UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Characterization of garlic (*Allium sativum*) extract and garlic vine (*Mansoa alliacea*) leaf extract, and their evaluation on the humoral response and antibacterial activity.

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniero Biomédico

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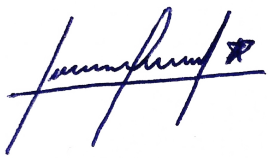

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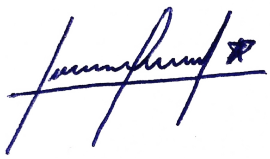

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Dedication

To my parents, Emith and Mario. Your selfless love and sacrifice have sustained me all these years. I hope I will measure up to your efforts.

To my brother, Ariel. You keep me grounded and inspire me to be better.

I dedicate this thesis to you.

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Resumen

Mansoa alliacea o ajo de monte, es una planta originaria de la Amazonía ecuatoriana que se reconoce por el olor a ajo de sus hojas y es utilizada por comunidades locales para tratar enfermedades. Sin embargo, existe un déficit de conocimientos sobre sus posibles aplicaciones biomédicas. En particular, sus propiedades inmunológicas casi no se han investigado y faltan estudios en modelos mamíferos. El presente trabajo pretende investigar la composición química y las actividades biológicas del ajo de monte, comparándolo con el ajo común (*Allium sativum*), debido a su aroma similar y a que el ajo está mejor estudiado.

Se prepararon tres extractos etanólicos a partir de las hojas de ajo de monte y los dientes de ajo: ajo de monte macerado (M-GV), ajo de monte por soxhlet (S-GV) y ajo macerado (M-G). El análisis fitoquímico cualitativo indicó el solapamiento de algunos fitocompuestos entre las dos especies. Los espectros FTIR y los cromatogramas de HPLC evidenciaron que compartían varios grupos funcionales, aunque M-GV exhibió una mayor diversidad. Los tres extractos no manifestaron citotoxicidad en células de fibroblastos embrionarios de ratón 3T3 a concentraciones de 5 y 10 g/mL. Además, no se registró actividad antimicrobiana contra las cepas *Escherichia coli* ATCC 25922 y *Staphylococcus aureus* ECBI-UITEY hasta la máxima concentración probada de 20 mg/mL de los extractos. DMSO al 5% mejoró mínimamente su actividad antimicrobiana pues mejoró la solubilidad de los extractos. Por último, el análisis de la respuesta humoral por el ensayo de ELISA indirecta mostró que M-GV fue el más inmunogénico, seguido de S-GV, lo que significa que el ajo de monte superó al ajo en inducir una mayor respuesta humoral. Además, se demostró la completa reactividad cruzada de los extractos por ELISA indirecta.

Palabras Clave: *Mansoa alliacea*, *Allium sativum*, respuesta humoral, actividad antimicrobiana, citotoxicidad.

Abstract

Mansoa alliacea, also known as garlic vine ("ajo de monte" in Spanish) is a plant native to the Ecuadorian Amazon that is easily recognized by the garlic-like odor of its leaves and is used by its local communities to treat a variety of illness. However, there is still an important knowledge deficit regarding this plant's potential biomedical applications. In particular, their immunological properties have been minimally researched, and there is a lack of data on mammalian models. The present work aims to investigate the chemical composition and biological activities of garlic vine, comparing it with common garlic (*Allium sativum*), due to their similar aroma and the fact that garlic is extensively studied.

Three ethanolic extracts were prepared from garlic vine leaves and garlic cloves: macerated garlic vine (M-GV), soxhlet garlic vine (S-GV) and macerated garlic (M-G). The qualitative phytochemical analysis indicated the overlap of some phytocompounds between the two species. The FTIR spectra and the HPLC chromatograms showed that they shared several functional groups, although M-GV exhibited more diversity. The three extracts showed no cytotoxicity on 3T3 mouse embryonic fibroblast cells at concentrations of 5 and 10 µg/mL. Furthermore, no antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ECBI-UITEY strains was registered up to 20 mg/mL concentration of the ethanolic extracts. 5% DMSO minimally improved their antimicrobial activity by improving the extract's solubility. Finally, the humoral response analysis showed that M-GV was the most immunogenic, followed by S-GV, which means that garlic vine surpassed garlic in inducing a higher humoral response. In addition, complete cross-reactivity of the extracts was demonstrated.

Keywords: *Mansoa alliacea*, *Allium sativum*, humoral response, antimicrobial activity, cytotoxicity.

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Chapter 1

Introduction

The use of plants has been key to the development of human communities. It is rather difficult to imagine an aspect of a person's life in which a plant would not be used. In fact, they have been utilized to improve human health and alleviate disease for centuries before the appearance of modern medicine [1]. Although traditional medicine is far from extinct, its continuity is fragile as it is generally transmitted from generation to generation orally. Surely, a lot of people around the world may not be aware of this enriching knowledge. Still, folk medicine is nowadays the primary source of health care for many communities, especially rural ones [2].

Many species are widely known for their medicinal purposes. Nevertheless, garlic must be one of the most popular and globally distributed. Hippocrates, 2300 years ago added it to its list of "medicinal food" directed to the prevention of diseases [3]. Since then, its various bioactivities beneficial to humans have been extensively studied. Nowadays, garlic can be found in many forms of treatment that promise to alleviate various illnesses. Many plants aspire to match the prolific nature of garlic. Consequently, *Allium Sativum* is included in this work in order to compare it to the lesser-known species.

Ecuador, as a biodiverse country, has managed to provide local communities with plenty of plant species to establish their traditional medical practices. Indigenous people of the Ecuadorian Amazon have their medicinal practices carried out typically by the shaman or healer [2]. The most used species by amazonian communities for medicinal purposes are *Croton lechleri* Müll ("sangre de drago"), *Ilex guayusa* Loes (guayusa), *Banisteriopsis caapi* (ayahuasca) and *Mansoa alliacea* ("ajo de monte") [4]. From them, the less recognized by

the general public may be the "ajo de monte" but it is equally utilized as treatment for various illness. Although it may not be as popular as other species its inclusion in traditional medicine of the Amazon suggest the need of more research about it.

1.1 Species overview

1.1.1 *Mansoa alliacea*

Mansoa alliacea is a species native to the Amazon rainforest, though not exclusive to it, that is highly valued by ethnic groups for its medicinal qualities. It takes many names but in Ecuador and Peru it is known as "ajo de monte" or "ajo sacha" due to the garlic-like odor of its leaves , while the more widely accepted names in English are garlic vine or wild garlic [5]. The taxonomic classification of this plant according to the Global Biodiversity Information Facility is Kingdom: Plantae, Phylum: Tracheophyta, Class: Magnoliopsida, Order: Lamiales, Family: Bignoniaceae, Genus: *Mansoa* DC., Species: *Mansoa alliacea* (Lam) A.H.Gentry [6].

In Pastaza, Ecuador, a survey revealed that garlic vine is among the most frequently used plants in their traditional medicine, employed to address colds, serve as an anesthetic, and alleviate muscle pain [4]. In Guiana, decoctions of stems and leaves are applied externally to treat symptoms like aches and muscle exhaustion [7]. Like this, other sources also show the role of garlic vine in the folk medicine of various communities as anthelmintic [8], analgesic [9, 10], antimalarial [5, 11], treatment for muscle pain [5, 4, 9], rheumatism [5, 10, 12], colds and fevers [5, 7, 13, 14], and other illnesses. The traditional uses of *M. alliacea* have sparked various investigations seeking for the biological activities that may be behind these beliefs.

1.1.2 *Allium Sativum*

Allium sativum, commonly referred to as garlic or "ajo" in Spanish, is well-known as a valuable spice and a prominent cure for a number of ailments across the world [15]. Kingdom: Plantae, Phylum: Tracheophyta, Class: Magnoliopsida, Order: Asparagales, Family: Amaryllidaceae, Genus: *Allium* L., Species: *Allium sativum* L. [16]. Originally from the Mediterranean and Central Asia, it is now in large-scale production all over the

world. [17].

Garlic is a good example of how the traditional knowledge is incorporated in modern medicine. Since ancient times Babylonians, Egyptians, Phoenicians, Greeks and Romans viewed garlic as a treatment for various illness like tumours, intestinal diseases, respiratory affections and more; additionally, it was used in epidemic diseases as tuberculosis and cholera before the appearance of antibiotics [18]. Garlic has a higher concentration of sulfur compounds (mainly allicin), which are responsible for its therapeutic properties [19]. Nowadays, garlic remedies can be found in capsules, oils, syrup, and so on. They are usually employed as treatments for high blood pressure, high cholesterol, coronary diseases, some types of cancer, diabetes, fungal infections, immune depression, rheumatism, asthma, and a lot of other affections [20, 21, 22, 23, 24, 25].

1.2 Chemical composition

Mansoa alliacea

In general, there are only a handful of studies that describe the chemical composition of this species. In a methanolic extract of the flowers of the garlic vine, allicin has been found, along with β-amyrin, apigenin, apigenin-7-glucoside, apigenin-7-glucuronide scutellarein-7-glucuronide, apigenin-7-glucuronyl glucuronide, apigenin-7-O-methyl glucuronide, cyaniding-3-rutinoside, ursolic acid, and luteolin [26]. Furthermore, volatile constituents, such as diallyl disulphide and of diallyl trisulphide have been discovered in garlic vine distilled oil [27, 28]. In another work, two cytotoxic naphthoquinones were obtained from the wood of *M. alliacea* by chromatographic purification: 9-methoxy-α-lapachone and 4-hydroxy-9-methoxy-α-lapachone [29].

More aligned with the objectives of this work, there is a study made with ethanolic extract in which they found by UHPLC and mass spectrometry (MS) the following phenolic compounds: chlorogenic acid, vanilic acid, caffeic acid, ρ-coumaric acid, ferulic acid and, trans-cinnamic acid, luteolin, apigenin and rutin [30]. Similarly, from an hydroethanolic extract of the leaves using a UHPLC-MS/MS system, all the phenolic compounds identified are consistent with the mentioned above [31].

Allium Sativum

The traditional use of garlic is more widespread and ancient compared to that of garlic vine. It has been investigated that the principal bioactives in garlic are the sulfur compounds like allicin and diallyl disulfide which are detected in high concentrations in its cloves [19]. More sulfur compounds are found in *A. Sativum* than in any other species of *Allium* [32]. The pungency and spicy flavor characteristic of this species are attributed to their presence [33].

To elaborate more, some sulfur containing compounds present in garlic are ajoenes (E-ajoene, Z-ajoene), thiosulfinates like S-allyl-cysteine sulfoxide (allicin), vinyldithiins (2-vinyl-(4H) -1,3-dithiin, 3-vinyl-(4H)-1,2-dithiin), sulfides (diallyl disulfide (DADS), diallyl trisulfide (DATS)) [33, 34, 35]. The smelly substance that causes garlic breath, allyl mercaptan, is produced when diallyl disulfide or allicin reacts with cysteine when S-allymercapto cysteine is present [36]. The primary precursor of allicin, which makes up over 70% of the total thiosulfinates found in crushed cloves, is allin [37]. It is important to note that *Mansoa alliacea* and *Allium Sativum* coincide in the presence of sulfides, which may explain the similarity of their odors.

In addition to sulfur compounds, garlic contains 17 amino acids and their glycosides, including arginine and others; enzymes like allinase, peroxidases, myrosinase; as well as trace minerals such as selenium, germanium, tellurium, and others that may contribute to its health benefits [38]. Overall, the components of *Allium Sativum* are varied and well researched.

1.3 Bioactivity

Mansoa alliacea

Extracts from this species have been evaluated for a range of activities. For instance, it is suggested that the plant may have anti-carcinogenic activity. In a study conducted with a water extract of its leaves, higher concentrations of the extracts seem to differentially target T3-HA mouse cancer cells, inhibiting their growth and diminishing their populations compared to the non-cancerous cell line [9]. Moreover, it was mentioned before that the leaves are often used as an analgesic for general or muscular pain. In an inflammatory pain

model, created to emulate arthritis, which was achieved through intraplantar injections of complete Freund's adjuvant (CFA), the treatment with *M. alliacea* water extract prevented and reversed the CFA-induced mechanical allodynia, thermal hyperalgesia but not the CFA-induced edema and myeloperoxidase activity [31]. Those are nociceptive parameters that indicates a moderate action of the extract against pain.

Further examples of bioactivities associated with the garlic vine have been explored. For example, at 500 mg/kg, the ethanolic extract of *M. alliacea* exhibited a notable hepatoprotective effect in a CCl_4 induced liver damage model on Wistar albino rats; more importantly, a much higher dose of the extract did not cause toxicity in the rats by measuring LD_{50} [39]. It also possess activities against fungi. For instance, there is a report of aqueous extract of *Adenocalymma alliaceum* (a synonym for *M. alliacea*) displaying antimycotic and antiaflatoxigenic activity against a wide range of fungi associated with biodeteroration of food [40]. Another interesting source, states that garlic vine ethanolic and aqueous extracts reduced the white blood cells count on concentrations that also not caused toxicity to Wistar rats [41]. This finding could lead to more elaborate research about the potential of this species as a leukemia treatment.

Allium Sativum

As mentioned before, this species is proven to have multiple medicinal properties. For example, garlic has antidiabetic properties showed by these compounds: allyl propyl disulfide, allicin and cysteine sulfoxide. They allegedly enhance the short-acting insulin production [42]. The garlic is also said to possess antihypertensive properties. On a study, this was approached by proving the capacity of *A. sativum* to inhibit the activity of an important enzyme for this disease, they believe that the compound in the garlic that contribute to that activity is Gamma-glutamylcysteine [25].

