PhD Project

Applicant
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Field
Biology

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Project Title
New products with agricultural and health applications: bringing value to the invasive plants *Gunnera tinctoria* and *Hedychium gardnerianum*

Keywords
Gunnera tinctoria, *Hedychium gardnerianum*, ethanolic extracts, bioactivities, insecticidal, fungicidal, anti-aging, antibacterial, antioxidants, natural compounds, waste biomass valorisation

Abstract

The search for new natural sources of bioactive compounds is an increasingly hot topic of interest, not only by the scientific community but also by different industries. For instance, in agriculture, the excessive use of synthetic pesticides has raised many concerns regarding their harmful effects in humans and their negative impact in the environment. Thus, more practical and affordable ecologically friendly alternatives are highly desired. Regarding medicine, the pursuit for bioactive compounds with potential for health applications never ceases, with several new drugs emerging each year with all kinds of activities: *e.g.* anti-aging, antibacterial, anti-inflammatory, antioxidant, antitumor, antiviral and neuroprotective, to name a few. Therefore, the quest for new products with natural origin and health benefits is a vast field of work with countless paths to follow. Plants offer a chemical diversity that allows for a variety of opportunities to discover new natural products with the desirable properties to develop new pesticides and drugs.

The conversion of waste biomass from invasive plants into useful products is an underexplored area with great potential and environmental benefits. Thus, two well established invasive plant species in São Miguel Island were selected to be studied in the present project: *i.e. Gunnera tinctoria* (Molina) Mirbel and *Hedychium gardnerianum* Sheppard ex Ker-Gawl. The criteria of plant choice were based on their prospective as excellent sources of plant biomass and unexplored bioactive compounds with potential for conversion into agricultural and health applications. In addition, there is scarce information about the active compounds responsible for the biological activities reported in literature for these plants, studies addressing both plants derived products in agricultural applications are non-existent and there is an absence of *G. tinctoria* rhizomes studies. Taking this into account, *G. tinctoria* and *H. gardnerianum* emerge as particularly interesting natural sources of biomass with appreciated underexplored properties that this PhD project will address, adding value to worthless and unwanted plants while simultaneously providing economic justification for their ecological control.

State of the Art

To succeed in nature, plants can modulate their behaviour as an evolutionary response to the environment [1], synthesizing several products and developing specialized morphological structures to try to ensure their survival over other plants [2] and against insects and other predators [3]. Compounds are, therefore, produced with specific properties that suppress the germination growth of other plants [4] or that have antinutritional, repellent, and/or toxic effects against insects [5]. Since the beginning of mankind history, humans have always observed these effects and started to take advantage of these diverse properties, using plants not only as food, construction material, energy source and clothes, but also as medicine and pesticide sources [6]. This exploitation of what plants have to offer continues to this day, with different industries looking for new natural sources of bioactive compounds [7].

For instance, in agriculture, the search for more ecologically friendly alternatives to synthetic pesticides is on high demand, with bio-pesticides being an increasing topic of investigation and interest [8]. To be a viable alternative to synthetic pesticides, bio-pesticides should avoid resurgent of new pests and have low phytotoxicity, being benign to the natural enemies of the targeted pest and causing no negative effects on crop yields [9,10]. Furthermore, they should be simple to prepare by the daily farmer (not requiring solvents which are either toxic or difficult to buy, nor complex equipment) and affordable (based on plant materials readily available and cheap) [11].

Addressing medicine, the investigation of natural products for health applications is an equally, or even greater, hot topic of interest by cosmetics, pharmaceutical and food industries, as well as by the scientific community [12,13]. Nature offers a vast array of chemical diversity that allows for drug development, with several plant compounds becoming new drugs or sources of inspiration for drugs [14,15]. Given this chemical diversity, it is possible to find compounds with all kinds of activities: *e.g.* anti-aging, antibacterial, antidepressant, anti-inflammatory, antioxidant, antitumor, antiviral, immunostimulant, hepatoprotective and neuroprotective [16]. As so, the quest for new products with natural origin and health benefits is a vast field of work with countless paths to follow.

