays		Peritoneal macrophages isolated and purified from ICR mice.	Nanoparticles significantly stimulated phagocytic activity, MHCII+, CD86+, and CD80+ expression. The levels of NO, iNOS, TNF-α, IFN-γ, IL-1β and IL-12 were enhanced. PEI-AHPP showed the best effects on the expression of co-stimulatory molecules, and secretions of NO, iNOS, and cytokines (Wusiman, He, et al., 2019).
		In vitro and *in vivo* assays		ICR mice. Splenic lymphocytes isolated from immunized mice.	H5N1-loaded PEI-AHPP formulation could induce highest hemagglutination inhibition (HI) titer, IgG-subtype, and cytokines, activated dendritic cells (DCs) in lymph nodes, and CD3e+CD4+ and CD3e+CD8a+ T cells in the spleen of immunized mice. PEI-AHPP could stimulate DCs to highly express MHCI and MHCII molecules and had good antigen slow-release effect at the injected site along with lymph node targeting (Wusiman et al., 2021b).
			Chitosan modified PLGA nanoparticles, polyethyleneimine modified PLGA nanoparticles, and ε-Poly-L-lysine modified PLGA nanoparticles.		The antigen loading capacity and stability of nanoparticles were improved by the surface cationic polymers modification, and these formulations generated a strong Th1-biased immune response. Among them, polyethyleneimine modified PLGA nanoparticles induced the strongest Th1-biased immune response (Wusiman et al., 2021a).

Table 6. Studies regarding anti-diabetic activities of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Anti-diabetic**	*In vivo* assays	Aqueous and ethanolic extracts.	Streptozotocin-induced Diabetes Mellitus rats.	Both extracts decreased elevated blood glucose levels and hyperlipidemia and suppressed oxidative stress caused by diabetes mellitus (Sheweita et al., 2016).
Alhagi camelorum			Alcoholic extract.		The alcoholic extract decreased the rate of hyperglycaemia and hyperlipidaemia associated with diabetes (Nabiyouni, Vaezi and Malekirad, 2019).

Table 7. Studies regarding anti-inflammatory and antipyretic properties of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Anti-inflammatory**	Biochemical assays	Methanolic extract of the air-dried root barks.	Liquid chromatography–mass spectrometry method.	Lupeol, a bioactive triterpenoid, has been isolated in considerable quantity. The anti-inflammatory property of A. maurorum can be correlated to this compound and can be used as a cheaper and ever-available source for the lupeol (Laghari et al., 2011).
		In vivo assays	Ethanolic extract.	Adult albino rats. Carrageenan-induced paw edema method.	The standard drug diclofenac sodium (30 mg/kg) and the total ethanol extract (1000 mg/kg) produced significant reduction of paw edema, as compared to the control rats. Also, the activity of the tested extract was higher than that of the standard drug (Awaad et al., 2011).
	Antipyretic		2% aqueous acetic acid extract.	Administration of the extract intraperitoneally in mice.	Administration of the extract induced time and dose dependent decreases in the rectal temperature. Doses of 0.5 g and 1 g/kg produced decreases of 4.3 and 7.3 ^{o}C, respectively (Marashdah and Farraj, 2010).
			Ethanolic extract.		Administration of the extract did not induce any changes in the rectal temperature. Administration of the extract in doses of 1 g/kg decreased the body temperature, but the temperature started to rise again (Marashdah and AL-Hazimi, 2010).
				Orally administered. Brewer's yeast-induced hyperpyrexia in rats.	Results presented no antipyretic activity comparable to the standard drug diclofenac sodium (Awaad et al., 2011).
Alhagi hedysurum				Yeast-pyrexic rabbits.	Used aspirin as a reference. The results showed no obvious antipyretic activity (Ikram et al., 1987).

Table 8. Studies regarding antimicrobial properties of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Antimicrobial**	*In vitro* assays	Nanoemulsion of essential oil	Agar well plate method. 6 antibiotic-resistant pathogenic bacteria.	Had higher inhibition against bacteria compared to free essential oil and had antibiofilm activity. MIC values were highest for A. baumannii and lowest for E. coli. Also had a remarkable effect the curing of R-plasmid of three antibiotic-resistant bacteria (Hassanshahian, Saadatfar and Masoumipour, 2020).
			Chloroformic and methanolic extracts	Disc diffusion method. 8 bacteria and 3 fungi.	Both extracts indicated antibacterial activity against all tested bacteria pathogens. Chloroformic extract had no antibacterial activity on B. subtilis. Methanolic extract presented the maximum antibacterial activity against X. campestris. None of the tested extracts had antifungal activity (Hadadi, Nematzadeh and Ghahari, 2020).
Alhagi canescens			Hexane, benzene extracts and essential oils	Modified agar diffusion method. Bacteria and fungi.	Benzene extract possessed the highest antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. Only, EO possesses a weak activity against fungi *C. albicans* (Nishanbaev et al., 2019).

Table 9. Studies regarding the benefits for nephrotoxicity and ureter of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Benefits for nephrotoxicity and ureter**	*In vivo* assays	Ethanolic extract (EE) of powdered roots.	Guinea-pigs. Addition of histamine to the isolated ureter.	Addition of histamine induced continuous contractions. The addition of the extract completely suppressed it and addition of another dose of histamine did not reverse it (Marashdah and AL-Hazimi, 2010).
			Glyceryl-n-tetracosan-17-ol-1-oate, isolated from ethanol extract of the roots.		This compound was found to enlarge and induce relaxations to the ureter and suppressed histamine induced spasms (Marashdah, 2014).
			2% aqueous acetic acid extract of powdered roots.		The addition of the extract completely suppressed histamine induced contractions and the addition of another dose of histamine did not reverse the inhibition effect (Marashdah and Farraj, 2010).
			Ethanolic extract of aerial parts.	Cisplatin-induced nephrotoxicity in rats.	The extract reduced the level of the renal function markers as well as the levels of sodium/potassium increased by cisplatin. Cisplatin also induced pathological damage in kidney, whereas treatment with this agent improved this condition (Changizi-Ashtiyani et al., 2016).
			Aqueous extract.	Norfloxacin-induced side effects in rats.	The extract significantly ameliorated norfloxacin-induced elevation in tissue malondialdehyde, and reduction in tissue antioxidant enzymes as well as reduced glutathione concentration. Concurrent administration of extract with norfloxacin significantly reduced serum alkaline phosphatase, aminotransferases, urea, creatinine, and uric acid and increased RBCs count, Hb concentration, PCV, leucocyte, and monocyte counts. Co-administration of extract with norfloxacin prevented the degenerative changes induced by norfloxacin alone in liver and kidney (Khalifa, Shalaby and Abdelaziz, 2020).

Table 10. Studies regarding antinociceptive and neuroprotective properties of *Alhagi* plants found in the search process of this review.

Specie	Bioactivity	Models and methodologies			Main results
Alhagi maurorum	**Antinociceptive**	*In vivo* assays	Ethanolic extract of powdered aerial parts.	Writhing and the hot plate tests in albino mice, peripherally and centrally.	The analgesic activities induced by the high dose of the extract were more effective than that induced by the standard drug metamizol. The hot plate result showed significant reduction of pain as compared to control (Awaad et al., 2011).
			Methanolic extract.	Acetic acid-induced writhing and tail-flick test in mice.	Oral administration of 400 mg kg−1 extract significantly inhibited the nociception to acetic acid-induced writhes with a protection of 85.5–61.3%. In the tail-flick test, in the same dose, produced a significant increase in the latency to response of tail to thermal stimulation (Atta and El-Sooud, 2004).
	Neuroprotective		Ethanolic extract (EE) of powdered roots.	Exposure of the frog's rectus abdominis muscle to the extract.	EE blocked the action of the neurotransmitter acetylcholine. The maximum reversal of antagonism was 27.7, suggesting that the extract blocked the action of ACh in a non-competitive manner (Marashdah and AL-Hazimi, 2010).
Alhagi sparsifolia		*In vitro* assays	Ethanolic extract.	MTT method and NO assay in LPS-induced N9 cells.	Syringgaresinol (IC50 2.68 μM) and 1, 3, 3, 4- tetramethylcyclopentene (IC50 2.63 μM) could be good leading compounds for the development of potential therapeutic agents against neurodegenerative diseases (Zhou et al., 2017).

Table 11. Studies regarding the phytochemistry of *Alhagi* plants found in the search process of this review.

Specie	Extracts	Methods/Assays	Results	Author
Alhagi maurorum	Methanol and dichloromethane root extracts.	UHPLC-MS analysis.	High total phenolic and flavonoid content. 18 secondary metabolites were identified, including flavonoids, saponins, phenolic and terpenoid derivatives.	(Saleem et al., 2020)
	Methanolic flowers and leaves extracts.	HPLC-DAD and LC-MS-APCI. Modified Folin–Ciocalteu reagent method.	The total phenolic contents of the leaves were higher than those of the flowers. The number of phenolic acids as well as their quantity are low in the extract of leaves compared to flowers.	(Laghari et al., 2012)
	Methanolic extract of whole plant.	CC, Mass spectrometry, 1D and 2D NMR spectroscopy.	Isorhamnetin-3-*O*-[-a-L-rhamnopyranosyl-(1-3)]-β-D-glucopyranoside, 3'-*O*-methylorobol and quercetin 3-*O*-β-D-glucopyranoside were isolated.	(Ahmad et al., 2010)
	Ethanol extract of the roots.	CC and TLC. ^{1}H, ^{13}C, 2D NMR and mass spectrometry.	Isolation of a new aliphatic ester, named glyceryl-*n*-tetracosan-17-ol-1-oate.	(Marashdah, 2014)
	Methanolic extracts of aerial parts.	TLC, KOH, UV.	The presence of unsaturated sterols, triterpenes, tannins, flavonoids, and carbohydrates and/or glycosides was confirmed. The tests for alkaloids, anthraquinones, lactones/esters, protein/amino acids and saponins were negative.	(Atta and El-Sooud, 2004)
			Results were positive for tannins, flavonoids, unsaturated sterols/triterpenes, carbohydrates, lactones, and proteins/amino acids. Traces of saponins were detected. Alkaloids and anthraquinones were not found.	(Atta and Mouneir, 2004)
	Diethyl ether (DE) and petroleum ether (PE) extracts of aerial parts.	GC–MS.	DE extract: presence of fatty acids. The main constituents were oleic acid, ethyl linoleate, myristic acid, ethyl oleate, and coumarins (Columbianetin, osthol and xanthotoxin). PE extract: presence of alkanes and some fatty acids. Abundant: hexatriacontane, tritriacontane and triacontane.	(Loizzo et al., 2014)
	Ethanolic extract of aerial parts.		Three flavone structures (2-phenyl-1,4-benzopyrone derivatives) with rate more than 50%.	(Shaker et al., 2010)
	Methanol extract from roots.	EI-MS, ^{1}H NMR and ^{13}C NMR, LC–MS-APCI.	Lupeol, a bioactive triterpenoid, has been isolated in considerable quantity.	(Laghari et al., 2011)
	Ethyl acetate extracts of roots.	^{1}H NMR and ^{13}C NMR, IR, ESI-MS, UV, TLC, CC.	A flavanenol was isolated and was determined to be 5,6,7,8,2',3',5',6'-octamethoxyflavan-3-en-4'-ol.	(Laghari et al., 2010)
	Crude extract, ethyl acetate fraction and n-butanol fraction of aerial parts.	UPLC-PDA-UV, UPLC-PDA-MS/MS.	The major phenolic compound was isolated from the ethyl acetate fraction, identified as isorhamnetin-3-O-rutinoside and quantified as 262.91 0.57 g/mg of the fraction. Analysis of this fraction also revealed tentative identification of 25 polyphenolic compounds.	(El-Zahar *et al.*, 2022)