Among its many valuable properties, one worth mentioning is its antiparasitic capability, as it can aid in the response to protozoan infections by preventing the parasite's RNA [43]. Furthermore, in addition to its antiparasitic properties, it is widely recognized for its anti-inflammatory attributes. For instance, in rat models of acute lung injury that were probably generated by lipopolysaccharides, it suppressed the inflammatory response by blocking NF-κ B and peroxisome proliferator-activated receptor γ (PPARγ) [44]. Those

are a few examples out of many that illustrate the varied bioactivity of *A. Sativum* products.

1.3.1 Antimicrobial activity

Mansoa alliacea

There are conflicting reports regarding the antimicrobial properties of this species. An ethanolic extract of garlic vine leaves showed inhibition zones against four bacterial strains: the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, and the Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, starting at concentrations of 10 ug/mL dissolved in dimethyl sulfoxide (DMSO) [45]. Whereas, in another study, an ethanolic extract of the leaves did not replicate the results against *Escherichia coli* and *Staphylococcus aureus* at 100 mg/mL (without DMSO), in fact, no inhibition was displayed; however, it did show moderated to low activity against the Gram-positive *Listeria monocytogenes* and the fungus *Fusarium oxysporum* [46]. In this work, the *M. alliacea* leaf extract that presented better range of antimicrobial activity was the ethyl acetate extract [46].

Another work that is worth mentioning is a study made on *Mansoa difficilis*, another species of the Bignoniaceae family. The methanolic extract dissolved in DMSO did not showed antimicrobial activity in a minimum inhibitory concentration assay; nevertheless, the hexane extract did exhibited moderate activity against a few bacteria strains such as *S. aureus*. This finding supports the idea of the leaf alcoholic extracts of the Bignoniaceae species not having antibacterial activity [47].

Allium Sativum

The ethanolic extract of garlic has great evidence of being an antibacterial agent against many bacteria, including *Escherichia coli* and *Staphylococcus aureus*, there is rather an extensive literature supporting the antimicrobial activity of the different presentations of garlic, the different type of extracts as ethanolic, hexane, and of its products [48]. In another work, they also found that the ethanolic extract inhibited some bacteria, however, it is important to highlight that they found inhibition zones for the bacteria using a concentration of the extract of 100 mg/mL or above [49].

Furthermore, there are even various studies that address this topic about its individual components like allicin, organosulfur compounds, among others to discover that they also possess antibacterial activity. For example, in the work of Choo, they explore the allicin molecule capacity to inhibit microbial growth and talk about allicin being an important antimicrobial agent in garlic by describing possible mechanism of inhibition and so on [50]. Finally, it was found that most of the reviews points out the positive presence of antimicrobial activity in the alcoholic extracts of *A. sativum*.

1.3.2 Cytotoxicity

Mansoa alliacea

There is not a lot to say about the cytotoxic effects of garlic vine as it has scarcely been studied. It is for this reason that this section will begin discussing about another species of the *Mansoa* genus, *Mansoa hymenaea* (DC.) A. Gentry. In this work relative to inflammation, antinociception and cytotoxicity of *M. hymenaea*, they found that the species methanolic extract indeed exerts a cytotoxic effect over a breast carcinoma (MDA-MB231) cell line, but fails to react with the other cancer cell lines; furthermore, not another type of cell line is tested to compare [51]. Another source states that *M. hymenaea* extracts show toxicity against a lung cancer cell line, also, out of the tested extracts, ethanolic extract is the most cytotoxic and they recommend further studies with other type of cell lines [52].

Now, in regards to the cytotoxic activity of *M. alliacea*, we first refer to a previous study that found the alcoholic extract to exhibit toxicity against the V-79 cell line and attributed it to two naphthoquinones they elucidated (4-hydroxy-9-methoxy-α-lapachone along with 9-methoxy-α-lapachone); however, it does not mention the concentration at which the alcoholic extract showed said toxicity [29]. Moreover, the flower methanol extract of *M. alliacea* (alternatively called *Adenocalymma alliaceum*) exhibited the capacity to inhibit the growth of MCF-7 (ER +ve) and MDA-MB-231 (ER –ve) breast-adenocarcinoma cells by means of apoptosis; the study focused exclusively on cancer research so no other cell lines were tried [53]. To give another example, the ethanolic extract demonstrated a minimum level of toxicity against a non cancer cell line but it was capable of protecting the pancreatic β-cells [54] from damage. Another source also corroborates this hepatoprotective effect in

studies of ethanol induced toxicity on HepG2, a hepatic cell line [55]. These are virtually the only reports on the interaction of *M. alliacea* extracts with cells, whether the effects are cytotoxic or cytoprotective. The lack of reviews on this topic demonstrate the premature state of this specific field.

Allium Sativum

Various examples can be brought up when talking about this species. For instance, aqueous extracts of Spanish and Polish garlic demonstrated to be cytotoxic to carcinoma SCC-15 cells possibly by stimulating reactive oxygen species (ROS) production [56]. Other work on tumorous cell lines says that aqueous extracts of some varieties minimized the viability of melanoma B16F10 cells [57].

Another work studies the cytotoxic ability of a non polar extract of *A. sativum* against a cornea cell line; it did not show cytotoxic effects at 3.90 mg/mL [58]. Furthermore, a study compared the activity of alcoholic extracts on normal and tumor cell lines by calculating their half maximal inhibitory concentration IC_50; the extracts were toxic to the various cell lines they worked with [59]. What is more important, several reviews have included the activity of *A. sativum* on cells [60, 61], implying the abundance and significance of the results of this plant on this topic.

1.3.3 Humoral response

Mansoa alliacea

Unfortunately, the report on this segment is rather short. The immunology research on this species is underdeveloped despite its many uses in traditional medicine. Other areas have advanced, but the immunological response to this agent has not yet been studied. The only work that could be mentioned is one made on *Arapaima gigas* fish. They fed them diets that include hydroalcoholic garlic vine extract and then they tested them by infecting them with *Aeromonas hydrophila*; the fish fed with higher concentrations of the extract exhibited higher levels of leukocytes, lymphocytes and respiratory burst compared to the group of control. This indicates that *M. alliacea* improved the immune response of the fish [62]. Similar experiments on mammals have not been published.

Allium sativum

In an antileishmanial activity study, a mixture of extracts of mixture of *Tridax procumbens* and *Allium sativum* produced an increase in IgG2a/IgG1 ratio on BALB/c and CD-1 infected mice, which they say indicates a raise in the Th1-type immune response [63].

One important work related to this specific topic is one where they studied immunogenicity an adjuvanticity potenital of garlic. The two lectins that they purified from the raw garlic extract, (obtained by being suspended in PBS, homogenized, filtrated and then centrifuged) demonstrated to stimulate the humoral response (anti-lectin IgG); however, the most important finding was that the lectins and the raw garlic extra showed to be good mucosal adjuvants by producing a 4 or 6 fold increase in the humoral response to the weak immunogenic ovoalbumin (anti-OVA IgG) [64]. Similarly, another source states that a garlic product (aged garlic fructans), intranasally administered, was a good immunoadjuvant because it incremented the IgG response to OVA compared to OVA alone [65].

Chapter 2

Problem Statement

Over centuries of human civilization plants demonstrated to be critical to people health. Even in modern medicine, plants and traditional medicine often serve to spark scientific research and advance medicine further. For instance, the history of popular aspirin begins with a plant, willow bark, as it was used as a painkiller by Sumerians, Egyptians, Greek and Roman physicians; from there, scientists took inspiration in its constituents and synthesized the active ingredient in aspirin [66].

Mansoa alliacea is a treasured species among the locals in the Ecuadorian Amazon for its diverse properties [4]. The existing gap of knowledge about this plant's biomedical potential, in some fields more than others, is substantial. Therefore, there is a growing need to unfold the contributions to medicine that *Mansoa alliacea* could potentially provide. Moreover, the fact that its odor is so similar to that of the garlic raises several questions about their similarities about not only their chemical composition but also their biological activity. It would be relevant to study those possible similarities. A more specific area in which garlic vine is severely understudied is immunology. As described before, not a single study has yet investigated into the humoral response that this plant may arise in mammalian models. Exploring this aspect would constitute a novel area of research.

Overall, it has been identified the need to expand the knowledge about *Mansoa alliacea* bioactivity, especially about its immune response, while also uncover the plant chemical composition that may be taking part in said activities. Besides, the interest to compare it to the common garlic, which has countless properties, arise from the expectation of potential shared bioactive compounds.

Chapter 3

Objectives

3.1 General objectives

The present work undertakes the task of exploring *Mansoa alliacea* and *Allium sativum* biomedical potential. First, by analyzing the chemical composition with different techniques in order to discover how similar they are and which constituents may be of interest. Then, by performing some assays like antimicrobial activity and cytotoxicity to assess its properties, but more importantly by evaluating their humoral response in a mammalian model to target the most obvious knowledge gap.

3.2 Specific objectives

- To obtain ethanolic extracts of garlic vine and garlic which are the most common in the literature.
- Compare extraction methods for the less-known species and identify the most effective approach to each activity.
- To perform phytochemical qualitative analysis to roughly assess the phytocompounds present in each extract.
- Follow with chemical characterization techniques such as HPLC and FTIR to further elucidate the similarities between species.
- Determine the antimicrobial activity of each extract against Gram positive (*S. aureus*

ECBI-UITEY) and Gram negative (*E. coli* ATCC 25922) bacteria strains by using the agar disk-diffusion disk method.

- To evaluate the cytotoxicity of the extracts of garlic and garlic vine at various concentrations on 3T3 fibroblast cells employing the Roche MTT based colorimetric assay.
- Evaluate the humoral response in a murine BALB/c mice model with the extracts and collect the hyperimmune sera with antibodies against the extracts.
- To perform indirect ELISA tests to assess the IgG response caused by each extract compared to a preimmune serum from non treated mice.
- Evaluate the cross-reactivity between extracts using an indirect ELISA assay and infer common epitopes.

Chapter 4

Hypothesis

Based on preliminary literature revision, it can be expected from *Mansoa alliacea* and *Allium sativum* to at least share a few chemical components like allicin or sulphides since they are related to the garlic's pungent odor that is also present in the leaves of *M. alliacea*. Regarding to antimicrobial activity, reports on garlic vine support the notion of it not having significant activity; however, garlic should have antibacterial activity. Now passing onto cytotoxicity, both species have mixed reports about this feature. Nevertheless, there seems to be more literature supporting a very low cytotoxic capacity so that will be expected. Then, based on its traditional uses *Mansoa alliacea* may present immunogenicity. Also, *Allium sativum* should be immunogenic as its separated components are. Finally, cross-reactivity at some level is reasonable to anticipate due to previous reports on similar chemical constituents.

Chapter 5

Methodology

5.1 Ethanolic extracts

5.1.1 Garlic vine

5.1.1.1 Maceration method

Leaves from *Mansoa alliacea* were dried out in the oven at 40°C for 2 weeks. Then, an electric mill was used to grind the leaves into small pieces. The crushed leaves were soaked into absolute ethanol (96%) and put in the sonicator for 1 hour at 25°C. After that they were left to macerate for 2 weeks long with constant agitation from a magnetic stirrer.

Once the maceration time was completed, the mixture was filtrated to separate the leaves from the ethanolic extract. Following that, the extract was rotoevaporated to remove a good amount of the solvent. Finally, the extract underwent two rounds of freeze-drying until it was completely dry. This ethanolic macerated garlic vine extract will be referred from now on as M-GV.

5.1.1.2 Soxhlet method

From the two selected species, *Mansoa alliacea* is the more understudied. Thus, an additional method was used to obtain the extract and analyze it further. To start, 8 grams of dried and crushed leaves of garlic vine were placed in the extraction tube inside of a thimble made of filter paper. The extractor was connected to a 100 mL boiling flask that contained 30 mL of ethanol.

The extract obtained was rotoevaporated to remove the solvent. Later, it was freeze-

dried two times until it was dry. This ethanolic garlic vine extract obtained by soxhlet will be known in this work as S-GV.

5.1.2 Garlic

5.1.2.1 Maceration method

The extract was obtained from peeled garlic cloves. They were minced and soaked into absolute ethanol (96%), after that they were put into the sonicator for 1 hour at 25°C. Then, they were macerated for 2 weeks long.

Following that, the mixture was filtrated to separate the cloves fragments from the liquid extract. After that, the extract was rotoevaporated to remove as much solvent as it could. Finally, the extract was freeze-dried two times to ensure the complete elimination of the solvent. This ethanolic macerated garlic extract will be called by the name M-G.

5.2 Chemical characterization

Determining what may be the composition of the extracts is one of the main elements of this thesis. For that, three different approaches were considered: phytochemical qualitative assays, Fourier transformed infrared spectroscopy (FTIR) and ultra-high-performance liquid chromatography (UHPLC). Those three techniques will be useful in the assessing of the chemical composition of each extract.

5.2.1 Phytochemical qualitative analysis

The analysis consisted of various assays that evaluated the absence or presence of determined chemical compounds in the extracts. These kind of tests could provide a broad idea of the composition of the phytochemcials in the plant.

5.2.1.1 Steroids

Steroids presence was determined by the Liebermann –Burchard test [67]. In a tube, 1 mL of each crude ethanolic extract was mixed with 1 mL of chloroform and 1 mL of acetic acid. Finally, a few drops of concentrated sulfuric acid (H_2SO_4) were added. A brown ring between the two layers and color green and the top shows the presence of steroids.

5.2.1.2 Terpenoids

The Slakowski's test was chosen for this task [68]. In a tube, 1 mL of chloroform was combined to 1 mL of each extract. Then, a few drops of (H_2SO_4) were added. A red brick color appears to exhibit terpenoids in the sample.