Considering the great capacity of plants to produce bioactive compounds, some species are able to thrive not only in their original habitat, but also colonize other locations and subdue native species from these regions, becoming aggressive invasive species with great negative ecological impact [17]. Therefore, eradicating these plants from the places where they don't belong and restoring an ecosystem is a mandatory, yet complex and arduous task, that generally is expensive and unfruitful [18]. Invasive plants waste biomass conversion into useful products is an underexplored area with great potential and environmental benefits, adding value to worthless and unwanted plants while simultaneously providing economic justification for their ecological control [19,20]. With this in mind, two well established invasive plant species in São Miguel Island [21] were selected to be studied in the present project due to their prospective as excellent sources of plant biomass and bioactive compounds with potential for conversion into agricultural and health applications: *i.e. Gunnera tinctoria* (Molina) Mirbel and *Hedychium gardnerianum* Sheppard ex Ker-Gawl.

G. tinctoria is a perennial shrub native from South America, also known as giant rhubarb due to its dimensions, with rhizomes of 25 cm in cross section and as long as 350 cm, leaves that can reach 200 cm in diameter and a height up to 200 cm [22], thus being easily a good source of biomass. In native areas, *G. tinctoria* is used as food in pastry, ice creams and salads [23]. In addition, decocts of roots, petioles or leaves are prepared to assist in the treatment of ulcer, uterus pains and liver injuries, as well as circulatory, high respiratory tract and urinary disorders, and as an anti-inflammatory, febrifuge and haemostatic remedy [24,25]. A couple of these folk

medicine have been corroborated by some investigation conducted regarding the biological activities of *G. tinctoria*.

Topical anti-inflammatory activity for arachidonic acid induced edema in mouse ears was reported for aqueous, methanolic and ethyl acetate *G. tinctoria* aerial parts extracts [26]. Recently, Sabando and colleagues presented promising results regarding wound healing treatment using hydrocolloid pectin-based films incorporated with a methanolic extract of *G. tinctoria* and *Ugni molinae* Turcz. leaves mixture. Edematous response was inhibited by about 50% and after 17 days of topical application the pressure ulcer wound was completely close without showing any adverse reaction [27]. In addition to antifungal activity against *Cryptococcus laurentii*, aqueous leaf extract of *G. tinctoria* also reported antioxidant capacity [28]. In another study, *G. tinctoria* methanolic leaf extract was demonstrated to have antioxidant properties by the DPPH assay at a concentration of 5 µg/mL, presenting an efficiency in the same order of magnitude as the reported for gallic acid [29]. Antitumour activity against HeLa cell line was assessed for dichloromethane and methanol extracts of leaves of *G. tinctoria*, obtained by hot and room temperature extraction [30]. All extracts were active against HeLa tumour cell line, with methanolic extract at hot temperature showing promising activities for antiproliferative (EC_{50} = 25.28 µg/mL) and cytotoxicity (EC_{50} = 49.47 µg/mL) assays. Antibacterial activity against human pathogens *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus* and *Pseudomonas aeruginosa* was also screened in the same study, with only a weak activity against *M. luteus* (EC_{50} = 169.15 µg/mL) being detected [30]. More recently, aqueous extract of petioles from *G. tinctoria* were demonstrated to have good antibacterial activity (MIC and MBC = 32 µg/mL). against *Helicobacter pylori* [31].

H. gardnerianum, widely known as conteira in the Azores archipelago, is a rhizomatous perennial herb native to the Central and Eastern Nepal, North Myanmar, Northeast India and Bhutan with bulky branching surface rhizomes originating stems which can extend up to 200 cm long, producing alternately arranged oblong leaves with 30 cm and numerous yellow-orange flowers in a terminal cylindrical spike above the foliage of 25 to 40 cm in length [32]. Unlike others *Hedychium* species that are vastly present in folk medicine and in the diet of various countries [33], besides ornamental purposes, other known uses for *H. gardnerianum* are as cattle food [34], and, more recently, as source of biomass for biomaterials production [35]. Despite the lack of traditional use, some investigation regarding the biological activities of *H. gardnerianum* have been conducted in recent years.