Alhagi pseudalhagi	Chloroform extracts of aerial parts.	HR-ESI-MS, ^{1}H and ^{13}C NMR, CD, HMBC and UV.	Isolation of a isoflavonolignan named pseudalhagin A, with the structure 8-(3-hydroxy-4-methoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl methanol)-2,3-dihydro-7*H*-1,4-dioxino[2,3-*h*]chrom-7-one. Pratensein, calycosin, 3′,7-dihydroxyl-4′,8-dimethoxylisoflavone, formonoetin and ononin were also isolated.	(Li et al., 2010)
	Ethanol extracts of epigeal parts.	Monosaccharide, GC–MS, and NMR analyses. HPGPC, MALDI-TOF-MS.	The structure of a polysaccharide, APP90-2, was elucidated. APP90-2 interacted with Congo-red and had an obvious honeycomb structure. APP90-2 consisted of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, and arabinose.	(Ye et al., 2021)
	Ethanol extract, petroleum ether extract and chloroform extract.	Chemical transformation and spectral (UV, IR, PMR and mass).	Five β-phenethylamine and one tetrahydroisoquinoline alkaloids isolated and identified as β-phenethylamine, N-methyl-β-phenethylamine, hordenine, 3,4-dihydroxy-β-phenethyltrimethylammonium hydroxide, N-methylmescaline and salsolidine.	(Ghosal and Srivastava, 1973)
	Methanolic extract of whole plant.	CC, TLC, PC, ^{1}H NMR, ^{13}C NMR, TMS, EIMS and FAB-MS.	Two flavanone glycosides: alhagitin and alhagidin. Their structures are respectively naringenin 5-methyl ether 4'-glucoside and hesperitin 7-galactosyl(1-2)[rhamnosyl(1-6)].	(Singh et al., 1999)
Alhagi-honey	Polysaccharide extract (PE).	Macroporous adsorption resin chromatography, DEAE cellulose chromatography, and Sephadex gel chromatography. Gel filtration.	AP1-1 is a homogeneous polysaccharide. It was found that its molecular structure is composed of mannose, glucose, galactose, and galacturonic acid with a molar ratio of about 1.1:1.9:3.9:2.1.	(Jian et al., 2014)
Alhagi sparsifolia	Ethanol extract of aerial parts.	Bio-guided fractionation and isolation by means of chromatographic methods and recrystallization.	15 flavonoids: isorhamnetin, quercetin, 3′, 7-dihydroxyl-4′, 8-dimethoxylisoflavone, formonoetin, 3′, 7-dihydroxyl-4′-methoxylisoflavone, 3′, 7-dihydroxyl-4′, 6-dimethoxylisoflavone, 3′, 4′, 7-trihydroxylisoflavone, Butin, quercetin-3-O-(2-β-D-xylopyranosylrutinoside, Typhaneoside, quercetin-3-O-(2′′, 6′′-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside, rutin, kaempferol 3-O-β-D-rutinoside isorhamnetin 3-O-β-D-rutinoside, quercetin-3-O-β-D-glucopyranoside; 6 lignans and 8 others.	(Zhou et al., 2017)
Alhagi canescens	Volatile compounds of hexane, benzene extracts and essential oils (EOs) from aerial parts.	Steam- and hydrodistillation methods. GC-MS analysis.	Palmitic acid, neophytadiene, cis-chrysanthenyl acetate and cis-geranyl acetate were the main components in the hexane and benzene extracts. Camphor, bicyclogermacrene, α-copaene, (-)-germacrene D and eucalyptol were the main components in the EOs.	(Nishanbaev et al., 2019)
Alhagi mannifera	Petroleum ether, methylene chloride, chloroform, acetone, and methanol extracts.	Spectral analysis.	A total of 165 compounds were identified in different extracts. Petroleum ether extract: 55 compounds; methylene chloride extract: 11 compounds; chloroform extract: 23 compounds, acetone extract: 47 compounds and methanol extract: 29 compounds.	(Jaradat *et al.*, 2021)

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Chapter 3: Phytochemical evaluation, antioxidant screening and anti-inflammatory evaluation of *Alhagi graecorum* methanolic extract

3.1- Introduction

Oxidative stress is a concept widely used in medical sciences in the past three decades. It holds an important role in a variety of common illnesses, among them neurodegenerative diseases like Alzheimer's and Parkinson's, since the occurrence of degenerative processes is correlated with the existence of an oversupply of free radicals, promoting oxidative processes that are damaging to the body (Aruoma, 1998; Munteanu and Apetrei, 2021).

Reactive Oxygen Species (ROS) are free radicals that include all highly reactive oxygen-containing molecules, such as hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, and various lipid peroxides (Boligon, 2014). Significant scientific evidence has shown that, under situations of oxidative stress, ROS are generated, and the balance between antioxidation (reduction) and oxidation is lost. So, the functioning of biological cells is compromised that can lead to a deficient cell operation (Aruoma, 1998; Amaeze et al., 2011). To protect the cells and organs against this problem, the human body has developed a complex antioxidant protection system that operates to neutralize the free radicals having a negative impact on biological cells (Boligon, 2014; Munteanu and Apetrei, 2021). Therefore, antioxidants are compounds capable of stabilizing or deactivating free radicals before they attack cells, and subsequently delay, control, or prevent oxidative processes (Halliwell et al., 1995).

Under a condition of oxidative stress, the human body generates a physiological response process named inflammation (Calder et al., 2013; Mogana et al., 2013). Chronic inflammation can contribute to chronic neurodegenerative diseases such as Alzheimer's disease, which may increase disease-associated morbidity, and involves the release of several mediators, resulting in the proliferation of fibroblasts and vascular endothelium, as well as lymphocytes, plasma cells, and macrophages (Mogana et al., 2013). Inflammation in injured cells is both initiated and maintained by the overproduction of prostaglandins and leukotrienes, which are produced by separate enzymatic pathways, namely, the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, respectively (Mogana et al., 2013).

Knowing this, it seems obvious that we should enrich our diet with antioxidants to protect us against harmful diseases. That's why there has been an increased interest by the food industry and preventive medicine in the development of "Natural antioxidants" from plant materials (Amaeze et al., 2011). Plants' high content of compounds with antioxidant properties (carotenoid, phenolic, flavonoid, anthocyanin derivatives,

unsaturated fatty acids, vitamins, enzymes, and cofactors) makes them interesting for use in prophylactic and curative phytotherapy (Munteanu and Apetrei, 2021) and as a result, they are becoming more and more popular all over the world (Nantz et al., 2006).

In vitro assays measuring antioxidant capacity are an accessible, fast, and typically automated way to screen and make an initial assessment of the plant extracts or even new possible antioxidant compounds, allowing the confirmation of antioxidant activity of plant samples within particular reaction systems (Badarinath et al., 2010; Munteanu and Apetrei, 2021).

Additionally, High-performance liquid chromatography (HPLC) is a versatile, robust, and widely used chromatographic technique for the isolation of natural products, that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of a sample (Boligon and Athayde, 2014). Thus, HPLC analysis is an essential tool that allows us to understand what kind of compounds are present in our samples, as well as determine if the plant is interesting for antioxidant use or not.

The present chapter aims to evaluate the phytochemical properties, antioxidant activities, and anti-inflammatory capacity of a methanolic extract from the leaves of *A. graecorum*. To evaluate the chemical composition of this extract, an HPLC-DAD was performed. To access the antioxidant capacity, a plant extract was submitted to a variety of *in vitro* assays, such as DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity, superoxide anion scavenging activity, nitric oxide scavenging activity, as well as iron chelating activity (ICA). Regarding anti-inflammatory activity, the COX-2 peroxidase endpoint assay was performed.

3.2- Materials

3.2.1- Reagents

The reagents used for the work performed in this chapter are listed in the Table 12.

Table 12. List of reagents used for the experiments performed in this chapter and its company.