5.2.1.3 Flavonoids (Alkaline reagent)

In a test tube, 1 mL of 2% NaOH solution and 1 mL of the crude extract of either from *M. alliacea* or *A. Sativum.* were combined. After a few drops of diluted hydrochloric acid were added, the bright yellow color that had formed went colorless, indicating the presence of flavonoids [68].

5.2.1.4 Flavonoids (Shinoda Test)

In a tube, a few drops of diluted hydrochloric acid were added to 1 mL of the extracts, then, 50 mg of magnesium followed. Colors like reddish pink, or brown color indicated the presence of flavonoids [68].

5.2.1.5 Alkaloids (Wagner's test)

To 1 mL of each of the different extracts, the Wagner's reagent was added drop by drop. If the sample was positive for alkaloids a reddish brown precipitate was formed at the bottom of the tube [68].

5.2.1.6 Carbohydrates

The Benedict reagent was utilized for this purpose [69]. Equal volumes of the reagent and the extract (1 mL) was mixed in a test tube and then heated for 5 minutes in a water bath. The formation of a brown precipitate indicate the presence of carbohydrates.

5.2.1.7 Saponins

The foam test was performed to assess the presence of saponnins [67]. A heavy shaking was applied to a test tube containing 1 mL of the crude ethanolic extracts of either garlic vine or garlic and 5 mL of distilled water. To determine if saponins were present it was necessary to look for the development of a stable foam.

5.2.1.8 Phenols

The ferric chloride test was used for this[67]. 1mL of a 2% $FeCl_3$ solution was combined with 1mL of the three crude ethanolic extracts in a test tube. Phenols and tannins were characterized by the mixture changing to a blue-green, black or less commonly, red color.

5.2.2 FTIR spectroscopy

The three extracts underwent analysis using Fourier transform infrared spectroscopy. The solid extracts obtained after the freeze-drying rounds were put in the sample holder for each analysis. The equipment utilized for this assessment was the Cary 360 FT-IR spectrometer with a diamond crystal and an attenuated total reflection (ATR) system in the 4000-650 cm^{-1} wavelength range. The solid samples interacted with the infrared radiation and the spectrometer collected the transmittance spectra.

5.2.3 UHPLC

The garlic vine and the garlic extracts were analyzed by ultra-high-performance liquid chromatography. The Dionex UltiMate 3000 UHPLC system with a UV/Vis detector at 220 nm wavelength and the Chromeleon software were employed. First, the three extracts were diluted again in type 1 water until approximately 10 µg/mL. Then, the solutions were filtered using a 0.22 µg filter unit. Flow rate was 1 mL/min and the injection volume was 20 µL. Moreover, the measurements were carried out at room temperature using a linear gradient elution of H_2O/CH_3CN (60:0) to (0:60) for 8 minutes.

5.3 Evaluation of the biological activity

5.3.1 Antimicrobial activity: agar disk-diffusion technique

The antimicrobial activity of the three extracts was tried out against two bacteria strains with the agar disk-diffusion method. One that is gram negative which was *Escherichia coli*, and one that is gram positive which was *Staphylococcus aureus*. The used strains were ATCC 25922 for *E. coli* and ECBI-UITEY for *S. aureus*.

5.3.1.1 Determining optimal bacterial concentration

The two strains were first cultured in Luria-Bertani (LB) liquid medium which was prepared and sterilized previously. For every 2 mL of medium, 20 µL of bacteria were added. Then, both culture tubes were left for 16 hours in a shaking incubator alongside a tube containing only LB medium to compare and ensure the cultures were not contaminated.

At this point, the bacterial density was not adjusted yet for optimal growth in an agar plate. For said task, the initial culture were measured for optical density OD_{600} at 625nm with the Thermo Scientific™ NanoDrop™ One to achieve a value of approximately 0.5 in the McFarland turbidity standards that corresponds to 1.5 x 10^8 colony forming units (CFU/ml). The absorbance registered in the Nanodrop must be between 0.08 to 0.1 using LB medium as blank. If the measurement was different from it the concentration was adjusted by the means of the dilution formula:

$$C_1V_1 = C_2V_2$$

Now that the absorbance of the solutions with the bacteria were set to at least 0.08 they were ready to execute the disk-diffusion technique on agar.

5.3.1.2 Agar disk-diffusion antimicrobial assay

This test consists in placing disks containing the solutions to be tested in a solid medium and then measure the inhibition zones. It assesses the sensitivity of the strains to the studied solutions. The experiment was made using the Mueller-Hilton (MH) agar. It was prepared and sterilized following the product instructions and placed in sterile plastic plates that were stored at 4 °C until needed. When the time came, they were labeled and marked for the disk placements.

In each plate, disks for each of the extracts (M-GV, S-GV, and M-G) were included, along with one containing antibiotic for positive control (ampicillin for *E. coli* and vancomycin *S. aureus*, both at 1 mg/mL). The extracts were diluted in type 1 water and assessed for antimicrobial activity against *E. coli* and *S. aureus* in 1 mg/mL, 10 mg/mL, and 20 mg/mL concentrations. After conducting several experiments, the need to dissolve the extracts with dimethyl sulfoxide (DMSO) was observed. Hence, one extra test was

made with a concentration of 20 mg/mL + 5% DMSO. The plate of this assay included an additional disk with 5% DMSO as another control.

Each bacteria strain was inoculated by soaking a sterile swab with the liquid bacterial cultures described earlier and seeding them in each agar plate. Afterwards, the disks, which were previously loaded with 10 µL of each component and dried, were placed carefully with sterile metal forceps onto the agar and pressed gently to ensure their adherence. The process was carried out under total asepsis using the Telstar AV-30/70 vertical laminar flow cabinet. The plates were put into the Heratherm™ Refrigerated Incubator for 24 hours at 37 °C after which the inhibitions zones were observed and measured with a caliper.

5.3.2 Cytotoxicity assay

This feature was tested with 5 µg/mL and 10 µg/mL for all three extracts (macerated-garlic vine, soxhlet-garlic vine and macerated-garlic). A positive and negative control were set to ensure the reliability of the assay. So one well was filled only with PFHM-II medium at 1X and other with 1X plus cells. Also, the whole assay was conducted in triplicate. Moreover, the design of the 96-well culture plate distribution is there to better illustrate it (Figure 5.1). Additionally, this experiment was carried out using the Cell Proliferation Kit I (MTT) by Roche Corporation following the manufacturer's protocol. The absorbance was read with the RT-2100C Microplate Reader. The procedure is described below.

A 3T3 fibroblast cell culture was preincubated in a medium supplemented with 1% of antibiotic and 10% of inactivated fetal bovine serum. Then, in order to culture the cells in the plate, they were seeded at a concentration of $5x10^4$ cells/well. That concentration was contained in the 50 µL destined for each well. For optimal growth of the cells, the wells were filled first with 50 µL of 1X medium, then, 50 µL of 4X medium, next, 50 µL of each extract solution and in the end, the 50 µL of cell culture. Cells were incubated at 37 °C and 5-6.5% CO_2 for 24 hours.

Subsequent to the incubation period, 20 µL of MTT reagent was added to each well. The plate was incubated again at 37 °C and 5-6.5% CO_2 for 4 hours. Measurements of spectrophotometrical absorbance were taken immediately using the microplate reader after the MTT reagent was added and after the 4 hours of incubation. After that, 80 µL of solubilization buffer were added into each well. The absorbance was also registered at

this final instance.

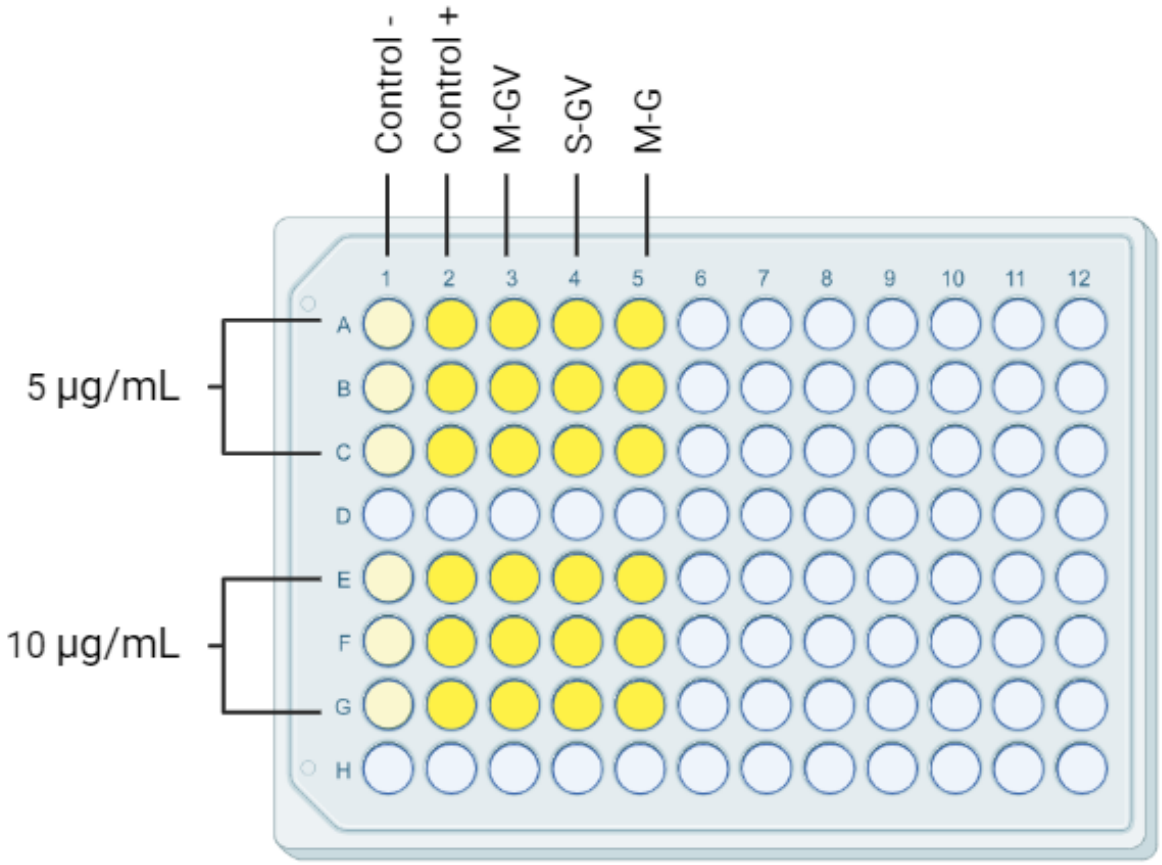


Figure 5.1: Distribution scheme for the cytotoxicity assay carried out in a 96-well plate. All the extracts were tested for two concentrations (5 µg/mL and 10 µg/mL). "Control -" refers to medium without cells and "Control +" to medium with cells and no treatment. Created with BioRender.com

5.3.3 Humoral response analysis

The experiment was performed to assess the humoral response using the extracts at 1mg/mL concentration as antigens: macerated garlic vine extract, garlic vine extracted with soxhlet and macerated garlic extract on BALB/c mice. Likewise, a control group of this animals was used to contrast the results.

5.3.3.1 Immunization of BALB/c mice and obtention of the hyperimmune sera

First, to obtain preimmune sera, blood samples from the control group (3 healthy male mice) were centrifuged in eppendorfs for 5 minutes at 3500 rpm with the Spectrafuge 24D. In contrast, to obtain the hyperimmune sera the mice were immunized with the extracts. To immunize each mouse, a mixture was prepared by combining 100 µL of each extract serving as the antigen with 100 µL of Complete Freund's Adjuvant (CFA). The mixture was then homogenized using a vortex until it formed an emulsion.

Each mouse of the three experimental groups was immunized via intraperitoneal with 200 µL of the emulsion. After the initial immunization, three additional doses were administered, each spaced one week apart. The protocol was repeated but the emulsions were prepared with Incomplete Freund's Adjuvant (IFA), instead of CFA, mixed with each extract at equal volumes. The mice were sacrificed in the fifth week in order to get blood from the coronary arteries. To obtain hyperimmune serum, the blood sample was centrifuged for 5 minutes at 3500 rpm after letting it rest for 20 minutes.

5.3.3.2 Indirect ELISA test using the plants extracts as antigens

First, the ideal conditions needed to be found so all the combinations between antigen concentrations (5, 10 and 20 µg/mL) and serum concentrations (1:100 and 1:200 dilutions) were tested. (Figure 5.2 shows the plates distribution). Besides, the absorbance was taken every 5 minutes until 50 minutes to find the ideal reading time. The tests followed the directions of the protocol by Voller and Bidwell [70].

To each well of the 96-well ELISA plates, 100 µL of the corresponding antigens diluted in carbonate-bicarbonate buffer (pH 9.6) were added and left to incubate overnight at 4 °C in a humid chamber. Then, the plates were washed by adding 200 µL of the washing solution (phosphate buffer solution (PBS) at 7.4 pH + 0.005% of Tween 20) per well and let it rest three minutes. The plates are washed three times in total. After that, the plates were blocked by adding 100 µL of bovine serum albumin diluted to 6% in PBS to each well. They were left to incubate in the Heratherm™ Refrigerated Incubator for 2 hours at 37 °C in the humid chamber.

After that, they were washed three times in the same way as before. Then, the hy-

perimmune sera and controls (PBS and preimmune sera) were added (100 µL per well) following the distribution showed in Figure 5.2. Then, they were incubated at 37 °C for 1 hour in the humid chamber. Later, three more washes were performed.