The essential oils from *H. gardnerianum* leaves (30 µL/dish) presented antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*, originating inhibition zones similar to the antibiotic tetracycline (50 mg/dish) used as control [36]. Another work, showed that the leaf essential oil of *H. gardnerianum* collected from four different locations had acetylcholinesterase inhibitory properties [37]. Values of IC_{50} ranged from 1.03 ± 0.14 mg/mL to 1.37 ± 0.27 mg/mL, comparable to the inhibitor standard compound used, *i.e.* α-pinene, that presented an IC_{50} value of 1.43 ± 0.07 mg/mL. The same study also assessed by DPPH assay that the same essential oils presented antioxidant activity, presenting EC_{50} values ranging from 8.46 ± 0.90 µg/mL to 31.14 ± 2.70 µg/mL, being better than the control compound BHT (EC_{50} = 31.00 ± 0.19 µg/mL) [37]. DPPH assay was also used to demonstrate the antioxidant effect of *H. gardnerianum* rhizomes ethanolic extract, presenting an IC_{50} value of 1.59 µg/mL, even better than the reference compounds ascorbic acid (IC_{50} = 8.37 µg/mL) or trolox (IC_{50} = 10.19 µg/mL) [38]. Furthermore, isolated compounds from hexane and dichloromethane extracts of *H. gardnerianum* rhizomes demonstrated potent cytotoxic activity against human small cell lung cancer (NCI-H187) and high selectivity index for non-cancerous Vero cells [39].

The information stated above regarding the uses and biological activities of the invasive plants *G. tinctoria* and *H. gardnerianum* provides encouraging indicators about their potential for further investigation and applications in different areas, *e.g.* agricultural, cosmetic, food and pharmaceutical industries. In addition, the lack of information in some cases are excellent opportunities to work on and fill that knowledge gaps. For instance, studies addressing *G. tinctoria* only focus on the leaves and petioles of the plant, with their rhizomes being left unexplored. Furthermore, there are no records of agricultural applications of extracts or compounds of both plants. In addition, little is known about the active compounds responsible for the biological activities reported for the aimed plants. Moreover, there is an urgent need to shed some light to uncover the reasons to why both plants are so invasive in their introduced habits. Based on these, *G. tinctoria* and *H. gardnerianum* emerge as particularly interesting natural sources of biomass with valuable underexplored properties that this PhD project will address.

Objectives

Given the negative impact of the invasive plants *G. tinctoria* and *H. gardnerianum* in Azorean biodiversity, the main aims of this work, and the writing of the thesis and its free divulgation and availability to anyone, is a) to enrich the general knowledge over these problematic species, b) to contribute for the understanding of why both plants are so invasive and c) to bring added value to their biomass waste.

To achieve those main goals, some overall points must be adressed:

1- Ethanolic extracts from different parts of both invasive plant species must be obtained in sufficient quantity to allow further tasks to be fulfilled;
2- The ethanolic extracts obtained must be assessed regarding their biological activities for agricultural applications (insecticidal, fungicidal and toxicity assessment activities) and for health applications (anti-aging, antibacterial, antifungal and antioxidant activities);
3- The chemical composition of the extracts must be elucidated to allow the identification of which compounds are responsible for the most relevant effects reported in the ethanolic extracts;
4- The results obtained throughout this PhD project must be disseminated to the scientific community and society in general.

Accomplishing these four points will set the base for future work regarding the valorisation of the biomass of these invasive species, allowing for a sustainable use of the Azorean natural resources in the quest for new natural products with agricultural and health applications.

Project Structure

The proposed plan was divided into five work packages (WP), each organized by different tasks for better work organization.