Name	Company
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Sigma-Aldrich
Ascorbic acid	Sigma-Aldrich

Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Hydrochloric acid (HCl)	Sigma-Aldrich
Ethanol	Carlos Erba
Methanol	Carlo Erba
Phosphoric acid	Ridel-de Haën
Potassium chloride	Panreac
Potassium phosphate monobasic	Ridel-de Haën
Quercetin (QE)	Sigma
Sodium hydroxide (NaOH)	Merck
Sodium nitroprusside Dihydrate (SNP)	Sigma-Aldrich
Tris	Sigma
Trolox	Sigma-Aldrich
FeSO4	Sigma-Aldrich
Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4" disulphonic acid sodium salt)	Sigma-Aldrich
Phosphate buffer	Panreac
Nitroblue tetrazolium (NBT)	Roche Diagnostics
1,4-Dihydronicotinamide adenine dinucleotide (NADH)	PanReac AppliCher
Phenazine methosulphate (PMS)	Sigma-Aldrich
Na_2EDTA	Sigma-Aldrich
Quercetin	Sigma-Aldrich
TMPD	Sigma-Aldrich
Araquidonic acid	Sigma-Aldrich
Bovine hematin chloride	Sigma-Aldrich
Indomethacin	Panreac

3.2.2- Plant leaves

The dry leaves of *A. graecorum* were collected from Boushehr Province, located in the south of Iran.

3.3- Methods

3.3.1- Extraction of plant biomass

The air-dried leaves of *A. graecorum* were ground and soaked in 80% Methanol for 7 days. The biomass was re-extracted 3 times, using 80% Methanol. After filtration, the solvent was evaporated from the extraction solution by vacuum-rotary evaporation at 40°C (Bucchi RE121+461). After this, the extract was stored at -80ºC and then freeze-dried (Bioblock Scientific, Christ Alpha 2-4 LD Plus) for 72 hours, resulting in a powder.

For the antioxidant assays and the cellular assays, the powder was resuspended in DMSO (100mg/mL), and for HPLC analysis the extract was resuspended in 80% Methanol (5 mg/mL).

3.3.2- Phytochemical characterization: High-performance liquid chromatography (HPLC)

To perform HPLC analysis, a sample was prepared using 1 mL of the previous non-evaporated extract (10 mg/mL). The extract was filtered using a hydrophobic polytetrafluoroethylene (PTFE) filter (Branchia, 0.45 µm, Spain) and stored in an HPLC vial, in the dark, at 4ºC until further analysis.

The samples were runed in an HPLC (HITACHI, LabChrom Elite, Japan) equipped with two pumps L-2130, an auto-sampler L-2200, a diode array detector model L-2455 and was monitored by the EZChrom Elite software controller. The compound separation was performed on a reverse phase LiChroCART column (LiChrospher 100. RP-18e. 5 µm, Merk, Germany) at 30 Celsius, present in an L-2300 Column Oven. Samples were injected at a 1 mL/min flow rate. The mobile phase consisted of acetonitrile (solvent A) and formic acid (0.1% v/v in water) (solvent B). The elution gradient is described in Table 2.

The detection of compounds was processed in a 250-600 nm range. Chromatograms at 260, 280, 325, and 350 nm were recorded and used to evaluate the phenolic compounds present in the sample.

Table 13. Elution specifications for HPLC analysis.

Time (min)	Solvent A (%)	Solvent B (%)
0	5	95
7,5	5	95
30	30	70
40	90	10
45	90	10
47	5	95
60	5	95

3.3.3- Biochemical assays

3.3.3-1. *In vitro* antioxidant activities

a) DPPH radical scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay is among the most frequently used methods and offers the first approach for evaluating antioxidant activity (Shahidi and Zhong, 2015). This molecule is characterized as a stable free radical due to the delocalization of the spare electron over the molecule and this way, the molecule does not dimerize, as the case of most free radicals. Additionally, this gives rise to a deep violet color with an absorption band in ethanol solution at around 517 nm (Alam et al., 2013).

In this assay, when DPPH is reduced by an antioxidant/reducing compound, the molecule changes to the corresponding pale-yellow hydrazine, losing its violet color. This implies a decrease in the absorbance of DPPH, which is proportional to the concentration of free radical scavengers (Boligon, 2014).

A stock solution of DPPH (0,4 mM in ethanol 100%) was previously prepared and stored at 4ºC, in the dark. Before starting, the solution was corrected to an absorbance between 0,9, and 1. The different concentrations (150, 100, 75, 50, 25, and 10 µg/mL) were prepared by diluting the extract (100 mg/mL DMSO) in ethanol 100%.

In a 96 well-plate, 140 µl of DPPH work solution (400 µM) was added to 10 µl of extract in different concentrations and to 10 µl of ethanol 100% for negative control. In addition, the blanks of the samples were also considered, adding 140 µl of ethanol 100% to the 10 µl of extract.

After incubation of 60 minutes in the dark, the absorbance at 515 nm (SpectraMax Plus 384, Thermo-Fisher Scientific) was read and the percentage of DPPH reduction was calculated using the following formula:

$$\%\, reduction = \frac{Abs\ EtOH - Abs\ (sample - blank)}{Abs\ EtOH} \times 100$$

Abs EtOH: Absorbance of the negative control

Abs sample: Absorbance of the sample

Abs blank: Absorbance of the blank

EC50 was established using the GraphPad Prism 8 software and compared to the positive control, Quercetin. Three independent assays were performed, and all measurements were done in triplicate.

b) Iron Chelating Activity (ICA)

The extract ability for chelating iron was evaluated using the ICA assay. Iron under cell physiological conditions originated the Fenton reaction which is an important source of oxidative stress. Ferrozine can form chelates with Fe^{2+}, resulting in a red colour complex with an absorption maximum of 562 nm. This reaction is restricted in the presence of other chelating agents which results in a decrease in the red color of the ferrozine-Fe^{2+}. Due to this, the measurement of the color reduction determines the chelating activity of the sample (Alam et al., 2013; Mladěnka et al., 2011).

The experiment was performed on a 96-well plate. 50 µl of FeSO4 and 50 µl of ferrozine were added to 50 µl of each concentration of the sample (1500, 1000, 500, 250, and 100 µg/mL) was added. For the blank, 50 µl of ultra-pure H_2O was added replacing the ferrozine, and for the negative control, the sample was substituted with 50 µl of ultra-pure H_2O. The different concentrations were prepared by diluting the extract (100 mg/mL DMSO) in ultra-pure H_2O.

After incubation in the dark for 15 minutes, the plate was read at 562 nm (SpectraMax Plus 384, Thermo-Fisher Scientific) and the percentage of iron chelating activity was calculated with the following formula:

$$\% ICA = \frac{Abs\ uH20 - Abs\ (sample - blank)}{Abs\ uH2O} \times 100$$

Abs uH_2O: Absorbance of the negative control

Abs sample: Absorbance of the sample

Abs blank: Absorbance of the blank

EC50 was established using the GraphPad Prism 8 software and compared to the positive control, EDTA (Ethylenediaminetetraacetic acid), considered a standard iron chelator. Three independent assays were performed, and all measurements were done in triplicate.

c) Superoxide scavenging activity

Superoxide radical anion ($O2^{\cdot-}$) is produced when one electron is donated to oxygen and can occur from several metabolic processes or subsequent oxygen activation by irradiation. This radical anion can be generated using a non-enzymatic reaction of phenazine methosulphate (PMS) in the presence of nicotinamide adenine dinucleotide (NADH), and this way, $O2^{\cdot-}$ can reduce nitroblue tetrazolium (NBT) into formazan, which is spectrophotometrically monitored at 560 nm (Boligon, 2014). The measurement of the color decrease determines the extract scavenging activity.

In a 96 well-plate, 50 µL of NADH (166 µM) was added to 50 µL of each sample concentration (500, 250, 100, 50, 25, and 12,5 µg/mL), followed by 150 µL of NBT (43 µM). The reaction was initiated when 50 µL of PMS (2,7 µM) was added to the mixture. For the blanks, PMS was replaced by 50 µL of PBS 1X and for the measurement of negative control, the sample was replaced by 50 µL of PBS 1X. All concentrations were prepared by diluting the stock concentration of the extract (100 mg/mL DMSO) in PBS 1X.

After incubation in the dark at room temperature for 7 to 10 minutes, the absorbance was measured at 562 nm (SpectraMax Plus 384, Thermo-Fisher Scientific) and the percentage of superoxide inhibition was calculated using the following formula:

$$\% inhibition = \frac{Abs\ PBS - Abs\ (sample - blank)}{Abs\ PBS} \times 100$$

Abs PBS: Absorbance of the negative control

Abs sample: Absorbance of the sample

Abs blank: Absorbance of the blank

EC50 was established using the GraphPad Prism 8 software and compared to the positive control, Quercetin. Three independent assays were performed, and all measurements were done in triplicate.

d) Nitric oxide scavenging activity

In vitro inhibition of nitric oxide radical (NO·) is also a measure of antioxidant activity. This method is based on the inhibition of nitric oxide radical formation from sodium nitroprusside (SNP) in buffer saline, measured by Griess reagent (Boligon, 2014). The compound SNP is known to decompose in aqueous solution at physiological pH (7.2) producing this radical. Under aerobic conditions, it can react with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using the Griess reagent (Alam et al., 2013). In the presence of scavengers, the absorbance of the chromophore is evaluated at 546 nm and the activity is expressed as % reduction of nitric oxide (Boligon, 2014).

The experiment was performed on a 96-well plate. In the first step, to 50 µl of each concentration of the sample (1500, 1000, 500, 250, 125, 50, 25, and 12,5 µg/mL) was added 50 µL of SNP (20 mM), and negative control was made with 50 µL of H3PO4 buffer (2%) and 50 µL of SNP. All concentrations were prepared by diluting the stock concentration of the extract (100 mg/mL DMSO) in H3PO4 buffer (2%). After 60 minutes of incubation, at room temperature and by the light, 50 µL Griess reagent was added to the samples and negative control. For the blanks, the Griess reagent was replaced by 50 µL of H3PO4 buffer (2%). After 10 minutes of incubation, in the dark, the absorbance was measured at 546 nm (SpectraMax Plus 384, Thermo-Fisher Scientific) and the percentage of reduction of nitric oxide was calculated using the following formula:

$$\%\ NO\ reduction = \frac{Abs\ control - Abs\ (sample - blank)}{Abs\ control} \times 100$$

Abs control: Absorbance of the negative control

Abs sample: Absorbance of the sample

Abs blank: Absorbance of the blank

EC50 was established using the GraphPad Prism 8 software and compared to the positive control, Ascorbic Acid. Three independent assays were performed, and all measurements were done in triplicate.