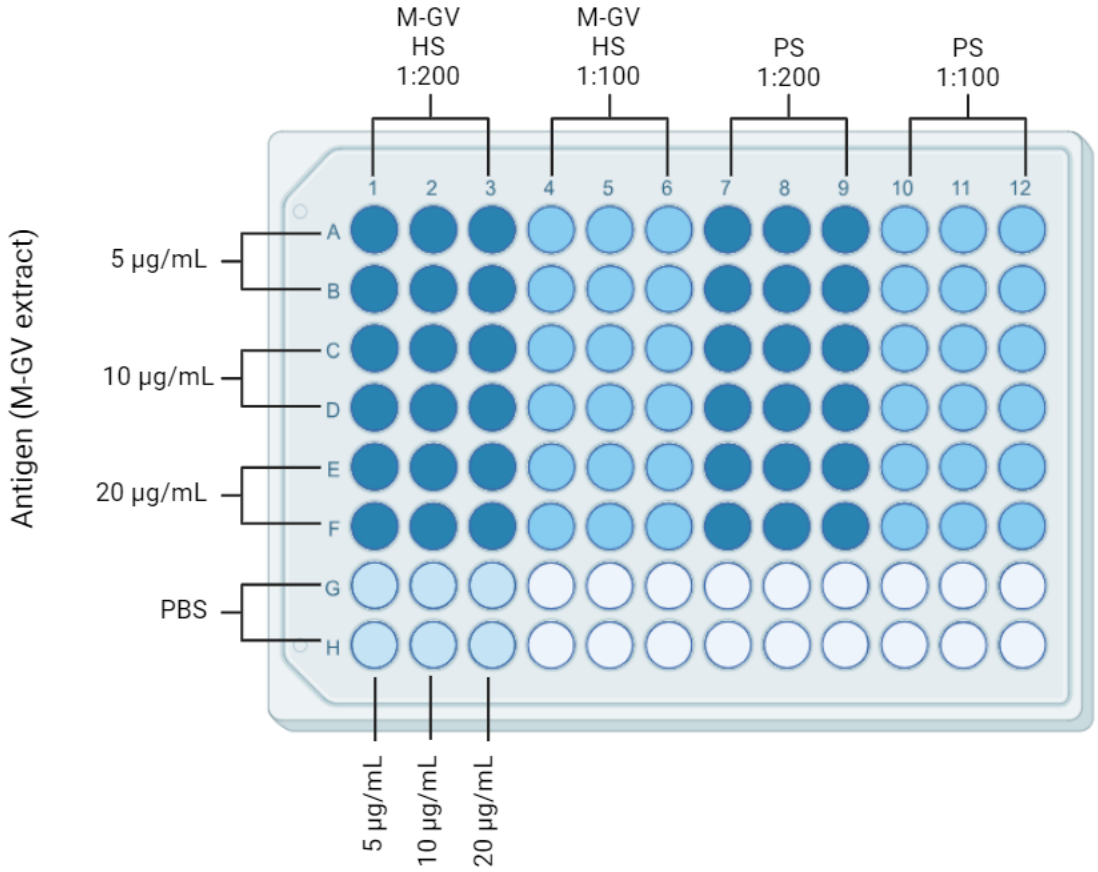


Figure 5.2: Distribution scheme for the standardization of the indirect ELISA test. Three different concentrations of the M-GV antigen were tried out against various concentrations of the hyperimmune and preimmune sera. The control in this experiment was PBS. The standardization was also performed for the other antigens (S-GV and M-G). Distribution was the same. (HS = hyperimmune serum; PS = preimmune serum). Created with BioRender.com

Following that, the conjugate anti-mouse IgG produced in goat with horse radish peroxidase (HRP), the secondary antibody, was added (100 µL per well). Then, they were incubated 1 hour at 37 °C. After that, three washes were required.

Finally, the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), also called ABTS substrate, was added (100 µL/well) after being activated with the required volume of peroxidase. It needed to incubate 20 minutes more in the dark at room temperature. After that, the absorbance was read at 405 nm every 5 minutes until 60 minutes. Between

readings the plates remained in the dark.

5.3.3.3 Cross-reactivity of the extracts using the hyperimmune sera

In this indirect ELISA assay, the antigens (M-GV, S-GV and M-G) were all tried against all the hyperimmune sera (M-GV HS, S-GV HS and M-G HS) and the controls to determine their cross-reactivity. The number of plates used were three and their distribution is described better in Figure 5.3. Furthermore, the ideal conditions previously obtained were used, which were 5 µg/mL for the three antigens, 1:200 dilution for each hyperimmune serum and reading time of 45 minutes. The protocol is exactly the same as the one in section 5.3.3.2.

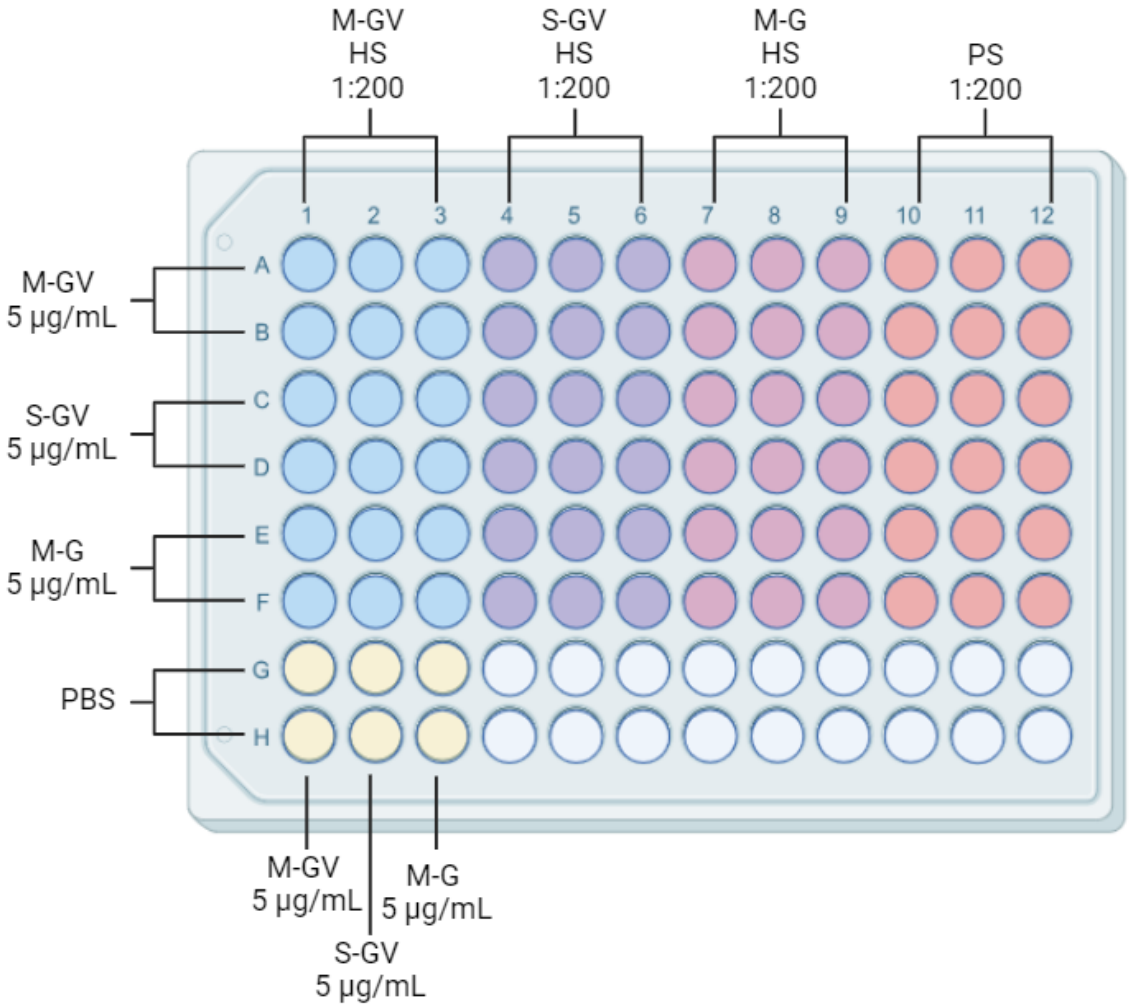


Figure 5.3: Distribution scheme for the cross-reactivity test. The choice of serum and antigen concentrations came from the standardization. (HS = hyperimmune serum; PS = preimmune serum). Created with BioRender.com

5.4 Statistical analysis

The analysis of variances (ANOVA) test is the most relevant statistical test for this work. ANOVA requires normality so the Shapiro-Wilks will be used to assess this information. In the case of a not normally distributed data, the Kruskal-Wallis test could be the ANOVA alternative. Lastly, when the ANOVA accepts the hypothesis that at least one of the variances is significantly different, a post hoc or multiple comparison test was employed (Turkey HSD) to determine which group or groups were the significantly different. The statistical analysis was applied specially to the MTT ctytotoxicity assay and the cross-reactivity assay. These statistical tests provide the confidence to interpret the results correctly.

Chapter 6

Results and Discussion

6.1 Chemical characterization

6.1.1 Phytochemical qualitative analysis

The phytochemical screening of the garlic vine and garlic macerated extracts are exhibited in Table 6.1. The screening of the garlic vine demonstrated the presence of flavonoids, alkaloids and phenolic compounds. On the other hand, the garlic extracts showed flavonoids only when tested with the alkaline reagent. Alkaloids, carbohydrates, and phenolic compounds were also identified alongside steroids, which were tested using the Salkowski test. In general, garlic macerated extract had greater phytocompound diversity. Also, garlic vine and garlic macerated extracts match in the presence of flavonoids, alkaloids, phenolic compounds and lack a visible presence of saponins and steroids. These findings disclose a similar chemical composition between both species.

Table 6.1: Phytochemical screening of *Mansoa alliacea* and *Allium sativum* macerated ethanolic extracts

Phytocompound	**Test**	**Species**	
		M. alliacea	***A. sativum***
Steroids	Lieberman-Burchard	-	-
Terpenoids	Salkowski	-	+
Flavonoids	Shinoda	+	-
	Alkaline reagent	+	+
Alkaloids	Wagner	+	+
Carbohydrates	Benedict	-	+
Saponins	Foam test	-	-
Phenolic compounds	Ferric chloride	+	+

Various sources agree that *M. alliacea* leaf ethanolic extract gives positive to flavonoids, alkaloids, proteins, glycosides and negative to saponins, whereas it is not clear about the presence of carbohydrates [39, 45, 71]. Also, a variety of phenolic compounds are present in *M. alliacea* ethanolic extract [30]. These reports agree with the results of this work.

On the other side, garlic ethanolic extract is supposed to present alkaloids, flavonoids and carbohydrates but the presence of tannins (polyphenols) and steroids is not clear in literature [72, 73]. In methanolic extracts of garlic alkaloids, flavonoids, saponins and proteins would be present, however tannins, steroids, and terpenoids may be present or not, the sources do not agree [74, 75]. All except for the results of the saponins test are replicated in this work. In regard of steroids and terpenoids, the tests were negative, and the test for phenols was positive.

6.1.2 UHPLC of the extracts

The chromatograms were obtained with a elution gradient of H_2O/CH_3CN (60:0) to (0:60) over 8 minutes. Figure 6.1 shows the UHPLC profile of the garlic vine macerated extract, Figure 6.2 corresponds to soxhlet garlic vine and Figure 6.3 to the garlic extract. Also, Table 6.2 presents the relative areas of each peak in M-GV, S-GV and M-G extracts. The relative area somehow quantifies the presence of the unknown compound in the sample relative to the other components.

Table 6.2: Summary of the peaks and its retention times (RT) of the HPLC chromatograms of the three different extracts

Peak	***M-GV***		***S-GV***		***M-G***	
	RT (min)	Area (%)	RT (min)	Area (%)	RT (min)	Area (%)
1	2.030	0.70	2.777	1.15	3.097	30.55
2	2.377	4.62	3.123	28.53	3.170	15.14
3	3.127	23.24	3.200	20.90	3.353	12.35
4	3.217	10.53	3.493	16.72	3.477	13.21
5	3.543	2.34	3.680	13.63	3.670	3.94
6	3.683	5.96	3.873	8.81	3.903	5.41
7	3.770	12.62	4.193	10.26	4.247	8.67
8	3.870	19.68	-	-	-	-
9	4.000	7.56	-	-	-	-
10	4.367	6.87	-	-	-	-
11	4.610	0.79	-	-	-	-
12	6.407	5.09	-	-	-	-

The most abundant constituent in each extract is around the same retention time for all of them. For M-GV, its 3rd peak at 3.127 min represents 23.24% of the sample. For S-GV, its 2nd peak at 3.123 min accounts for 28.53%. Similarly, M-G 1st peak at 3.097 min represents 30.55% of the extract. Other compound that seems to occur in all the extracts is the one around 3.68 min (5.96% for M-GV, 13.63% for S-GV and 3.94% for M-G). More so, from the seven peaks in the soxhelt garlic vine and garlic extracts are all close in retention time except for the first one. Whereas, although the M-GV extract showed more peaks, which allegedly means that it has a more diverse composition, it did have six peaks that are roughly similar in retention time to those in of S-GV and M-G (3, 4, 5, 6, 8 and 10). In spite of the fact that there is not information in literature that may point to the exact identity of those peaks or the others, those six peaks mean that the extracts may share 6 unidentified compounds.