<u>WP 1 – Biomass collection and extractions</u>

Objectives

To collect specimens of *G. tinctoria* and *H. gardnerianum* in order to obtain ethanolic extracts of their different parts (rhizomes, stems and leaves, flowers and fruits) for the subsequent tasks and work packages.

Task 1.1 – Collection of plant materials and its processing in the laboratory

An authorization will be requested to the competent regional authorities to harvest the species in question and a voucher of each will be deposited in the herbarium of the Azores University. In order to obtain all the different parts of both plant species, the collection of plant materials will take place in different phases of their life cycle, always in the same spots of forest in São Miguel Island, thus this task will be repeated several times. In the laboratory, the collected plant materials will be cleaned and separated according to their species and plant part. In addition, part of the samples will be airdried at room temperature, while other part will be used fresh.

Task 1.2 – Ethanolic extraction optimization for each plant part

A small sample (50 g) of dried and fresh plant material (*e.g.* leaves) will be milled and sieved to 60-40 mesh and then extracted by different methods using always ethanol as the solvent. The choice of a less toxic solvent, *i.e.* ethanol, for the extractions allow a greener, more sustainable and environmentally friendly scientific investigation.

Extraction method A – Three cycles of maceration at room temperature for 24h with continuous agitation and solvent renewal after each cycle.

Extraction method B – Three cycles of maceration at room temperature for 1h (15 minutes subjected to high power ultrasonic probe + 45 minutes of continuous agitation) with solvent renewal after each cycle.

Regardless of the extraction method, at the end, the extract is filtered using a porous plate funnel, a kitasato and a manual vacuum pump, and the solvent is evaporated to dryness and recovered using a rotary evaporator under vacuum.

Deliverables

- Acquisition of enough plant material from each different part of each species to be used in the ethanolic extractions;
- The ethanolic extracts of the different parts of *G. tinctoria* and *H. gardnerianum* that will be used in the other work packages.
- At least 5-10 g of extract from each morphological part/species will be obtained.

<u>WP 2 – Assessment of potential for agricultural applications</u>

Objectives

The level of activity of the ethanolic extracts of *G. tinctoria* and *H. gardnerianum* will be assessed against insects (*Ceratitis capitata* and *Sitophilus zeamais*) and fungi (*Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum*), pathogenic species that affect agricultural activities). In addition, the level of toxicity of the ethanolic extracts of both plant species will be assessed against other plants. Later on, fractions of the more active extracts and isolated compounds will also be tested, against the same pathogenic species using the same procedures (bioguied approach).

Task 2.1 – Insecticidal activity

The agricultural pests used in this laboratory test will be *Ceratitis capitata* and *Sitophilus zeamais*, both responsible annually for a major economic downturn in the agricultural sector in fruit and corn production, respectively [40,41]. Therefore, it is necessary to find an environmentally friendly solution in the fight against them.

Ceratitis capitata

Each sample (ethanolic extracts, fraction or isolated compound) will be evaluated regarding its effect on mortality and oviposition of the fruit fly *C. capitata* using artificial fruits made from agar. This protocol was adapted and improved based on the work of Salles [42] and Furtado *et al*. [43]. Briefly, the artificial fruits are put in cups and brushed with the different samples at different concentrations, with attention to evenly spread the liquid over the fruit surface. Then, 13 flies (3 males and 10 females) are selected and added to the cup. Mortality is checked every 24 hours. After 72 hours, mortality is checked and the artificial fruits are removed from the cups to verify the oviposition in each one.

Sitophilus zeamais

The insecticidal activity against *S. zeamais* will be performed based on Rosa *et al.*, [44] methodology, with some modifications. Briefly, 10 adult weevils are placed in 5 cm diameter Petri dishes containing one filter paper disc (Whatman Nº 1) impregnated with 100 µL of different samples at different concentrations. Mortality is checked every 24 hours and the assay ends after 72 hours. Weevils are considered dead if they do not respond to prodding with a needle.