3.3.3.2- In vitro anti-inflammatory activity: Peroxidase assay for measurement of COX-2 activity

COX-2 peroxidase endpoint assay was performed according to the method previously described with slight modifications (Mogana et al., 2013). This method evaluates the extract's ability to inhibit COX-2 activity *in vitro*. Briefly, 20 µL of two different concentrations of *A. graecorum* extract (100 µg/mL and 10 µg/mL) were plated in a 96-well plate, then 20 µL of 0.01 U/µL of COX-2 enzyme solution was added, followed by 180 µL of endpoint assay mix solution (bovine hematin chloride 100 µM, Arachidonic Acid 10 mM, N, N, N′, N′-Tetramethyl-p-phenylenediamine (TMPD) 17 mM, and Tris-HCl buffer 1 mM, pH 8.1). A blank for each concentration was made, containing only 20 µL of sample and 200 µL of uH_2O. For the negative control, the samples were replaced by 20 µL of DMSO. As a positive control, Indomethacin was used to compare the effect of the extract with a well-known COX-2 inhibitor. After 5 minutes of incubation in the dark at room temperature, the absorbance was measured at 590 nm (SpectraMax Plus 384, Termo-Fisher Scientific). All solutions were prepared daily and kept protected from the light. Results were expressed as percentage of inhibition of COX-2 relative to the negative control, calculated using the following formula:

$$\%\ COX-2\ inhibition = \frac{Abs\ negative\ control - Abs\ (sample - blank)}{Abs\ negative\ control} \times 100$$

Abs negative control: Absorbance of the negative control

Abs sample: Absorbance of the sample

Abs blank: Absorbance of the blank

Data treatment was made using the GraphPad Prism 8 software and compared to the positive control, Indomethacin. Three independent assays were performed, and all measurements were done in triplicate.

3.4- Results and discussion

3.4.1- Phytochemical characterization: High-performance liquid chromatography (HPLC)

HPLC was executed for the *A. graecorum* methanolic extract towards understanding the relation of the biochemical and biological activities with the chemical profile.

HPLC is a versatile, robust, and widely used chromatographic technique for the isolation of natural products, that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of a mixture (Piana et al., 2013; Boligon and Athayde, 2014). Several authors describe the use of HPLC for the characterization and quantification of secondary metabolites in plant extracts, mainly phenol compounds, steroids, flavonoids, and alkaloids (Boligon and Athayde, 2014). The solvent usually flows through a column with the help of gravity, but in the HPLC technique, the solvent will be forced under high pressures of up to 400 atmospheres, leading to a better separation of the compounds in the mixture (Thammana, 2016). HPLC technique is usually composed of a stationary phase, a mobile phase, and a detector. Pumps will be used to pass the mobile phase (pressurized liquid solvent, including the sample mixture) through the column (stationary phase), and the detector will show the retention times of the different molecules present in the sample. This separation happens because of the interaction of each sample component with the column and the solvent (both stationary and mobile phases). The difference in the molecular structure of the compounds will cause different degrees of affinity with the column and the solvent, consequently, their flow rates will be different, leading to the chromatographic separation of components (Thammana, 2016).

A. graecorum methanolic chromatogram is shown in Figure 4. The phenolics quantification, in mg Quercetin Equivalents/g extract, was performed using the chromatograms at 350 nm and the results obtained are summarized in Table 14.

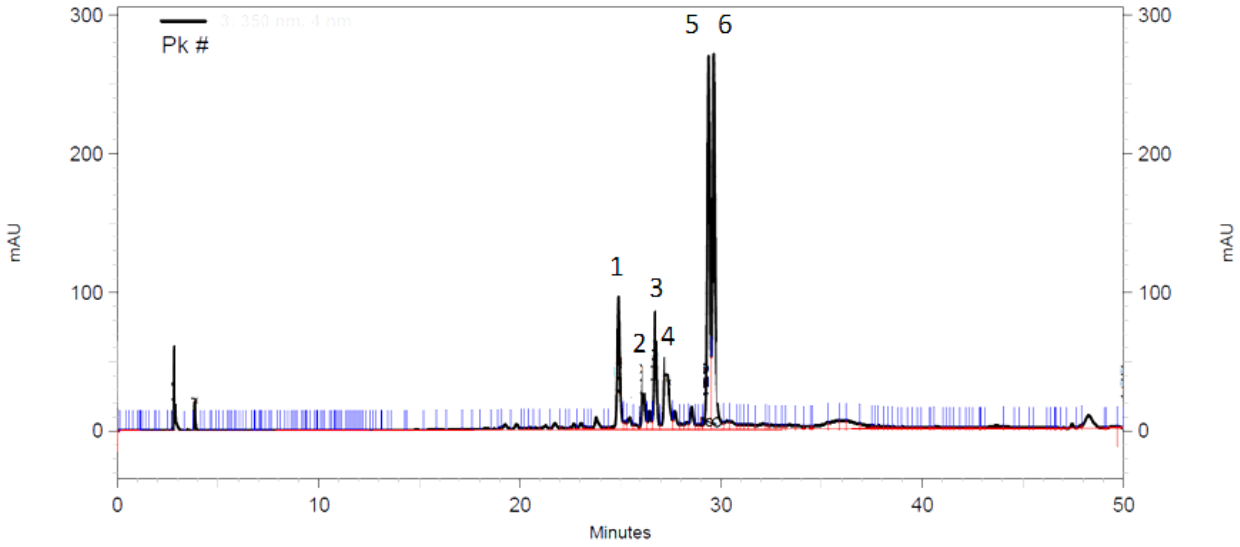

Figure 2. HPLC chromatogram of *A. graecorum* methanolic extract, recorded at 350 nm. The identification of the compounds identified in the Figure are listed in the Table 14.

Looking at the *A. graecorum* extract chromatogram it is possible to identify 6 major compounds (Figure 4). The compounds found were identified as flavonoid derivatives, as the UV-visible spectra obtained for each peak of the plant's chromatograms were tightly identical to those obtained for the pure compounds, apigenin, and quercetin (all flavonoids). Based on the obtained results, the major contributors to the bioactivities reported for *A. graecorum* methanolic extract might be flavonoid derivatives (expressed as quercetin derivatives).

Table 14. Distribution and content of identified compounds in HPLC analysis at 350 nm of *A. graecorum* methanolic extract, expressed in mg QE (quercetin equivalents)/g dry weight.

Number	**Compound**	**QE (mg/g dry weight)**
1, 5, 6	Quercetin derivatives	3,3
2, 3, 4	Apigenin derivatives	0,18
	Total	3,48

Flavonoids are an important class of natural products that are associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications (Panche et al., 2016). This is because of their antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties coupled with their capacity to inhibit several enzymes, such as cyclo-oxygenase (COX) (Panche et al., 2016).

Knowing this, it is expected that the flavonoid content present in this extract will give a good antioxidant and anti-inflammatory potential to the plant. Therefore, knowing that the content in flavonoid derivatives can be related to the antioxidant potential of the plant extract, the extract was tested as a radical scavenger, iron-reducing agent, and inhibitor of COX-2, which are well-known antioxidant and anti-inflammatory properties. Results are presented in the following sections.

3.4.2- Biochemical assays

3.4.2-1. *In vitro* antioxidant activity

a) DPPH radical scavenging activity

In this study, the ability to scavenge DPPH radicals by *A. graecorum* extract was evaluated. The data obtained are represented in Figure 5 and the EC50 values are presented in Table 15.

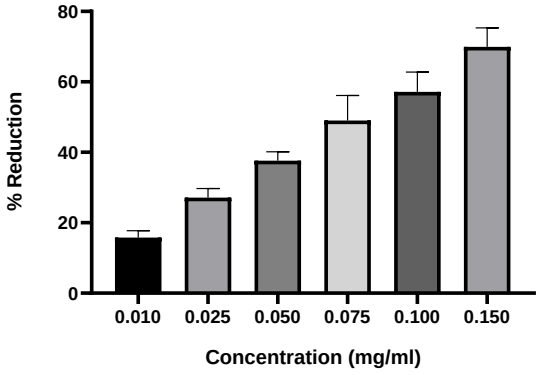

Figure 3. DPPH scavenging- activity (%) of *A. graecorum* methanolic extract. Values are represented in mean ±SD.

The screening revealed that this plant can reduce DPPH radical in a concentration-dependent manner, which proved that the extract/compounds present in the extract can donate an electron or hydrogen that could react with DPPH radical. Having this in mind the extract demonstrated good antioxidant activity.

Table 15. Effectiveness values (EC50) obtained on DPPH assay of the extract compared with control Trolox. The results are expressed as mean±SD.

	EC50 (μg/mL)
A. graecorum	78,03±4,32
Trolox	12,49±1,39

Trolox is the water-soluble analog of α-tocopherol (a type of vitamin E) and is known for its high radical scavenging activity and is therefore frequently used as a model compound. It is commonly used as a reference antioxidant (Oehlke et al., 2011).

As a result, the extract showed to be less effective than the control Trolox, once the concentration required to reduce 50% DPPH free radicals was higher than Trolox. However, it is important to keep in mind that we are comparing a single compound with an extract, which is a mixture of various compounds. It is expected that a single antioxidant compound will have stronger activity than a mixture of compounds because the electron transfer between the isolated compounds and DPPH occurs more easily (Vieira et al., 2011).

The antioxidant effect on DPPH radical is because of the compound's ability to donate a hydrogen or transfer an electron. It is predicted that the extract scavenging ability is due to the presence of flavonoid derivatives, as we see by the HPLC analysis, which are well-known antioxidants.

Even though the DPPH assay has been widely used and is a valuable tool to screen the antioxidant activity of plant extracts, this is not a biological radical, which means that it is not present in nature or in our organism (Kedare and Singh, 2011). Due to this fact, it is important to take into account other methods that also study the antioxidant activity of the extracts for biological radicals, such as superoxide and nitric oxide (Vieira et al., 2011). These assays were performed, and the results are presented in the following sections.

b) ICA (Iron Chelating Activity)

In this assay, the *A. graecorum* extract capacity to chelate iron was assessed and compared to a standard iron chelator, EDTA. The results are represented in Figure 6 and the EC50 values are presented in Table 16.