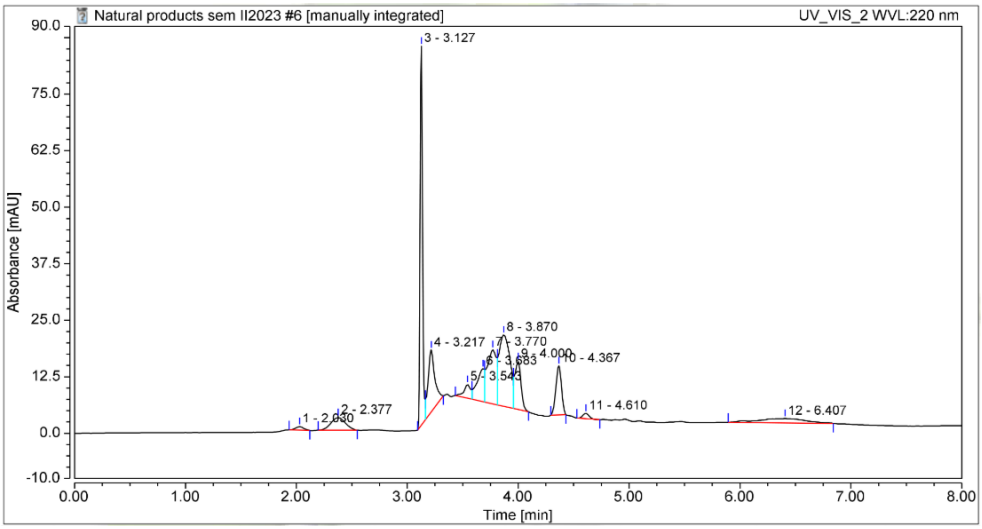


Figure 6.1: UHPLC profile of the garlic vine macerated extract.

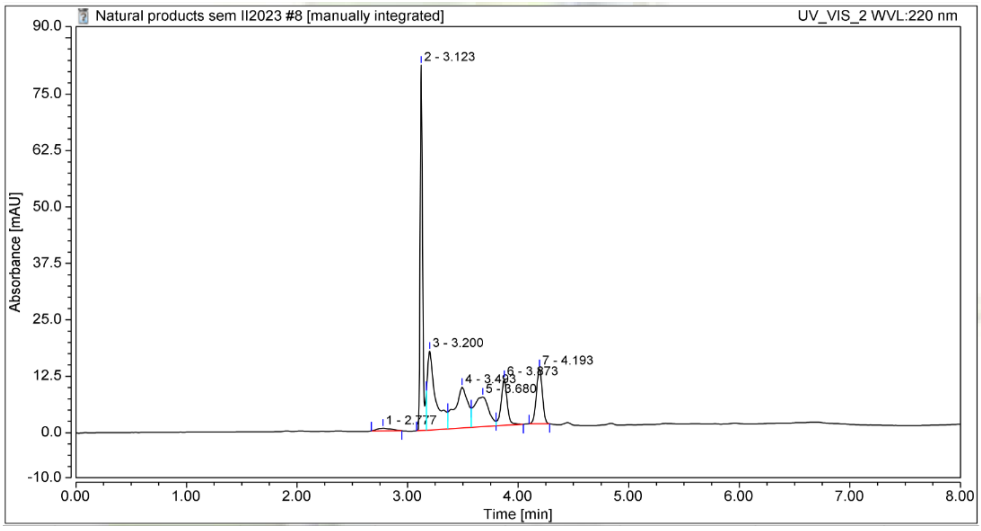


Figure 6.2: UHPLC profile of the garlic vine soxhlet extract.

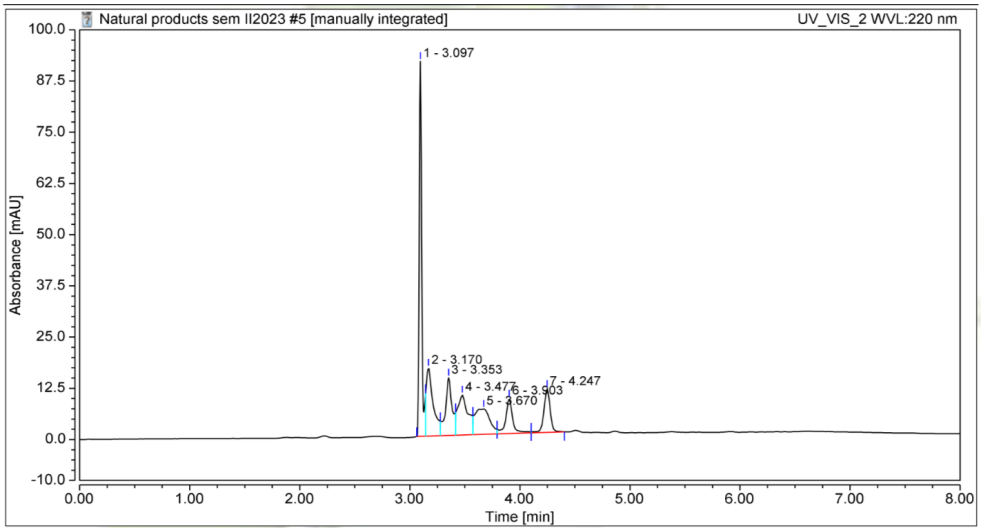


Figure 6.3: UHPLC profile of the garlic macerated extract.

6.1.3 FTIR of the plants extracts

The FTIR spectra of the extracts exhibited a certain degree of similarity (Figure 6.4), specially between the macerated and soxhlet *Mansoa alliacea* extracts compared to the macerated *Allium sativum* extract. Table 6.3 presents the most important peaks identified for each extract and paired them with their respective functional groups and vibrations. Overall, the principal functional groups identified are the same for every extract.

Table 6.3: FTIR analysis of the macerated garlic vine (M-GV), soxhelt garlic vine (S-GV) and macerated garlic (M-G) ethanolic extracts.

Extracts	**Wavenumber (cm^{-1})**	**Functional groups**	**References**
M-GV	3236	OH- stretching	[76, 77, 78]
	2924; 2852	-C-H stretching	[76, 77]
	1603	C=O stretching	[78, 77]
	1389	O-H bending	[79]
	1026	S=O stretching	[80]
S-GV	3360	OH- stretching	[76, 77, 78]
	2920; 2850	-C-H stretching	[76, 77]
	1618	C=O stretching	[78, 77]
	1387	O-H bending	[79]
	1029	S=O stretching	[80]
M-G	3258	OH- stretching	[79]
	2932	-C-H stretching	[79]
	1621	C=O stretching	[79]
	1404	O-H bending	[79, 81]
	1018	S=O stretching	[80]

The peak found around 3300 cm^{-1} is paired with a stretching OH- and it can be attributed to polyphenolic compounds or also be linked to flavonoids and non flavonoids [79, 76]. The -C-H in the peaks around 2900 cm^{-1} indicate the presence of aromatic compounds and the peak around 1600 cm^{-1} disclose the presence of C=O stretching related to carbonyl and carboxylic groups of peptide linkages[79]. The band found around 1400 cm^{-1} accounts for OH- bending in carboxylates [81]. Finally, the more important functional group founded in both species is the S=O (sulfoxides) because it denotes the presence of organosulfur compounds like S-allyl-cysteine sulfoxide (allicin) [79]. These compounds may beneficial to the bioactivity of the extracts considering the recognized qualities of allicin and the others organosulfur compounds.

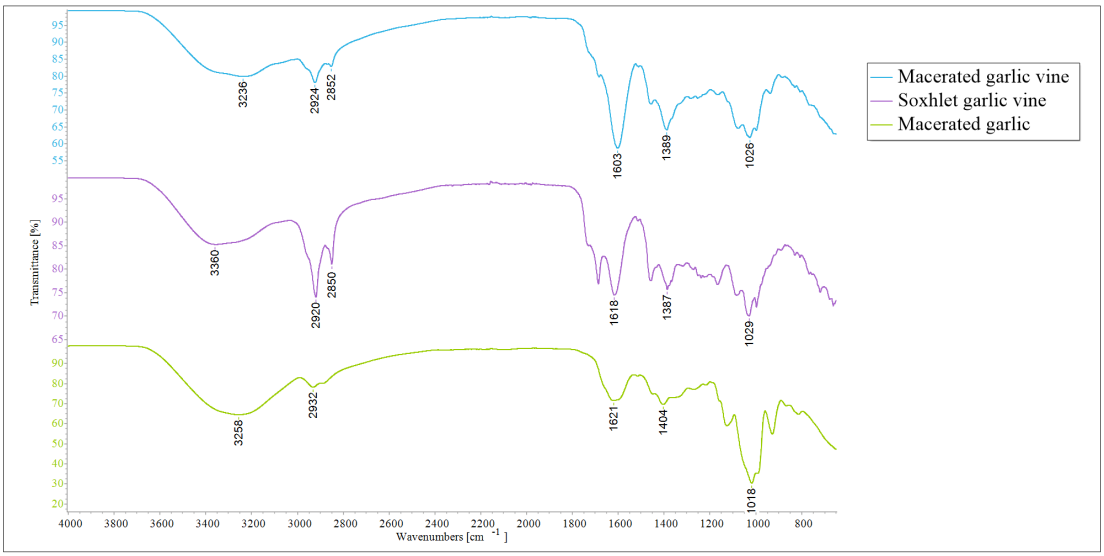


Figure 6.4: FTIR stacked spectra of the macerated garlic vine (M-GV), soxhlet garlic vine (S-GV) and macerated garlic (M-G) extracts.

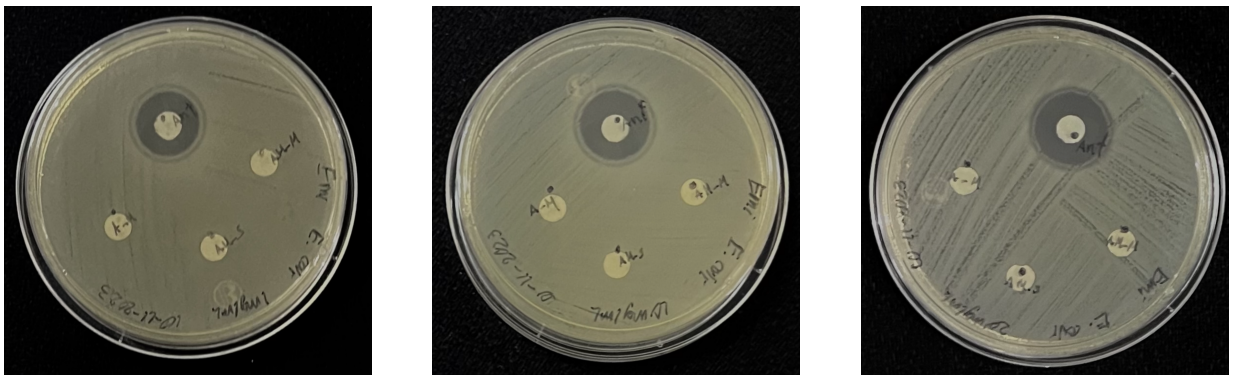
6.2 Biological activity

6.2.1 Antimicrobial activity assay

The agar disk-diffusion was performed in the Gram-positive *S. aureus* and the Gram-negative *E. coli* with different concentrations of the two extracts of *Mansoa alliacea* and the *Allium sativum* one. Besides, the antibiotics ampicillin for the Gram-negative bacteria and vancomycin for the Gram-positive bacteria were used as a control. The halo of vancomycin on *S. aureus* turned out considerable bigger than the one of ampicillin against *E. coli*. Certainly, it was attributed to the outer membrane present in Gram-negative bacteria but not in the Gram-positive one. Little to non antimicrobial activity was shown by the garlic and garlic vine extracts (Figure 6.5 and 6.6). Regarding the literature, there is a report of the ethanolic extract of *M. alliacea* inhibiting those bacteria species [45]. In other work, however, state that they did not achieve similar results even with 100 mg/mL concentration [46]. Our results agree more with the garlic vine absence of antimicrobial activity on *S. aureus* and *E. coli*.

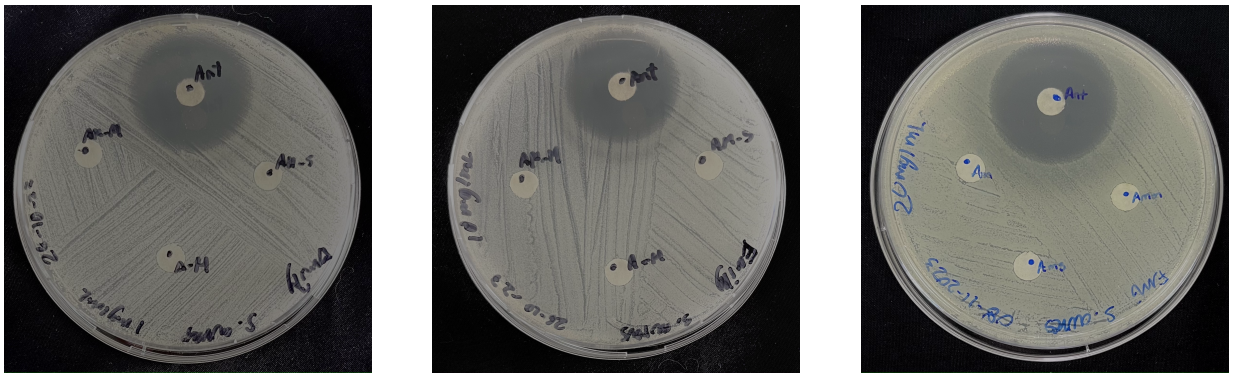
On the other hand, the lack of inhibition zone by *A. sativum* does not correspond with the previous studies mentioned earlier in introduction (section 1.3.1) which demonstrated antimicrobial activity. A low concentration of the extract may be the reason behind those

results. In the work of Enejiyon and coworkers they evaluated the same type of extract and they found antimicrobial activity at 100 mg/mL [49]. The maximum concentration in our work is only 20 mg/mL, five time less than that. This may be the reason to observing no antimicrobial activity when the control do show it (See Figures 6.5 and 6.6).



(a) 1 mg/mL (b) 10 mg/mL (c) 20 mg/mL

Figure 6.5: *In vitro* antimicrobial assay on *E.coli* ATCC 25922 using different concentrations of the extracts (1, 10 and 20 mg/mL). One disk with ampicillin as a control is included in each plate. Agents can be recognized in the plate the following way: AM-M ("ajo de monte macerado") = M-GV; AM-S ("ajo de monte soxhlet") = S-GV; A-M ("ajo macerado") = M-G; and, Ant = antibiotic (ampicillin).