Task 2.2 – Fungicidal activity

Fungicidal activity will be assessed by evaluation of the ethanolic extracts, fractions and isolated compounds against the phytopathogenic fungi that cause plagues in crops: *i.e. Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum*. The antifungal assay will be performed by the agar disk diffusion method based on Simionato *et al.* [45]. The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) will be determined.

Task 2.3 – Antigerminative assessment

2.3.1. Impact on crops

The different samples will be evaluated regarding their toxic effect on the germination of *Lactuca sativa* L. and *Solanum lycopersicum* L. (well-known agricultural products with great and rapid germination). Germination bioassays was adapted from Hernández-Herrera *et al.* [46] and Adam *et al.* [47]. Briefly, 10 seeds are placed on a Whatman Nº 5 filter paper treated with 5 mL of each sample at different concentrations in sterilized 90 mm Petri dishes. The plates are then incubated for a week and at the end, the percentage germination (production of a radicle ≥ 10 mm) is recorded.

2.3.2. Impact on other plants

In addition to the toxicity assessment for crops, given the widely known invasive status of *G. tinctoria* and *H. gardnerianum*, the toxicity effect of the different samples will also be assessed against seeds of invasive and native plants.

Deliverables

- Results of this WP will provide important data regarding the potential use of the selected plants in agricultural applications, *e.g.* insecticide and fungicide products;
- The toxicity assessment over germination of crop plants will give indicators of the potential impact of the use of these new insecticide and fungicide products on the plants that they are trying to protect from pests;
- The toxicity assessment over germination of invasive and native plants will give a contribution in the understanding of why both plants are so invasive and the impact of these new insecticide and fungicide products on the surrounding plants of the crop site.

WP 3 – Assessment of potential for health applications

Objectives

To assess the anti-aging (inhibition of acetylcholinesterase, butyrylcholinesterase, collagenase, elastase, hyaluronidase and tyrosinase), antibacterial (*Bacillus subtilis* and *Escherichia coli*), antifungal (*Candida albicans*), antioxidant (ABTS, β-carotene, DPPH) potential of the different extracts (and later fractions and isolated compounds) of *G. tinctoria* and *H. gardnerianum*. Assessment of these activities will bring value to both plant species since there is a great interest by cosmetic, food and pharmaceutical industries in finding new sources of active products.

Task 3.1 – Anti-aging activity

The aging process is interconnected with the action of several enzymes, thus, various anti-enzymatic assays will be carried out to assesses the inhibition potential of the different extracts, fractions and isolated compounds against acetylcholinesterase, butyrylcholinesterase, collagenase, elastase, hyaluronidase and tyrosinase [48,49].

Task 3.2 – Anti-bacterial activity

The antibacterial assay will be performed by the agar microdilution method as described by Golus *et al.*, [50]. Cultures of Gram (+) *Bacillus subtilis* and Gram (-) *Escherichia coli* will be used as targeted organisms for the different samples. The minimal inhibitory concentrations will be determined.

Task 3.3 – Anti-fungal activity

The antifungal assay will be performed for each different samples by the agar microdilution method for the yeast (*Candida albicans*) as described by Gong *et al.* [51] and the minimal inhibitory concentration (MIC) will be determined.

Task 3.4 – Antioxidant activity

Plant antioxidants can act through various pathways, thus different *in vitro* antioxidant assays will be used to evaluate the antioxidant capacity of the different extracts, fractions and isolated compounds, namely: ABTS [52], β-carotene [53] and DPPH [54].

Deliverables

- Results of this WP will provide extensive data that will bring value to both plants and support their potential for use in health applications by cosmetic, food and pharmaceutical industries.

WP 4 – Bio-guided fractionation of extracts for isolation and characterization of the bioactive compounds responsible for relevant biological activities

Objectives

To isolate and identify the compounds in the extracts responsible for relevant effects with agricultural and health applications.