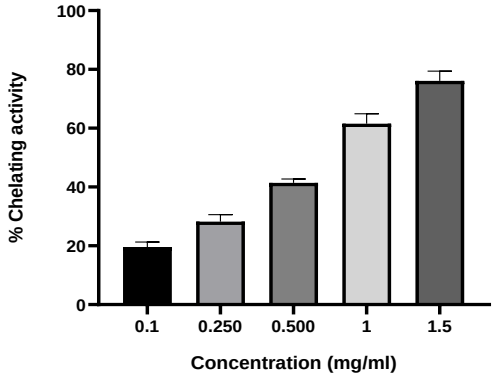

Figure 4. Iron chelating activity (%) of *A. graecorum* methanolic extract. Values are represented in mean ±SD.

Chelating agents are molecules that form stable bonds with metallic atoms, making them more stable, soluble, and resistant to disassociation. EDTA is a chelating agent that binds to calcium, lead, iron, copper, and other metal ions forming soluble complexes (Ibad et al., 2016).

Table 16. Effectiveness values (EC50) obtained on ICA assay of the extract compared with control EDTA. The results are expressed as mean±SD.

	EC50 (µg/mL)
A. graecorum	698,20±32,42
EDTA	0,024±0,0001

Local brain iron overload is frequently observed in common neurodegenerative disorders such as Parkinson's or Alzheimer's diseases and is a hallmark of other rare conditions of "neurodegeneration with brain iron accumulation" (Galaris et al., 2019). The presence of unshielded redox-active iron favours Fenton chemistry which involves a reaction between H_2O_2 and Fe^{2+} (Genaro-Mattos et al., 2015). HO• is the major product of the Fenton reaction and is short-lived but extremely reactive. For instance, iron attached to membrane phospholipids catalyzes the initiation of lipid peroxidation chain reactions, which have been associated with necrotic or ferroptotic types of cell death. Furthermore, iron associated with DNA induces mutations or single and double-strand breaks, while iron loosely attached onto proteins can promote H2O2-dependent redox signalling (Galaris et al., 2019).

Regarding the ability to chelate the iron of the extract, it proved to be less active compared to the effect shown in the other biochemical assays. Although the extract was able to chelate iron in a concentration-dependent manner, higher concentrations were needed to reach the EC50 and as such, this result was much higher than the EC50 of the control used, EDTA.

c) Nitric oxide scavenging activity

In this assay, the plant extract was tested for NO-scavenging activity and compared with a known positive control, ascorbic acid. The data of NO scavenging activity obtained for the investigated extract are represented in Figure 7 and the EC50 of the extract and the control used, Ascorbic Acid, are summarized in Table 17.

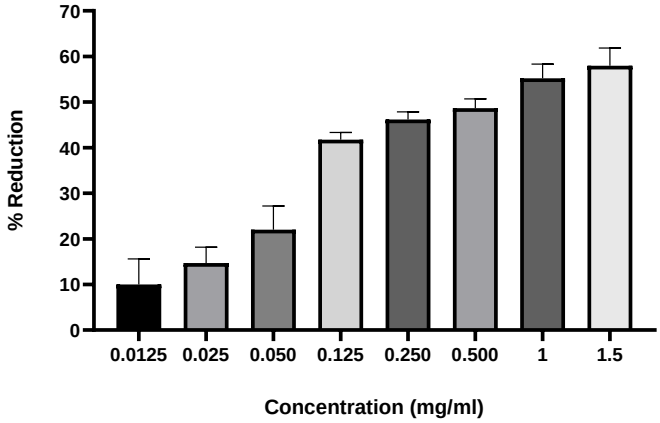

Figure 5. Nitric oxide scavenging activity (%) of *A. graecorum* methanolic extract. Values are represented in mean ±SD.

As a highly reactive radical, NO• itself directly damages normal functional tissues, and it can also react with O2•- to form an even stronger oxidant, peroxynitrite (OONO-). Moreover, NO• that is excessively produced by inducible nitric oxide synthase (iNOS) in abnormal situations, is considered a key mediator that can induce the generation of pro-inflammatory cytokines, leading to chronic inflammation as well (Junmarkho and Hansakul, 2019). Therefore, the strong inhibitory activities against NO• and O2 •- in the body have the potential for blocking the accumulation of both radical species and their subsequent reactions, which partly cause continued oxidative stress and chronic inflammation implicated in various neurological diseases (Junmarkho and Hansakul, 2019).

Table 17. Effectiveness values (EC50) obtained on nitric oxide assay of the extract compared with control Ascorbic Acid. The results are expressed as mean±SD.

	EC50 (µg/mL)
A. graecorum	296,8±43,8
Ascorbic Acid	922,1±56,3

Ascorbic Acid, also known as Vitamin C, efficiently scavenges toxic free radicals and other reactive oxygen species (ROS) formed in cell metabolism, thus it can be considered an effective antioxidant (Arrigoni and De Tullio, 2002).

The extract was capable of inhibiting the accumulation of NO radical, in a concentration-dependent manner, demonstrating a much stronger action than the control, ascorbic acid, obtaining an EC_{50} much lower than this compound.

d) Superoxide scavenging activity

In this assay, the plant extract was tested for superoxide scavenging activity and compared with a known positive control, quercetin. The data of superoxide scavenging activity obtained for the investigated extract are represented in Figure 8 and the EC50 values of the extract and the control used, quercetin, are summarized in Table 18.

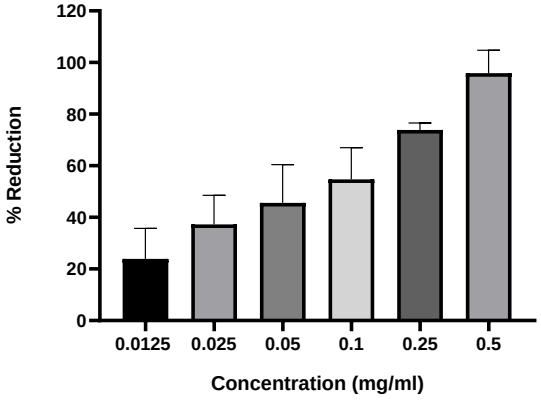

Figure 6. Superoxide scavenging activity (%) of *A. graecorum* methanolic extract. Values are represented in mean ±SD.

Superoxide anion (O_2^-), one kind of ROS, is the single-electron reduction product of oxygen molecules and is closely related to many inflammatory processes. Excessive superoxide anion results in abnormal cell apoptosis and even a series of diseases, among them neurodegenerative diseases (Jie et al., 2022). Interest in the scavenging ability of the superoxide anion is largely due to its role in the production of the highly reactive hydroxyl radical in the presence of metal ions (Boligon, 2014).

As a result, the extract was capable of inhibiting the accumulation of superoxide anion, in a concentration-dependent manner, however, showed to be less effective than the control Quercetin, once the concentration required to reduce 50% of superoxide anions was higher than Quercetin. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and binds transition metal ions (Baghel et al., 2012).

Table 18. Effectiveness values (EC50) obtained on superoxide scavenging assay of the extract compared with control Quercetin. The results are expressed as mean±SD.

	EC50 (µg/mL)
A. graecorum	42,79±10,26
Quercetin	27,28±5,7

It is predicted that the extract scavenging ability is due to the presence of flavonoid derivatives, as we see by the HPLC analysis and discussed in the other antioxidant assays, which are well-known antioxidants.

3.4.2.2- In vitro anti-inflammatory activity: Peroxidase assay for measurement of COX-2 activity

There are two cyclo-oxygenase isoforms: an inducible cyclo-oxygenase (COX-2), predominating at sites of inflammation, which is expression is enhanced by cytokines, growth factors, and other inflammatory stimuli; and one house-keeping enzyme, COX-1, constitutively expressed throughout the body and of particular importance for gastrointestinal protection (Hawkey, 2001).

Inflammation in injured cells is both initiated and maintained by the overproduction of prostaglandins and leukotrienes, which are produced by separate enzymatic pathways, namely, the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, respectively. Both the prostaglandins as well as the leukotrienes are on-demand biosynthesized from arachidonic acid (AA). AA is then further metabolized by the COX and 5-LOX enzyme systems to a variety of mediator molecules, including prostaglandin (PG) E2, thromboxanes (TXs) (TXA2), prostacyclins (PGI2), and highly inflammatory leukotrienes such as leukotriene (LT) B4, LTC4, and LTD4. Conventional pharmacological management of inflammatory diseases involves treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors that block the formation of PGs (Mogana et al., 2013).

One major strategy for developing treatment options for the treatment of neurodegenerative diseases is to slow the rate of neuronal loss through the suppression of inflammatory mechanisms. Substantial evidence exists that these processes contribute to neuronal loss, suggesting that an anti-inflammatory regimen may offer some degree of neuroprotection for patients (Aisen, 2002).

COX-2 activity was significantly inhibited by *A. graecorum* extract, and the effect was more powerful than the control used, Indomethacin. The results obtained for the COX-2 peroxidase endpoint assay are represented in Figure 9.

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) with very effective antipyretic, analgesic, and anti-inflammatory activity (Lucas, 2016).

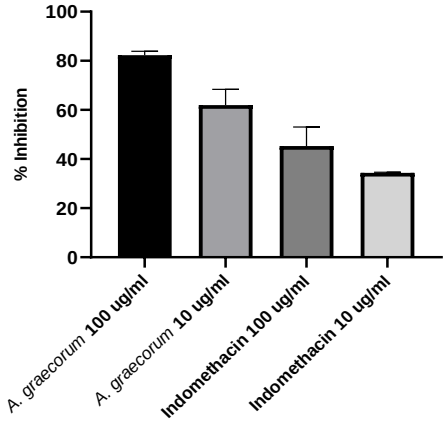

Figure 7. COX-2 inhibition (%) of *A. graecorum* extract at concentrations of 100 and 10 µg/mL, compared with the control Indomethacin, at the same concentrations.