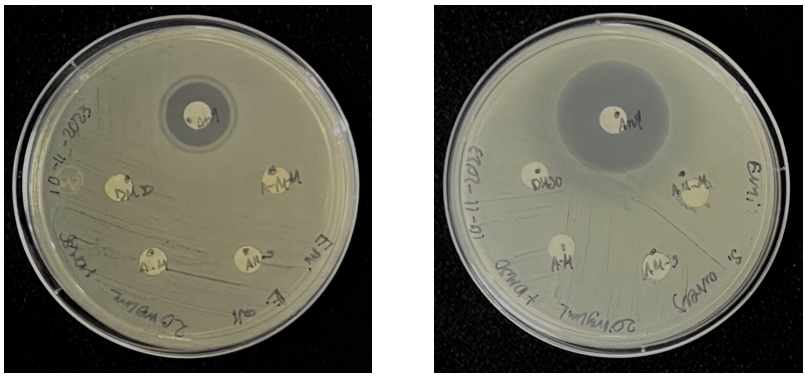
(a) 1 mg/mL (b) 10 mg/mL (c) 20 mg/mL

Figure 6.6: *In vitro* antimicrobial assay on *S. aureus* ECBI-UITEY using different concentrations of the extracts (1, 10 and 20 mg/mL). One disk with vancomycin as a control is included in each plate. Agents can be recognized in the plate the following way: AM-M = M-GV; AM-S = S-GV; A-M = M-G; and, Ant = antibiotic (vancomycin).

In a study that reports on garlic vine having antimicrobial activity, they use DMSO to dissolve their extract [45]. It may be that DMSO contributed with the antimicrobial activity and not the extract. To test this, M-GV, S-GV and M-G extracts were diluted

in just 5% DMSO. Also, a disk was added to the plates and loaded with a control of 5% DMSO as well.

The DMSO by itself did not show any antimicrobial activity (Figure 6.7) on both strains. Nevertheless, against *S. aureus* the M-GV extract at 20 mg/mL + DMSO did show a very small and irregular zone of inhibition Figure 6.7b. Similarly, the S-GV + DMSO presented an even smaller zone of inhibition. This did not happen with the *E. coli* strain. DMSO improved lightly the antimicrobial activity by dissolving the bioactive constituents of the extracts and making them more available since DMSO has a broad solubilizing capacity [82, 83]. Therefore, a higher concentration of DMSO may improve the antimicrobial activity by improving the dissolution of the extracts. However, DMSO is by itself an antibacterial agent at 5% and higher concentrations according to literature [84, 85]. That does not agree with our results at the same concentration. In addition, DMSO have proven to be toxic to human cell lines at lower concentrations than 5% so it should not be safe to use in biomedical applications [86, 87].



(a) *Escherichia coli* (b) *Staphylococcus aureus*

Figure 6.7: *In vitro* antimicrobial assay on *E.coli* ATCC 25922 and *S. aureus* ECBI-UITEY at a concentration of 20 mg/mL of each extract to which 5% DMSO was added. Each plate included one disk with antibiotic (ampicillin and vancomycin respectively) and one with only 5% DMSO. Agents may be recognized in the plate in the following way: DMSO = Dimethyl sulfoxide; AM-M = M-GV; AM-S = S-GV; A-M = M-G; and, Ant = antibiotic (ampicillin or vancomycin).

Overall, none of the extracts showed significant antimicrobial activity against *E.coli* ATCC 25922 and *S. aureus* ECBI-UITEY strains in concentrations up to 20 mg/mL. DMSO by itself does not register activity, but the addition of 5% DMSO to the extracts seemed to have aided the garlic vine extracts in inhibiting the bacteria by better dissolving

the active ingredients. Still, the inhibition zone with extract + 5% DMSO is really too small and difficult to measure (See Fig. 6.7b). The lack of clear inhibition zones by DMSO, which is antimicrobial at even lower concentrations than 5% [84], indicates some level of resistance from the strains, specially the *S. aureus* ECBI-UITEY strain that was isolated from a hospital patient.

6.2.2 MTT cytotoxicity assay

It was pertinent to analyze if any of the extracts would be cytotoxic. They were tried at concentrations of 5 µg/mL and 10 µg/mL with a positive (cell culture in supplemented medium) and negative control (medium alone). Also, in this assay the absorbance was measured at three different times in in order to determine the experiment optimal time of reading (0 h, 4 h and 4 h (after the solubilization buffer) at which the MTT reagent showed better results.

The best reading time was 4 hours (after the solubilization buffer) and the additional data can be found in appendix A. Furthermore, Figure 6.8 shows the results of the cytotoxicity assay at 4 hours (after the solubilization buffer). It is observed that the negative control, which was medium alone, has a smaller absorption which compared to the evaluated extracts is expected. The absorbances reported to each of the extracts at the different concentrations show little differences, specially in 10 µg/mL, where the S-GV treatment seemed to have resulted in less alive cells in relation to the positive control, and M-G the opposite (See Fig. 6.8). The positive control being a cell culture with no treatment. Nonetheless, statistically this differences may not be significative.

The statistical test that was used was the Kruskal-Wallis non parametric test. There is no significant difference in the viability of the cells between the cells treated with the extracts and the positive control (5 µg/mL: p=0.6955; $p > 0.05$ and 10 µg/mL: p=0.2960; $p > 0.05$). Therefore, none of the extracts is cytotoxic and they are safe for *in vivo* assays in experimental animals. This coincides with previous reports about garlic and garlic vine alcoholic extracts which state the low or no cytotoxicity of them [58, 54].

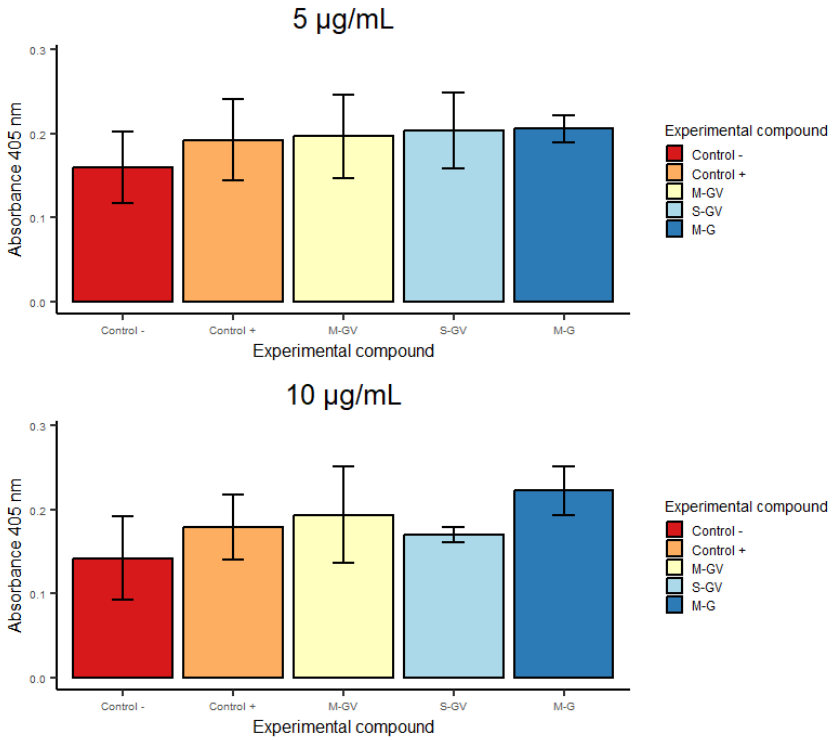


Figure 6.8: Cytotoxic assay on 3T3 fibroblast cells. It depicts the cytotoxic effect of macerated garlic vine (M-GV), soxhlet garlic vine (S-GV) and macerated garlic (M-G) extracts at 5 µg/mL and 10 µg/mL. It includes a negative, which is medium alone, and positive control, which is the same cell culture with no treatments.

6.2.3 Evaluation of the humoral response by indirect ELISA assays

The sera obtained from the immunization of the mice over 4 weeks with 1 mg/mL M-GV, S-GV and G-M extracts were evaluated afterwards with indirect ELISA tests using them as primary antibodies and the extracts as antigens to evaluate if the treatment with the extracts induced a significant immunoresponse dependent of antibodies (humoral response).

6.2.3.1 Standardization of the assay

First, the assay was tested with 5 µg/mL, 10 µg/mL and 20 µg/mL for each extract as antigens, interacting with the serum from immunized mice at 1:100 and 1:200 dilutions. Also, the absorbance was read every 5 minutes until 50 min to determine the optimal reading conditions of the assay. In the next figures, it is showed the evaluation of the indirect ELISA through the 50 minutes.

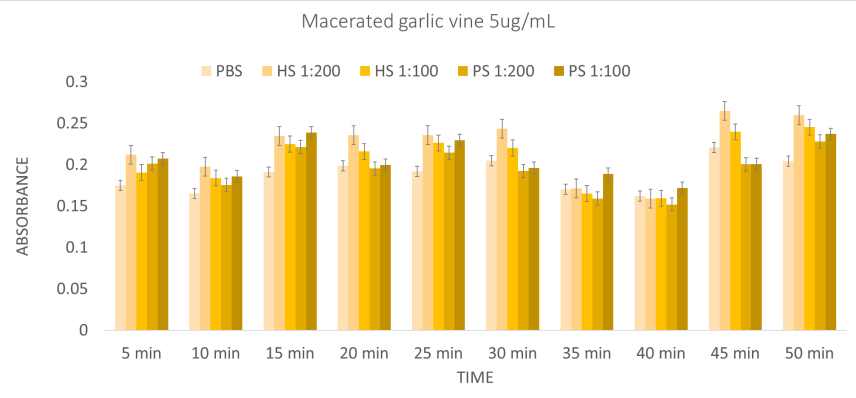


Figure 6.9: Indirect ELISA assay with the macerated garlic vine (M-GV) extract at 5 µg/mL interacting with its hyperimmune serum (HS M-GV) diluted to 1:100 and 1:200, adding PBS and preimmune serum (PS), which was also diluted to 1:100 and 1:200, as controls.

Figure 6.9 illustrate the experiment with M-GV extract at 5 µg/mL concentration, which was the one that gave the best results. The best conditions of this indirect ELISA assay can be observed with a hyperimmune and preimmune serum dilutions of 1:200 and at the time of reading of 50 minutes.

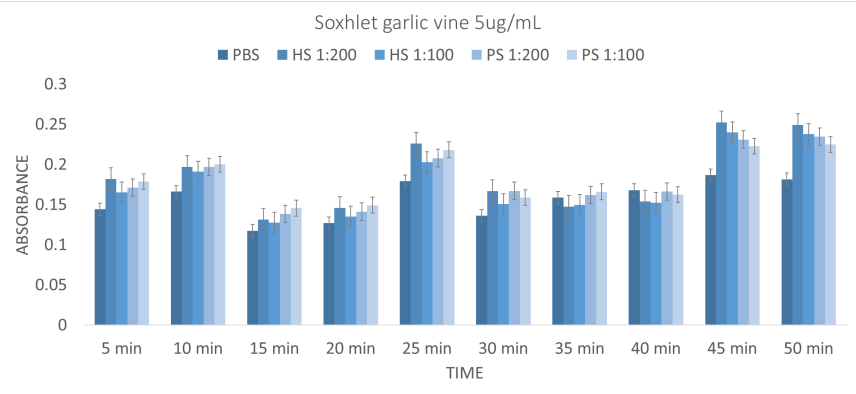


Figure 6.10: Indirect ELISA assay for the soxhlet garlic vine (S-GV) extract at 5 µg/mL interacting with its hyperimmune serum (HS S-GV) diluted to 1:100 and 1:200, adding PBS and preimmune serum (PS), which was also diluted to 1:100 and 1:200, as controls.

Similarly, Figure 6.10 presents the results of the assay with S-GV extract at 5 µg/mL concentration because it was best than 10 and 20 µg/mL. The best conditions were set to hyperimmune and preimmune serum dilutions of 1:200 and reading time of 45 minutes.

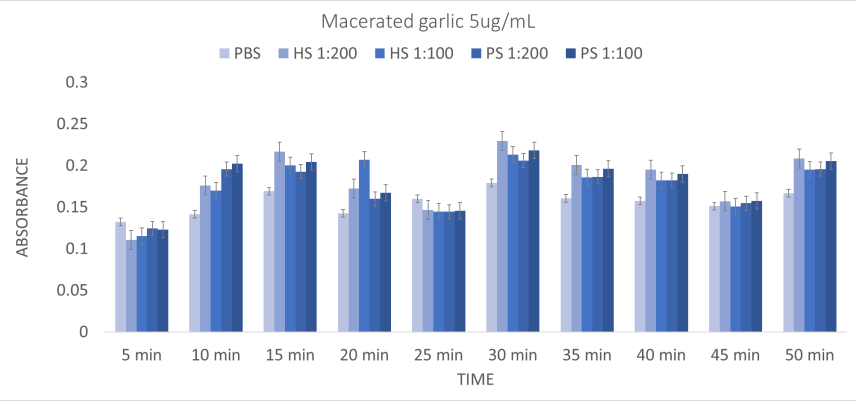


Figure 6.11: Indirect ELISA assay for the macerated garlic (M-G) extract at 5 µg/mL interacting with its hyperimmune serum (HS M-G) diluted to 1:100 and 1:200, adding PBS and preimmune serum (PS), which was also diluted to 1:100 and 1:200, as controls.