Task 4.1 – Fractionation of the extracts, purification of fractions and compound isolation

The results of WP 2 and 3 will indicate which extracts show the more relevant effects in the various biological assays. In order to identify the pure compounds responsible for such effects, the more promising extracts will be fractionated trough liquid-liquid separation with increasing solvent polarity (hexane, ethyl acetate, aqueous phase). The obtained fractions will be subjected to bioassays (see tasks in WP 2 and 3), to elucidate which ones are the more promising and deserve further study.

The more relevant fractions will be further fractioned by preparative silica gel column chromatography and eluted with a gradient system of solvents of increasing polarity. The originated sub-fractions will be collected and checked for their purity using analytical thin layer chromatography. In case of unpurified sub-fractions, preparative thin layer chromatography using several solvent mixtures will be used to obtain more pure compounds. The isolated compounds will be subjected to the tasks of WP 2 and 3 to clarify if they are responsible for the previously biological effects reported on the fractions and extracts.

Task 4.2 – Structural elucidation of isolated compounds and identification

Resorting to spectroscopic methods, in particular mass spectrometry (MS) and nuclear magnetic resonance (NMR), the structure of all isolated natural compounds will be established. The structural elucidation will be determined by the analysis of 1D (^{1}H and ^{13}C) and 2D (COSY, DEPT, HMBC, HSQC, H2BC and NOESY) NMR techniques spectres and confirmed by mass spectrometry spectre analysis.

Deliverables

- Elucidation of the compounds responsible for the biological effects.
- Identification of new compounds in *G. tinctoria* and *H. gardnerianum*.

WP 5 – Divulgation of results

Objectives

To disseminate the results to the supporting (FRCT) and collaborating (UAc and ce3c-GBA) institutions, industries of interest, scientific community and society in general.

Task 5.1 – Scientific papers

The results obtained will allow for the redaction and publication of scientific papers in indexed journals with relevant impact factor.

Task 5.2 – Scientific congresses and meetings

Participation in scientific congresses and meetings will allow for wide dissemination of the results as well as for fruitful discussions over different thematic.

Task 5.3 – Outreach activities

The project, its development and more prominent results will be regularly communicated in initiatives directed to the general public, such as newspaper and magazine articles and interviews, presence in social media and opportunities to discuss science in informal settings.

Task 5.4 – Literature review & Thesis writing

The literature review for writing this thesis and its free divulgation and availability to anyone will enrich the general knowledge over the studied plants.

Deliverables

- At least 4 scientific papers published in indexed international journals.
- Participation in at least one scientific congress or meetings per year, with a poster or oral communication in each meeting.
- At least one article or interview each year in a magazine or newspaper, and at least 4-6 posts in the Faculty's Facebook and Instagram.
- PhD Thesis with all the data that enrich the general knowledge over the studied plants.
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Chronogram

Year	2022		2023												2024												2025												2026										
Month	Nov	Dez	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
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WP1 - T1.1 - Collection of plant materials and its processing in the laboratory

WP1 - T1.2 - Ethanolic extraction optimization for each plant part

WP2 - T2.1 - Insecticidal activity

WP2 - T2.2 - Fungicidal activity

WP2 - T2.3 - Antigerminative assessment

WP3 - T3.1 - Anti-aging activity

WP3 - T3.2 - Anti-bacterial activity

WP3 - T3.3 - Anti-fungal activity

WP3 - T3.4 - Antioxidant activity

WP4 - T4.1 - Fractionation of the extracts, purification of fractions and compound isolation

WP4 - T4.2 - Structural elucidation of isolated compounds and identification

WP5 - T5.1 - Scientific papers

WP5 - T5.2 - Scientific congresses and meetings

WP5 - T5.3 - Outreach activities

WP5 - T5.4 - Literature review & Thesis writing

WP1 - Biomass collection and extractions

WP2 - Assessment of potential for agricultural applications

WP3 - Assessment of potential for health applications

WP4 - Bio-guided fractionation of extracts for isolation and characterization of the bioactive compounds responsible for relevant biological activities

WP5 - Divulgation of results