This effect can be correlated to the flavonoid content of the extract, especially to the quercetin derivatives. The bigger fraction of flavonoid derivatives of the extract are quercetin derivatives, and there are already reports of quercetin's ability to inhibit COX-2 activity. Xiao et al. (2011), demonstrated that quercetin can significantly suppress COX-2 mRNA and protein expression and prostaglandin production.

3.5- Conclusions

The phytochemical analysis of the *A. graecorum* methanolic extract showed that this plant is mainly composed of flavonoids. Quercetin and apigenin derivatives were the main compounds found.

Overall, the extract showed positive results for antioxidant activities, except on iron chelating activity, where it showed to be less effective. In all antioxidant assays, the extract did not exhibit better outcomes than the controls used, except in the nitric oxide scavenging assay, where the extract exhibited a stronger action than ascorbic acid.

The extract also showed anti-inflammatory properties, inhibiting the COX-2 activity, more efficiently than the control (indomethacin).

These properties may be essentially due to the high flavonoid content, thus this class of compounds is well-known for its antioxidant and anti-inflammatory features.

The studies conducted in this chapter proved that *Alhagi graecorum* can be used as a source of compounds with pharmacological properties, namely antioxidants, which could be helpful in neuroprotection and other oxidative stress-related conditions.

Chapter 4: *In vitro* assessment of *Alhagi graecorum* methanolic extract cytotoxicity and cytoprotection of BV2 and HepG2 cell lines against t-BHP oxidative insult

4.1- Introduction

Microglia are the resident phagocytes and inherent immune cells of the brain. Over the past few decades, there has been an increased interest in these cells, due to their recognized importance in homeostasis, as well as in numerous pathologies of the central nervous system. It is now widely accepted that these brain cells are likely to contribute to the mechanisms of neuronal damage and cognitive loss (Giulian, 1999). Furthermore, activated microglia have been associated with a variety of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Giulian, 1999) (Gao et al., 2003), as discussed in Chapter 1.

The BV2 cell line is an immortalized murine microglial cell line that reproduces primary microglia with high fidelity and is frequently employed as the *in vitro* model for screening and evaluation of anti-neuroinflammatory agents (Henn et al., 2009).

The use of *in vitro* microglia cultures, like BV2 cells, presents advantages for a primary approach in neuroprotective features since the increased yield and homogeneity of cells in culture allow data output in a shorter time compared to most *in vivo* experiments. Additionally, it presents a beneficial tool to study the activation state, releasable factors, motility, and other crucial components that characterize these cells, which cannot be sufficiently examined *in vivo* (Stansley et al., 2012).

On the other hand, HepG2 is a well-recognized and established standard hepatocyte cell line to explore studies on drug metabolism, cytotoxicity, genotoxicity, hepatotoxicity, and a tool for chemical risk assessment *in vitro* (Kaur et al., 2018). Also, this cell line has been extensively exploited to examine antioxidative, hepatoprotective, anti-hepatoma, hypocholesterolemic, anti-steatosis, bioenergetic homeostatic, and anti-insulin-resistant properties. These cells are comparable to human hepatocytes advocating their use as a cell-based model in drug discovery, thus the mechanism of action of various botanicals and bioactive constituents has been reported using these cells (Kaur et al., 2018).

Based on the *in vitro* results obtained for *A. graecorum* methanolic extract, namely a high content in flavonoid derivatives, great anti-radicalar scavenging activity, iron chelating power, and COX-2 inhibition capability (Chapter 3), it is expected that this extract could be successful in reducing oxidative stress and oxidative damage in cell lines.

This chapter exhibits the *in vitro* cellular studies performed using HepG2 and BV2 cell lines.

The goal of this research was to screen the extract cytotoxicity, as well as assess the extract's protective effect on both cultures, subjecting them to t-BHP (tert-Butyl hydroperoxide), a compound capable of inducing oxidative stress and damage in cells.

There are already some reports showing the protective effects of *Alhagi* plant extracts in cellular cultures and tissues (Chapter 2). However, not much is known about their influences on the CNS and their neuroprotective properties. Also, despite their use in folk medicine, there are few studies concerning toxicological and safety considerations regarding these plants (Chapter 2).

4.2- Materials

The reagents used for the work performed in this chapter are listed in Table 8.

Table 19. List of reagents used for the experiments performed in this chapter and its company.

Name	**Company**
Sodium bicarbonate (Na_2CO_3)	Sigma-Aldrich
Resazurin	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich
Antibiotic (10.000 Units of penicillin, 10 mg of streptomycin, and 25μg amphotericin)	Sigma
Fetal bovine serum (FBS)	Sigma
Tert-butyl hydroperoxide (t-BHP)	Sigma
Trypsin-EDTA solution	Sigma

4.3- Methods

4.3.1- Cell culture

a) Principle

BV2 cells were derived from rat/mice-immortalized murine neonatal microglia and HepG2 cells were isolated from a human hepatocellular carcinoma. Both cell lines were used as *in vitro* models.

b) Subculturing cells

BV2 and HepG2 cells were grown in T-25 flasks (25 cm^2 polystyrene flasks) and maintained in Dulbecco´s modified Eagle´s medium (DMEM) (13.4 g/L DMEM D5648 sigma, 3.7 g/L sodium bicarbonate, pH 7.1-7.3) supplemented with 10 % fetal bovine serum (FBS) and 1% of antibiotics. Both cultures were maintained at 37°C in a humidified incubator (Sanyo Electric Co.) containing 5% CO_2.

BV2 cells were subcultured three times a week using 1x sterile PBS and 0,05% trypsin, while HepG2 cells were subcultured once a week using 1x sterile PBS and 0,25% trypsin.

c) Plating cells

According to the number of cells obtained in one T-25 flask, a certain volume of the cell resuspension was added to DMEM complete medium, in order to plate 200 µL of cell suspension in each well and obtain a cell density of $2x10^5$ cells/mL per well in a 96-well plate.

4.3.2- Cellular assays

a) Resazurin assay

Resazurin assay was used as a measurement of cell viability. This assay is based on the reduction of resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) to resorufin and di-hydroresorufin by viable cells, so this way, the oxidized blue nonfluorescent compound is reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000). This conversion occurs intracellularly, where the oxidized form of the resazurin enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from numerous cytochromes (Al-Nasiry et al., 2007; Vega-Avila and Pugsley, 2011).

Since resazurin is non-toxic to cells and is stable in culture media, continuous measurement of cell proliferation *in vitro* can be achieved. Toxic insults that impair cell viability and proliferation also affect the capacity to reduce resazurin, and the rate of dye reduction is directly proportional to the number of viable cells present (O'Brien et al., 2000).

The fluorescent signal is monitored using a 530-560 nm excitation wavelength and 590 nm emission wavelength while the absorbance signal is monitored at 540 nm and 630 nm (Vega-Avila and Pµgsley, 2011).

After incubation time, the medium was vacuumed from the 96 well-plate, and added a 10% resazurin (2,5 mM) solution diluted in DMEM, 100 µL per well. Cells were incubated for 3 hours at 37°C in a 5% CO_2 incubator (Sanyo Electric Co.) and then the fluorescence was measured at 530 nm excitation and 590 nm emission using a BioRad iMark microplate absorbance reader. Untreated cells, with medium only, were used as a control, and the percentage of viability was calculated according to the following formula:

$$\%\, Cell\ Viability = \frac{fluorescence\ of\ sample}{fluorescence\ of\ control} \times 100$$

The results are shown as the means ± standard error of the mean (SEM) of three independent experiments performed in triplet for each experimental condition.

b) Cytotoxicity assay

Alhagi extract was tested in both cell lines to evaluate the cytotoxicity range. Five concentrations were used (500 µg/mL, 250 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL) and the extract effect was assessed for 4 hours and 24 hours.

Cells were seeded into a 96 well-plate, incubated overnight, and exposed to the different concentrations of the extract the next day. After an incubation of 4 hours and 24 hours, cell viability was determined by resazurin assay.

c) Cytoprotective assay

To determine the extract antioxidant properties, BV2 and HepG2 cells were incubated with a toxic insult, t-BHP, which induces oxidative stress and significant cell death.

Cells were seeded in a 96 well-plate and incubated overnight. On the next day, cells were exposed to four different concentrations of extract: 250 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL. Two different conditions were evaluated: co-incubation with extract and t-BHP for 4 hours and a pre-incubation with extract for 20 hours plus a co-incubation with t-BHP for 4 hours.

A concentration of 0,5 mM t-BHP solution was used in the HepG2 cell line and a concentration of 0,4 mM in the BV2 cell line. After the incubation time, cell viability was measured using resazurin assay.

4.4- Results and discussion

4.4.1- Cytotoxicity assays

Cytotoxicity of *A. graecorum* extract was evaluated on BV2 and HepG2 cells using resazurin assay.

Cell viability results regarding the extract cytotoxic effect of 500, 250, 100, 50, and 25 µg/mL concentrations in BV2 cells, after 4 hours of incubation, are shown in figure 10 A). As observed, *A. graecorum* extract did not decrease the viability of microglial cells when they were incubated with the concentrations of 50 µg/mL and 25 µg/mL, therefore not showing a toxic effect at these concentrations. On the other hand, at 100 µg/mL concentration the extract reduced cell viability by 6%, eliciting a slightly toxic effect, at 250 µg/mL concentration the extract significantly reduced cell viability by 19%, and at 500 µg/mL concentration the extract significantly reduced cell viability by 29%, compared with the control group, provoking a toxic effect in this cell line.