Finally, Figure 6.11 shows the results with M-G extract at 5 µg/mL concentration, the best out of them. The best conditions were determined at 1:200 for hyperimmune and preimmune sera and reading time of 30 minutes. The additional data of this standardization for 10 µg/mL and 20 µg/mL of each extract can be seen in appendix B.

6.2.3.2 Indirect ELISA test of the plant extracts

For the analysis of the ELISA results, first the Shapiro-Wilks test was used to prove the normality of the data. They all follow a normal distribution. Overall, PBS, as the negative control, is the lowest among all groups indicating good blocking conditions (6% albumin). It can be observed that for every antigen the hyperimmune sera (1:200) interacted more with it than the preimmune serum by exhibiting a greater absorbance (Fig. 6.12). The ANOVA test demonstrated that M-GV and S-GV hyperimmune serums were significantly different than the preimmune serum, which was the control (M-GV: p=0.00381; S-GV: p=0.001128; $p < 0.05$). This somehow agrees with the study made on *Arapaima gigas* fish that registered an increase in the immune response due to a macerated ethanolic extract of garlic vine incorporated in their diets [62]. On the other side, the M-G hyperimmune serum did not was statistically different even though it seemed liked it (p=0.27268; $p > 0.05$). This information contradicts the studies of Padiyappa and Chandrashekar that found garlic products to be immunogenic and even presented adjuvant effects [64, 65]. Those studies were made on purified products from garlic and not plain alcoholic extracts so it is possible that explains the results of the present work.

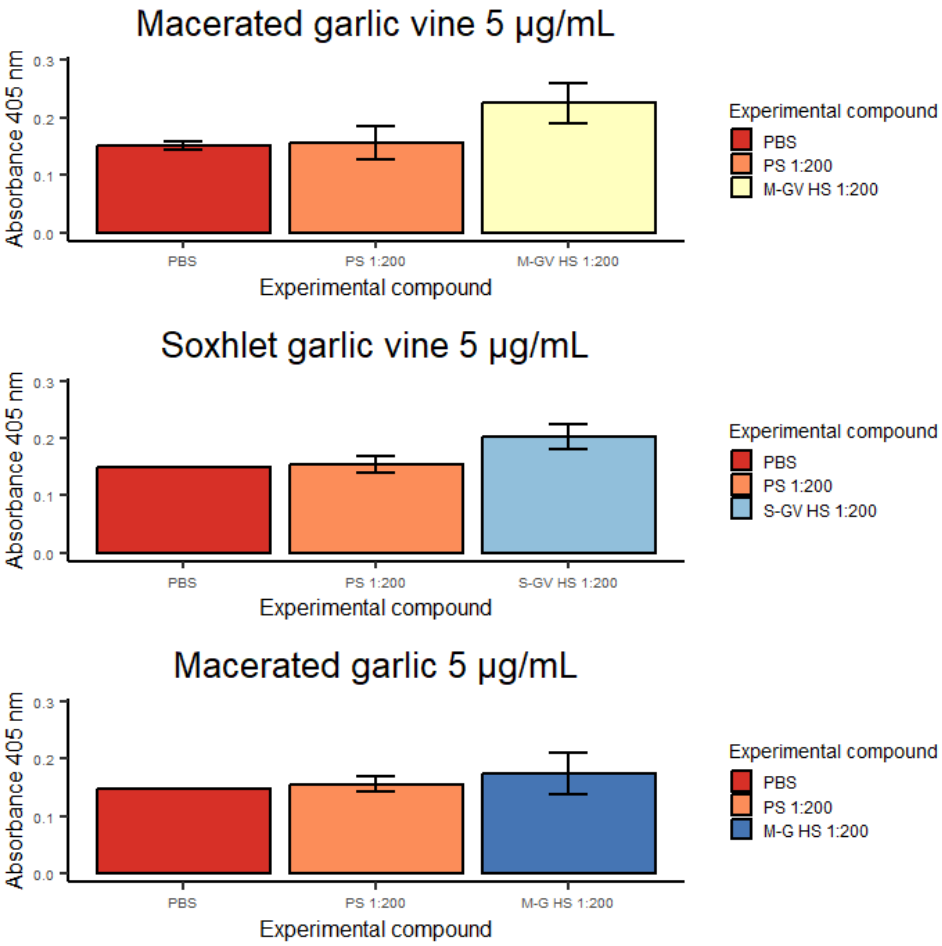


Figure 6.12: Indirect ELISA assay with the optimal conditions for each extract. On the X-axis are the experimental compounds, including the serums and PBS as control, that interacted with each antigen (M-GV, S-GV and M-G). whereas in the Y-axis is the absorbance at 405 nm.

The macerated garlic vine ethanolic extract is the more immunogenic out of them since it produced 1.5 times more IgG molecules than the control group and 1.3 times more than the garlic extract based on the absorbance values. Therefore, the less immunogenic is the macerated garlic extract. In addition, the absorbance value of M-GV hyperimmune serum seems to be higher than S-GV (See in Fig. 6.12. However, it is not significantly different (p=0.22502; $p > 0.05$). That means that the method of extraction did not make a difference in the humoral response of the mice. The additional compounds on the M-GV HPLC profile found in section 6.1.2 that could not be found in the other extracts profiles were not relevant to immunogenicity. All things considered, it can be confirmed with statistical confidence that immunization with the extracts resulted in a greater humoral

response (IgG), except for the M-G extract, as indicated by the increased absorbance. Overall, the data shows that garlic vine (*M. alliacea*) is more immunogenic in contrast to the common garlic (*A. sativum*). It would be interesting to take advantage of the immunogenicity of garlic vine and test if those extracts could have adjuvant qualities.

6.2.3.3 Cross-reactivity of the extracts

The cross-reactivity assay was made after with the optimal conditions to determine if the similar chemical composition that has been found in the two species (*M. alliacea* and *A. sativum*) would cause a crossed reaction in the immune system. The outcome of this assay is showed in Figure 6.13.

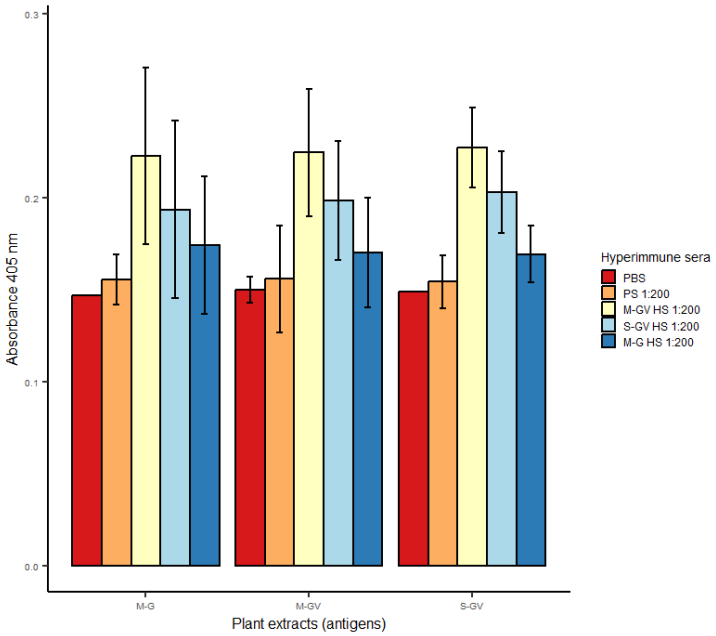


Figure 6.13: Indirect ELISA cross-reactivity assay. On the X-axis are the M-GV, S-GV and M-G extracts at 5 µg/mL, which acted as antigens against the obtained hyperimmune serums, whereas in the Y-axis is the absorbance at 405 nm. It incorporated controls of PBS and preimmune serum

It is observed in Figure 6.13 that the M-GV hyperimmune serum reacted similarly to all three of the extracts (antigens). Similarly, this was repeated for S-GV and M-G hyperimmune sera. Statistically, after normality verification using the Shapiro-Wilk test, the ANOVA test indicated that there were no significant differences in absorbance values (M-GV HS: p=0.9755; S-GV HS: p=0.9044 and, M-G HS: p=0.9499; p> 0.05).

Therefore, there were no significant differences in how each hyperimmune serum interacted with M-GV compared to how it recognized S-GV or M-G extracts. This suggests complete cross-reactivity between the extracts, as every hyperimmune serum recognized each antigen equally. As previously analyzed in the chemical section of this thesis, a good amount of the constituents of both species appear to be the same. Specially, it is inferred from the shared presence of the S=O bond revealed by the FTIR spectroscopy and the literature that the organosulfur compounds that concede both species their odor are candidates for being the common epitope and responsible for their biological activities [27, 28, 32, 33]. Furthermore, any differences in the chemical components that were exposed before did not cause the extracts to be more or less recognized by the IgG molecules.

Chapter 7

Conclusions

Mansoa alliacea and *Allium sativum* have similar chemical composition. The phytochemical qualitative assays of both species, only varied in the presence of carbohydrates or steroids. In the HPLC technique, the macerated garlic vine, soxhlet garlic vine and macerated garlic vine extracts had in common six peaks in the chromatogram that would suggest six compounds they share in their composition. However, the maceration method resulted in more peaks that indicate a more diverse composition. Also, FTIR spectroscopy showed the three of them practically having the same bands of absorption and were interpreted to represent the same functional groups. Among them S=O or sulfoxide is the most important finding because it points to the presence of allicin or other organosulfur compounds beneficial to biological activities. Thus, the method of extraction did not influence on the FTIR spectra.

Furthermore, none of the extracts showed antimicrobial activity against *E. coli* ATCC 25922 and *S. aureus* ECBI-UITEY strains in concentrations up to 20 mg/mL. The addition of 5% DMSO improved minimally the inhibition zones by the garlic vine extracts. The lack of inhibition by the DMSO as control indicated certain resistance from the bacterial strains.

Regarding the cytotoxic feature of the extracts, the results of the assay showed that the treatment with 5 µg/mL and 10 µg/mL of the extracts (M-GV, S-GV and M-G) did not cause a significant difference with respect to the control group. None of the extracts showed cytotoxicity on the 3T3 fibroblastoma cells used in this assay, suggesting that they are likely to be safe in *in vivo* assays.

The standardization of the indirect ELISA test set the optimal conditions to 5 µg/mL for every antigen (extract), 1:200 dilution factor for all of the hyperimmune sera (primary antibodies), but the best reading times were 50 minutes for M-GV, 45 minutes for S-GV and 30 minutes for M-G. Also, the blocking by 6% of albumin showed good results. The assay uncovered that two out of the three extracts induced a greater humoral response, M-GV and S-GV, but M-G did not raise statistically significant differences. Additionally, the macerated garlic vine extract was the more immunogenic, having a 1.5 fold increase in anti-IgG response with respect to the control group. The indirect ELISA assay did not show significant differences between M-GV and S-GV values which suggest that the method of extraction did not have an effect on the humoral response.

Finally, the cross-reactivity assay showed that every hyperimmune sera recognize the three different antigens (extracts) equally. Therefore, there is a complete cross-reactivity between the extracts, explained by the similar chemical compositions found in this work.

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Appendices

Appendix A

Determination of the optimal reading time for the cytotoxicity assay

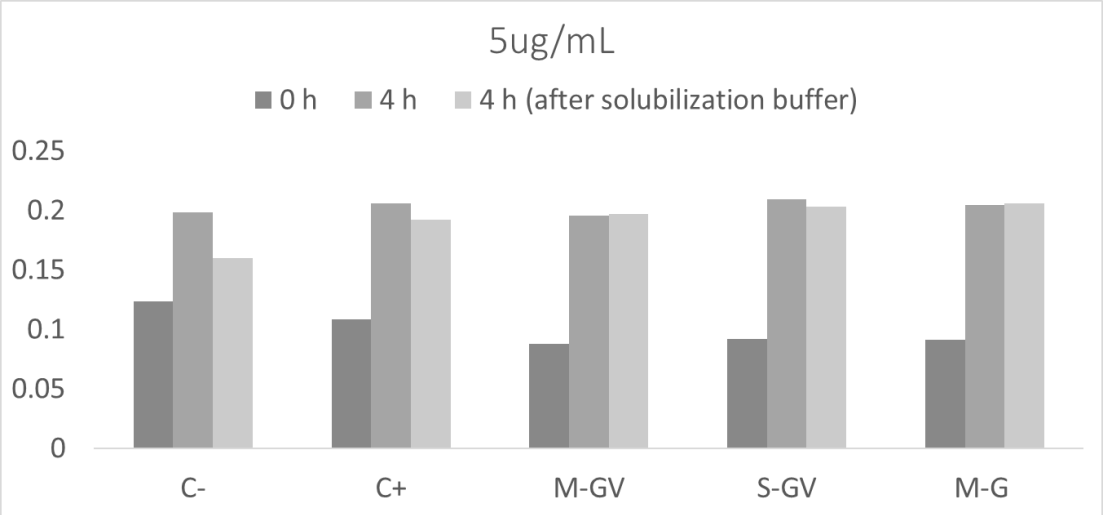

Figure A.1: Results of the cytotoxicity assay for all the extracts at 5 µg/mL. The absorbance was measure at 3 different times of the experiment to see which would be more fit to analysis. Positive and negative controls were included as well