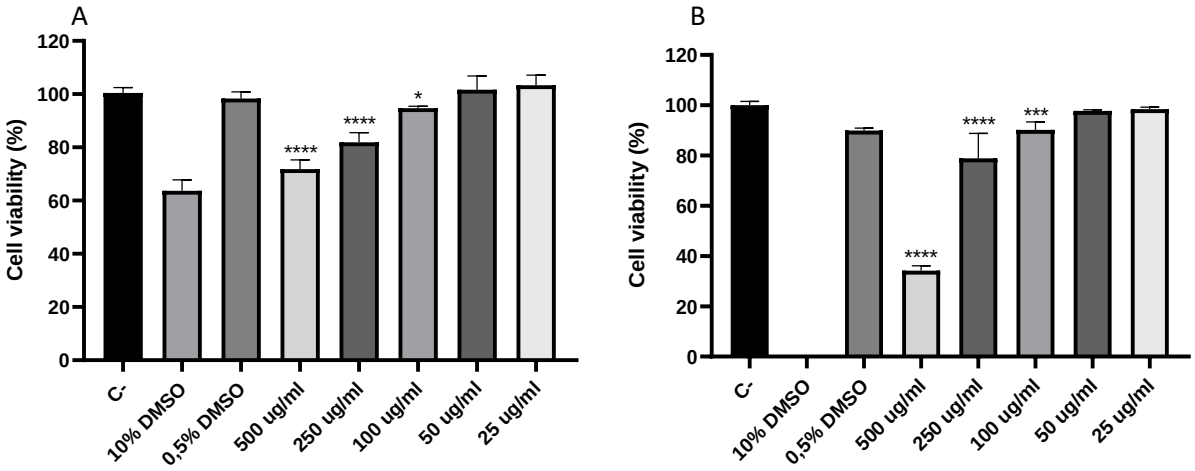

Figure 8. Effect of *A. graecorum* methanolic extract treatment on BV2 cell line. **A)** extract incubation of 4 hours. **B)** extract incubation of 24 hours. Values represent mean± standard deviation (SD) of three independent experiments. Statistical differences are presented *p<0,1, **p<0,01, ***p<0,001, **** p < 0.0001 vs. control (cells subject to medium without treatment).

Cell viability results regarding the extract cytotoxic effect of 500, 250, 100, 50, and 25 µg/mL concentrations in BV2 cells, after 24 hours of incubation, are shown in figure 10 B). As observed, similar to the 4 hours incubation, *A. graecorum* extract did not decrease the viability of microglial cells when they were incubated with the concentrations of 50 µg/mL and 25 µg/mL, therefore not showing a toxic effect at these concentrations. Otherwise, at 100 µg/mL concentration the extract reduced cell viability by 10%, at 250 µg/mL concentration, the extract significantly reduced cell viability by 21%, and at 500 µg/mL concentration the cell viability was reduced by 66%, compared with the control group, inciting a toxic effect in this cell line.

Cell viability results regarding the extract cytotoxic effect of 500, 250, 100, 50, and 25 µg/mL concentrations in HepG2 cells, after 4 hours of incubation, are presented in figure 11 A). As observed, *A. graecorum* extract did not decrease cell viability when incubated with the concentrations of 50 and 25 µg/mL, thus not revealing a toxic effect in these cells at these concentrations. At 100 µg/mL concentration the extract reduced cell viability by 8%, eliciting a little toxic effect, at 250 µg/mL concentration the extract reduced cell viability by 13%, and at 500 µg/mL the cell viability was significantly reduced by 28%, less than in BV2 cells but still significantly showing toxic effects in this cell line.

Cell viability results regarding the extract cytotoxic effect of 250, 100, 50, and 25 µg/mL concentrations in HepG2 cells, after 24 hours of incubation, are presented in figure 11 B). Similar to the previously mentioned 4 hours incubation assay, *A. graecorum* extract did not decrease cell viability when incubated with 50 µg/mL and 25 µg/mL concentrations, thus not revealing a toxic effect in these cells at these concentrations. At 100 µg/mL the extract reduced cell viability by 6%, and at 250 µg/mL the extract reduced cell viability by 58%, showing a significant toxic effect in this cell line at both concentrations.

To date, the study of Hawar et al. (2022) was the only one found to have information about the cytotoxicity of *A. graecorum* in cell lines. An aqueous extract showed a significant reduction of human breast cancer cells (MCF-7) viability when incubated in concentrations of 50 µg/mL or above, only from a concentration of 25 µg/mL the extract did not exhibit toxicity (Hawar et al., 2022).

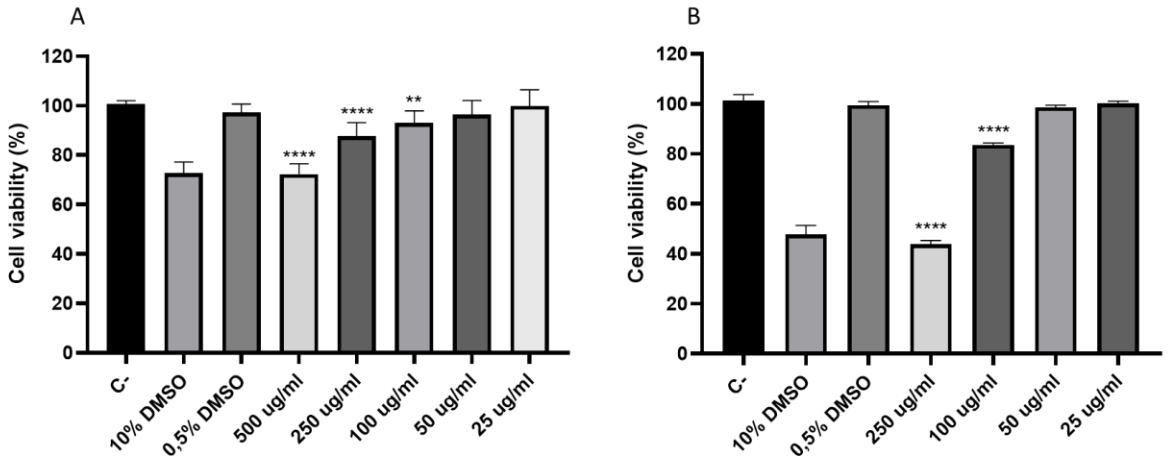

Figure 9. Effect of *A. graecorum* methanolic extract treatment on HepG2 cell line. **A)** extract incubation of 4 hours. **B)** extract incubation of 24 hours. Values represent mean± standard deviation (SD) of three independent experiments. Statistical differences are presented *p<0,1, **p<0,01, ***p<0,001, **** p < 0.0001 vs. control (cells subject to medium without treatment).

4.4.2- Cytoprotective assays

Plant extracts and specially containing flavonoids can provide both a short/direct protection and a long-term/indirect protection against oxidative stress via a variety of mechanisms (Wang et al., 2010). A direct protection includes acting as antioxidants themselves, directly neutralizing toxic ROS through the donation of hydrogen ions. An indirect protection involves inducing antioxidant enzymes or modulating cell signalling pathways (Wang et al., 2010).

To evaluate the *A. graecorum* extract cytoprotective effect against t-BHP oxidative insult, BV2, and HepG2 cells were incubated with the extract and t-BHP for 4h, assessing a direct cytoprotective effect, and pre-incubated with extract for 20h and more 4 hours with t-BHP, assessing an indirect cytoprotective effect. Cell viability was measured using resazurin assay.

Cell viability results regarding extract cytoprotective effect of 250, 100, 50, and 25 µg/mL concentrations in BV2 cells, after 4 hours of co-incubation with extract and t-BHP, are presented in figure 12 A). As shown, the positive control treated only with t-BHP decreased cell viability by 59%, but the extract was able to significantly inhibit t-BHP toxicity and therefore protect cells when incubated in 100, 50, and 25 µg/mL concentrations. The 250 µg/mL concentration was not able to protect cells against oxidative stress, obtaining only a percentage of cell viability of 52%, less than the positive control.

With a concentration of 100 µg/mL, cell viability was preserved by 80%, with 50 µg/mL the percentage of cell viability was 85%, and with 25 µg/mL the extract was able to protect 88% of cells.

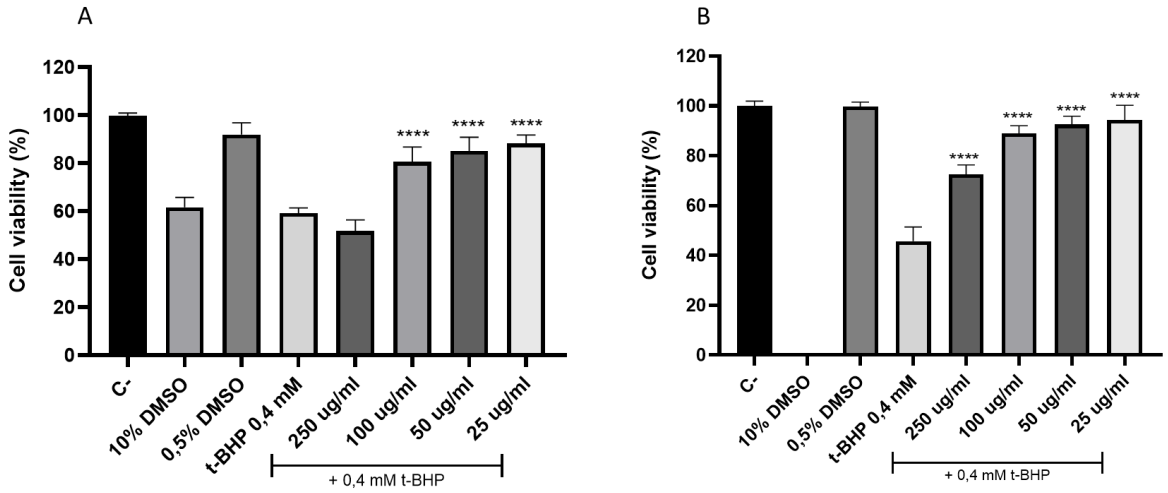

Figure 10. Effect of *A. graecorum* extract at 250, 100, 50 and 25 µg/mL against t-BHP insult. BV2 cells were treated with extract for **A)** 4 hours of co-incubation with t-BHP and **B)** 20 hours, then were co-incubated with t-BHP for 4 hours. Data are represented as means ± SD with one-way ANOVA. Statistical differences are presented **** $p < 0.0001$ vs. control (cells subject with t-BHP treatment).

Cell viability results regarding extract cytoprotective effect of 250, 100, 50, and 25 µg/mL concentrations in BV2 cells, after 20 hours of pre-incubation with extract and more 4 hours with t-BHP, are presented in figure 12 B). Very similar to the previous condition, the positive control treated only with t-BHP decreased cell viability by 46%, but the extract was able to significantly protect cells in all concentrations. With a concentration of 250 µg/mL the percentage of cell viability obtained was 73%, with 100 µg/mL cell viability was preserved by 89%, with 50 µg/mL the percentage of cell viability was 93%, and with 25 µg/mL the extract protected 94% of cells.