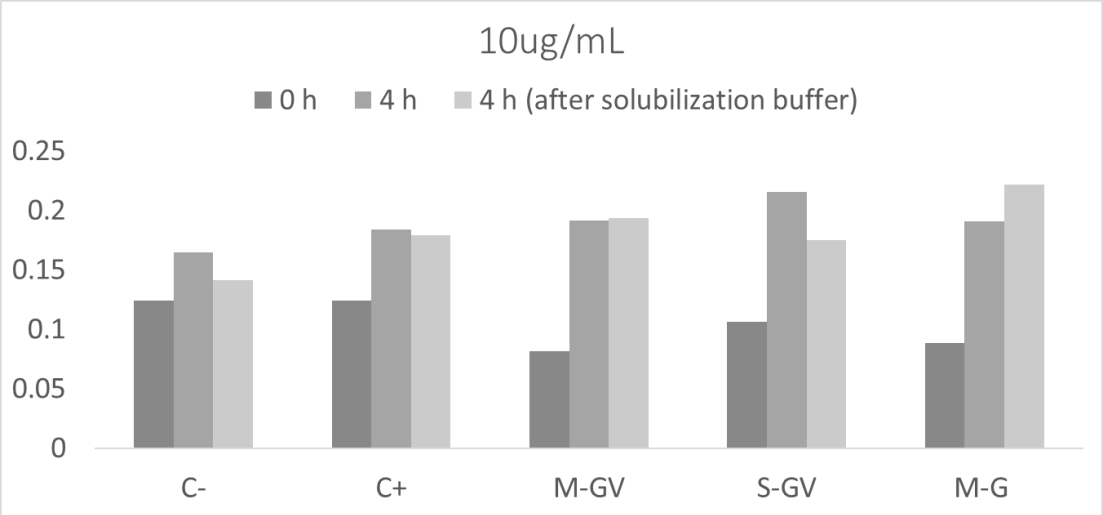

Figure A.2: Results of the cytotoxicity assay for all the extracts at 10 µg/mL. The absorbance was measure at 3 different times of the experiment to see which would be more fit to analysis. Positive and negative controls were included as well

Appendix B

Standardization of the indirect ELISA assay

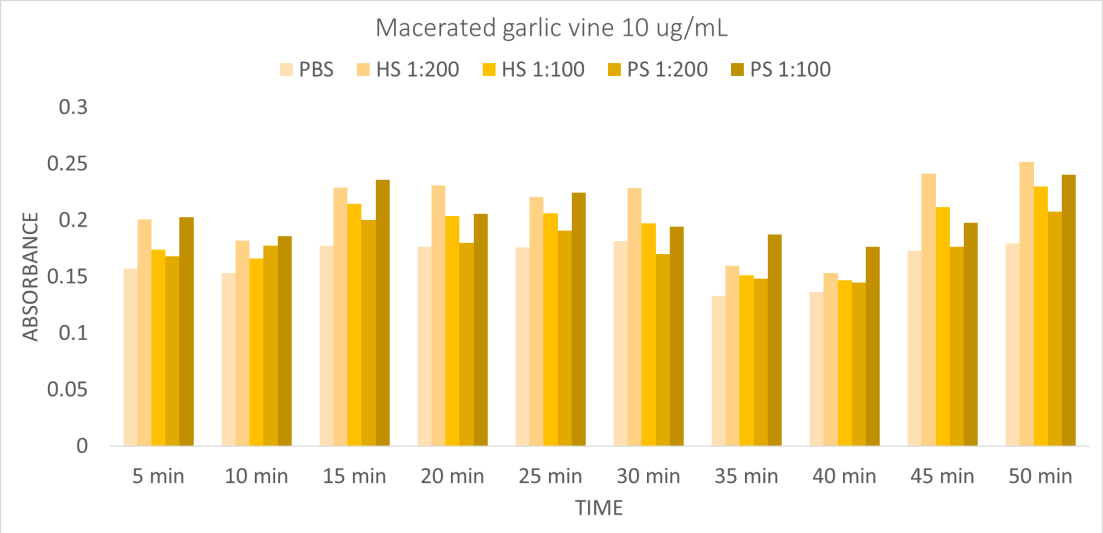

Figure B.1: Results of the indirect ELISA assay for the macerated garlic vine (M-GV) extract at 10 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.

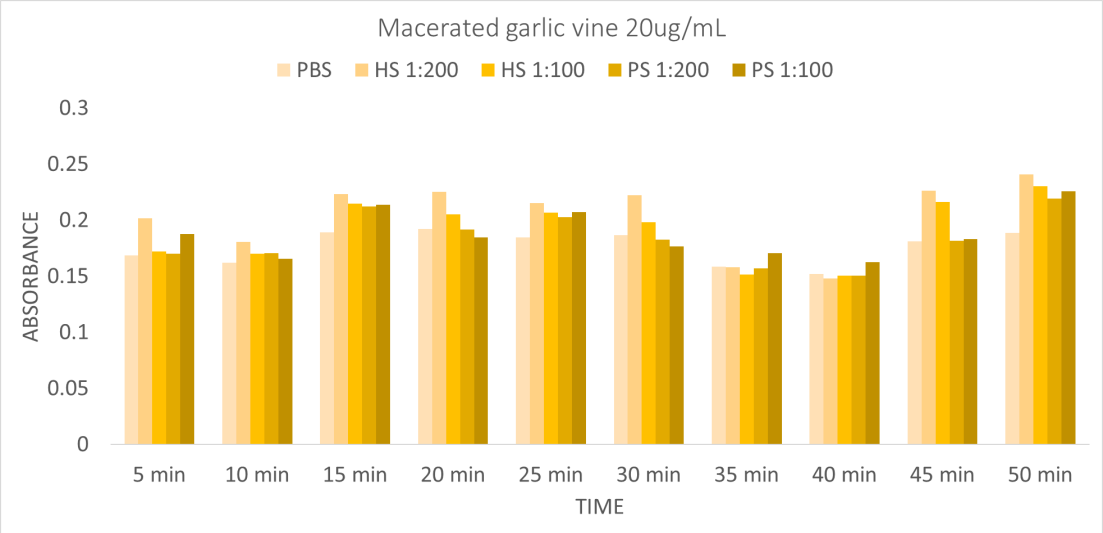

Figure B.2: Results of the indirect ELISA assay for the macerated garlic vine (M-GV) extract at 20 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.

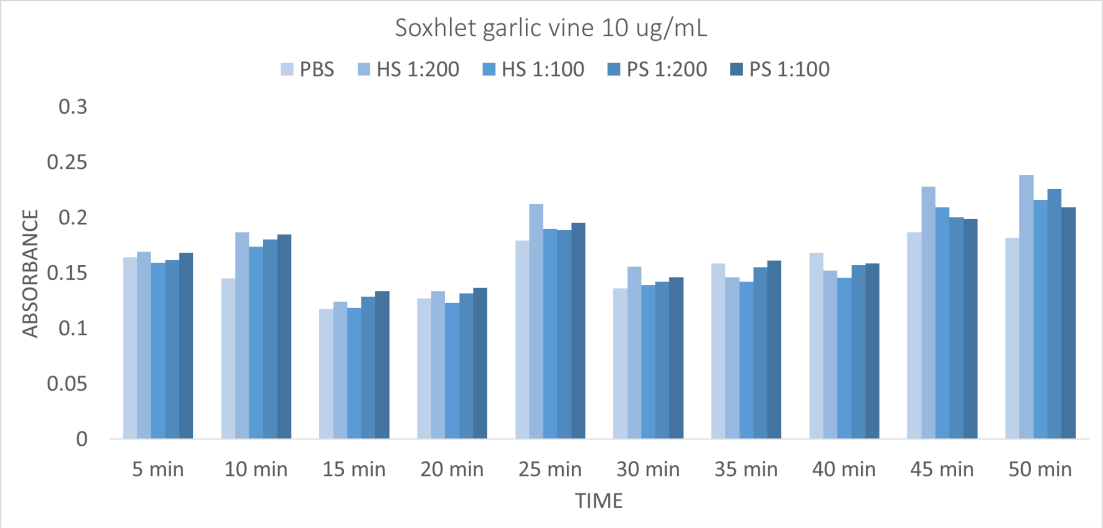

Figure B.3: Results of the indirect ELISA assay for the soxhlet garlic vine (S-GV) extract at 10 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.

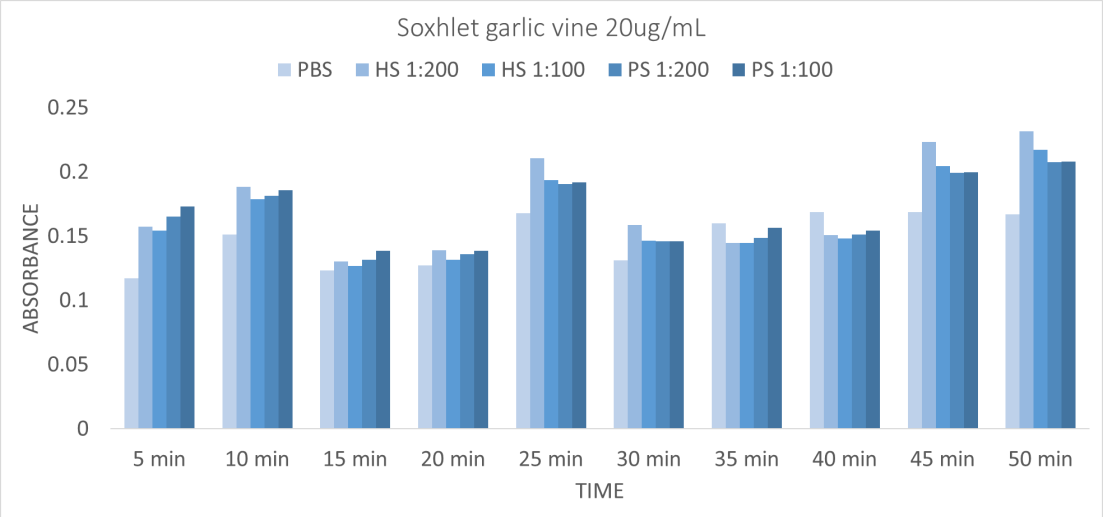

Figure B.4: Results of the indirect ELISA assay for the soxhlet garlic vine (S-GV) extract at 20 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.

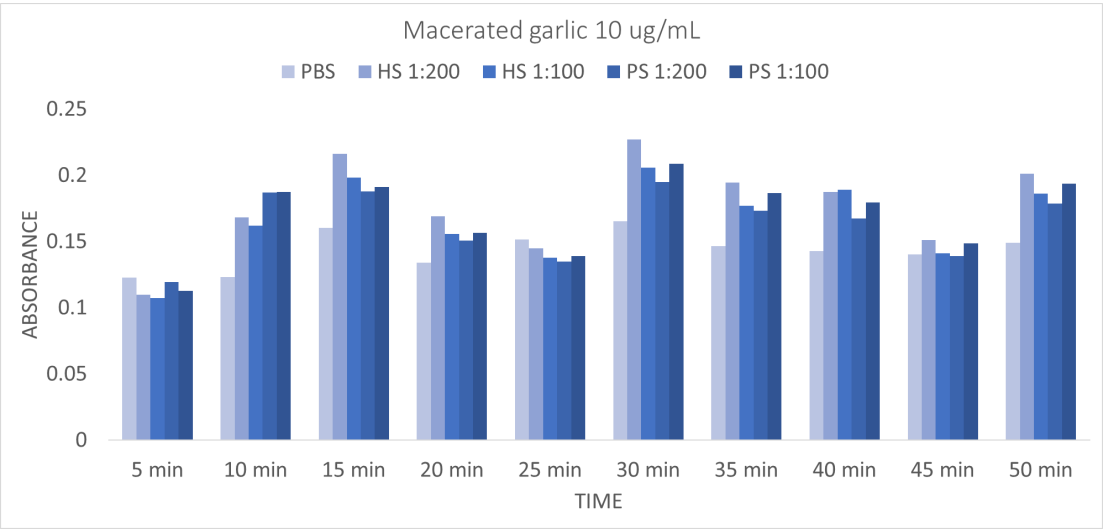

Figure B.5: Results of the indirect ELISA assay for the macerated garlic (M-G) extract at 10 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.

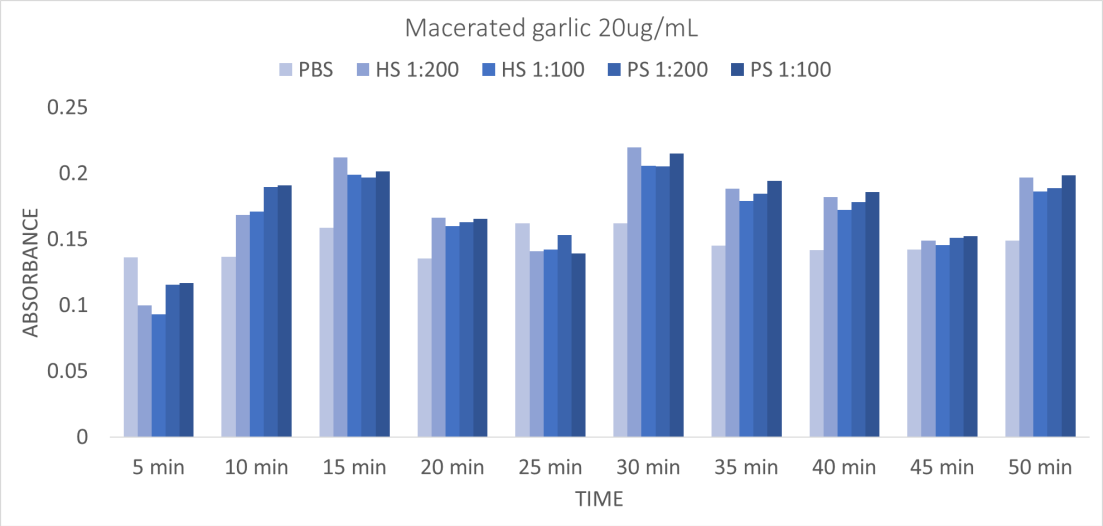

Figure B.6: Results of the indirect ELISA assay for the macerated garlic (M-G) extract at 20 µg/mL interacting with its hyperimmune serum diluted to 1:100 and 1:200, adding PBS and preimmune serum (also diluted to 1:100 and 1:200) as controls.