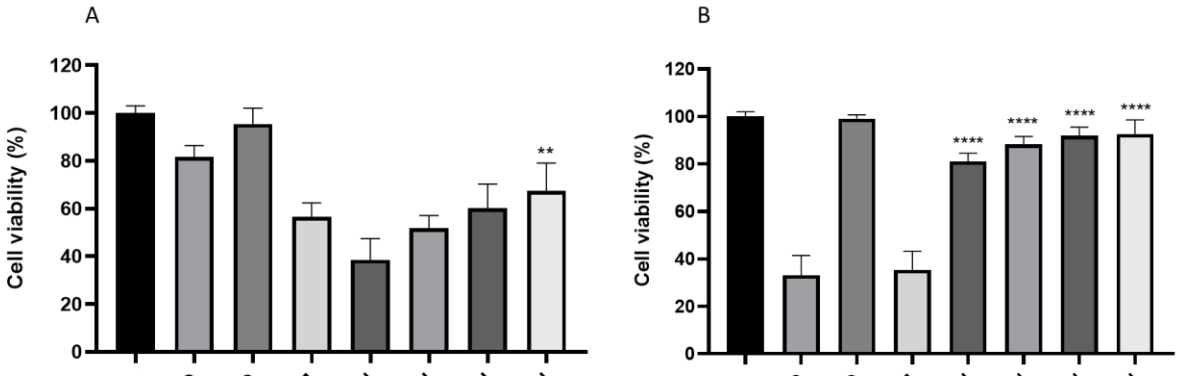

Figure 11. Effect of *A. graecorum* extract at 250, 100, 50 and 25 µg/mL against t-BHP insult. HepG2 cells were treated with extract for **A)** 4 hours of co-incubation with t-BHP and **B)** 20 hours, then were co-incubated with t-BHP for 4 hours. Data are represented as means ± SD with one-way ANOVA. Statistical differences are presented **** $p < 0.0001$ vs. control (cells subject with t-BHP treatment).

Cell viability results regarding extract cytoprotective effect of 250, 100, 50, and 25 µg/mL concentrations in HepG2 cells, after 4 hours of co-incubation with extract and t-BHP, are presented in figure 13 A). As shown, the positive control treated only with t-BHP decreased cell viability by 57%, but the extract was able to significantly inhibit t-BHP toxicity and therefore protect cells only with the 25 µg/mL concentration, with a percentage of cell viability of 68%. With a concentration of 100 µg/mL, cell viability was preserved by 52%, less than the positive control. In incubation with 50 µg/mL concentration, the percentage of cell viability was 60%.

Cell viability results regarding extract cytoprotective effect of 250, 100, 50, and 25 µg/mL concentrations in HepG2 cells, after 20 hours of pre-incubation with extract and more 4 hours with t-BHP, are presented in figure 13 B). Differently from the previous condition, the positive control treated only with t-BHP decreased cell viability by 35%, but the extract was able to significantly protect cells in all concentrations. With a concentration of 100 µg/mL cell viability was preserved by 88%, with 50 µg/mL the percentage of cell viability was 92%, and with 25 µg/mL the extract was able to protect 93% of cells.

tert-Butyl hydroperoxide (t-BHP) is commonly used as a model substance for the evaluation of mechanisms of cellular alterations resulting from oxidative stress in cells and tissues. There are two pathways by which t-BHP is metabolized and both induce oxidative stress. The first, provided by cytochrome P450, leads to the production of peroxyl and alkoxyl radicals. These radicals initiate lipoperoxidation of membrane phospholipids with subsequent alterations to membrane fluidity and permeability. The other pathway employs glutathione peroxidase, and t-BHP is detoxified to *tert*-butanol, glutathione is depleted by oxidation to its disulfide form (glutathione disulfide). Lipoperoxidation, depletion of glutathione, and the onset of mitochondrial permeability transition are general mechanisms involved in cell injury caused by oxidative stress (Kučera et al., 2014).

As expected, the extract was able to protect both cell lines against t-BHP, with more efficacy when pre-incubated for 20 hours. These results show that the extract has a direct effect, visible by the results on the co-incubation with the t-BHP assay. This means that it has scavenging properties, as was proved in the antioxidant assays presented in Chapter 3, and can neutralize the radicals produced by the t-BHP exposure. However, it has better outcomes when pre-incubated for 20 hours, indicating that the extract possibly has a more powerful indirect effect, for example, by increasing the activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalases (CAT) and Glutathione Peroxidases (GPX) (Huchzermeyer et al., 2022).

To date, these experiments were never performed for this *Alhagi* species, so there is no information in the literature to compare the obtained data. However, other studies performed with other plants of pharmacological interest, suggested that the indirect protection effect is usually correlated with increasing the expression levels of cytoprotective enzymes like heme-oxygenase-1 (HO-1), peroxiredoxin 4 (PRDX4), the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM), and NAD(P)H quinone oxidoreductase 1 (NQO1), as was shown by Carvalho et al. (2014). Also, the study of Magalhães et al. (2022) demonstrated that *L. ruthenicum berries* extracts achieved a much higher and significant cytoprotective effect against t-BHP insult when pre-incubated for 21 hours, and this indirect effect was due to decreasing IL-1β, IL-6, TNF, iNOS and COX-2 gene expression, and in decreasing NO production in BV2 cells.

Only two experimental studies have so far concerned *Alhagi's* neuroprotective effects, and both performed *in vitro* assays. The first one utilized frog tissue and proved that an ethanolic extract can block the action of the neurotransmitter acetylcholine (Marashdah and AL-Hazimi, 2010). The second one made use of LPS-induced N9 microglial cells and revealed two good leading compounds for the development of potential therapeutic agents against neurodegenerative diseases (Zhou et al., 2017). This literature and the work performed presented in Chapter 3 and 4 makes case for *Alhagi* plants having powerful neuro-potentialities and compounds that can inhibit neuronal inflammation.

4.5- Conclusions

The *A. graecorum* methanolic extract exhibited cytotoxicity in both cell lines, at concentrations of 250 and 100 µg/mL. At concentrations of 50 and 25 µg/mL, the extract did not exhibit toxicity, proving to be safe for use at these concentrations. This happened equally in the 4 hours incubation assay and the 24 hours incubation assay.

The extract proved to be capable of protecting both cell lines against t-BHP insult, with better outcomes when pre-incubated for 20 hours, proving to have an effective indirect effect (possibly through increasing the activity of antioxidant enzymes).

Overall, the results presented in this work demonstrated the potentiality of *A. graecorum* extracts in protecting neuroinflammation/oxidative stress and could be used for a therapeutic effect *in vivo.*

Chapter 5: Conclusions and future prospects

Oxidative stress and inflammation are major pathophysiological features of neurodegenerative disorders. These diseases are incurable to date and are becoming increasingly prevalent in the global population, especially due to aging. There are no effective therapeutics to treat neurodegenerative diseases, despite the significant attempts to find treatments that can reduce the incapacitating symptoms of conditions such as Alzheimer's disease and Parkinson's disease. Due to this, it is important and of interest to find possible therapeutic approaches to prevent and treat these illnesses.

Natural products are already known for their active compounds and pharmacological bioactivities and are a potential approach to developing new ways of combating neuroinflammation and oxidative stress. Therefore, natural products have been demonstrated that can prevent and treat neurodegenerative diseases modulating microglia cells and inhibiting or reducing chronic inflammation as well as oxidative stress.

Through the presented work, the antioxidant, anti-inflammatory, and cytoprotective properties of a methanolic extract of *A. graecorum* leaves were evaluated, as well as the extract's phytochemistry.

The extract showed to have high flavonoid content in the HPLC analysis, due to the strong presence of quercetin and apigenin derivatives. On the antioxidant screening, the extract had good outcomes in the nitric oxide scavenging, obtaining an EC50 lower than the control used (ascorbic acid), proving, in a concentration-dependent manner, that can inhibit the NO radical accumulation. The extract also revealed strong DPPH scavenging activity, proving to be capable of donating an hydrogen and neutralizing the DPPH radical, but not as strong as the control used (Trolox). Additionally, superoxide scavenging activity was also demonstrated by the extract, although not as high as the control used (quercetin). Finally, the extract exhibited low iron chelating activity, obtaining an EC50 much higher than the control (EDTA). The extract also showed anti-inflammatory properties, inhibiting the COX-2 activity, more efficiently than the control (indomethacin). These properties may be essentially due to the high flavonoid content, thus this class of compounds is well-known for its antioxidant and anti-inflammatory features.

Regarding cellular assays, incubation of *A. graecorum* methanolic extract at concentrations of 250 and 100 µg/mL exhibited cytotoxicity in both BV2 and HepG2 cell lines, but at concentrations of 50 and 25 µg/mL, the extract did not exhibit toxicity. This happened equally in the 4 hours incubation assay and the 24 hours incubation assay. In addition, the extract exhibit protection in both cell lines against t-BHP insult, with better outcomes when pre-incubated for 20 hours, proving to have an effective indirect effect, possibly through

increasing the activity of antioxidant enzymes. Since the extract showed good outcomes in the assays performed in Chapter 3 and knowing its flavonoid content, it was expected that the extract incited protection from oxidative stress, due to its antioxidant and anti-inflammatory features.

Overall, *A. graecorum* showed to have promising potentialities for possible future therapeutic approaches in neuroinflammation treatment. To continue this work and develop more information about these bioactivities, it is proposed to: i) develop more sensitive techniques to characterize the phytochemistry of the extract, as HPLC-MS, providing a structural identity of the individual components; ii) study anti-inflammatory protection against LPS insult in BV2 cell line, with measurement of NO production; iii) perform an RT-PCR assessing the expression of several of pro-inflammatory genes (IL-6, iNOS, TNF-α, COX-2, among others) in BV2 cells; iv) to evaluate the activity of enzymes as Acetylcholinesterase (AchE), SOD, CAT, and GSH to elucidate the mechanisms of extracts antioxidant activity.

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