Universidade de Lisboa

Faculdade de Farmácia

Discovery of New Antimicrobial Drugs Active Against Resistant Pathogens Agents: The Case Study of Portuguese *Asphodelus* Species

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Orientadoras: Prof. Doutora Olga Maria Duarte Silva
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Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia (Farmacognosia e Etnofarmacologia)

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2024

STATEMENT

All the research presented in this thesis was conducted under the supervision of Prof. Dr. Olga Maria Duarte Silva, Associate Professor at the Department of Pharmacy, Pharmacology and Health Technologies, Faculty of Pharmacy, Universidade de Lisboa, together with co-supervision of Prof. Dr. Maria Manuela Marin Caniça, Head and Principal Investigator at National Reference Laboratory of Antibiotic Resistances and Healthcare-Associated Infections, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge and Prof. Dr. Maria Beatriz da Silva Lima, Dean and Full Professor at the Department of Pharmacy, Pharmacology and Health Technologies, Faculty of Pharmacy, Universidade de Lisboa.

Maryam Malmir was awarded with a PhD scholarship (SFRH/BD/125310/2016), from Fundação para a Ciência e a Tecnologia (FCT) and has participated in the conceptualization and implementation of all studies as well as the analysis, interpretation of data and preparation of all the manuscripts forming the present dissertation.

To all those who have an unwavering commitment to unraveling the mysteries of nature's pharmacy

THESIS STRUCTURE

The present work is organized in six different sections (chapters). Chapter 1 consisted of a general introduction to antimicrobial resistance (AMR), the roles of medicinal plants and natural products as antimicrobials, and the significance of the genus *Asphodelus* and its Portuguese endemism as potential source of antimicrobial agents against resistant pathogens. This chapter is finalized by presenting the rationale and objectives of this PhD project.

Chapter 2 provided a comprehensive literature review on the taxonomy, ethnomedical, phytochemical and pharmacological data related to the genus *Asphodelus* L., aimed to compile an extensive list of the plant's parts, extract types and the identified secondary metabolites and their biological potentials. These data were needed for the selection of the raw materials, their preparations, creating the tests and assembling the pathogen panel as a component of the current study.

In Chapter 3, divided into two parts, Chapter 3.1, and Chapter 3.2, the monographic quality parameters and pre-clinical safety assessment, along with the phytochemical analysis, identification and quantification of the main marker secondary metabolites of both *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root tubers extracts are presented.

Although root tubers are the main plant parts traditionally used for the treatment of skin-related disorders and infections, leaves have also been used as food, and traditionally as medicine, for treating ulcers, urinary tract, and inflammatory disorders. Therefore, Chapter 4 presented their phytochemical profile, identification and quantification of the main marker secondary metabolites, together with the antimicrobial, antioxidant, and pre-clinical safety assessments.

In Chapter 5 the main findings derived from the studies presented in the previous chapters, are analyzed, and discussed in the context of the existing literature and conclusions drawn from the whole study are summarized.

Chapter 6 cited all the bibliographic references used in the document and formatted according to the style of the published articles.

SCIENTIFIC CONTRIBUTIONS

The results obtained in this thesis were described in the following scientific achievements, listed by order of publication date, the latest being the first listed:

Book Chapter

1. Malmir M., Serrano R., Silva O. Anthraquinones as potential antimicrobial agentes– A review. In: Mendez-Vilas A, editor. "Antimicrobial Research: Novel bioknowledge and educational programs" (Microbiology Book Series Nº 6). Formatex Research Center, Badajoz, Spain, August 2017, Pp. 55–61. ISBN-13: 978-84-947512-0-2

Full Papers

1. Malmir M., Lima K., Póvoas Camões S., Manageiro V., Duarte M.P., Paiva Miranda J., Serrano R., Moreira da Silva I., Silva Lima B., Caniça M., Silva O. Bioguided identification of active antimicrobial compounds from *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root tubers. Pharmaceuticals 2023, 16(6), 830, Impact Factor: 5.215, DOI: 10.3390/ph16060830
2. Malmir M., Lima K., Póvoas Camões S., Manageiro V., Duarte M.P., Paiva Miranda J., Serrano R., Moreira da Silva I., Silva Lima B., Caniça M., Silva O. Identification of marker compounds, and *in vitro* toxicity evaluation of two Portuguese *Asphodelus* leaf extracts. Molecules 2023, 28, 237. Impact Factor: 4.927, DOI: 10.3390/molecules28052372
3. Malmir M., Serrano R., Lima K., Duarte M.P., Moreira da Silva I., Silva Lima B., Caniça M., Silva O. Monographic quality parameters and genotoxicity assessment of *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root tubers as herbal medicines. Plants 2022, Vol. 11(22): 3173. Impact Factor: 4.658, DOI: 10.3390/plants11223173

4. Malmir M., Serrano R., Caniça M., Silva Lima B., Silva O. A Comprehensive review on the medicinal plants from the genus *Asphodelus*. Plants 2018, Vol.7 (20), Impact Factor: 4.658, DOI: 10.3390/plants7010020

Peer-reviewed abstracts

1. Malmir M., Lima K., Manageiro V., Moreira da Silva I., Caniça M., Silva Lima B., Silva O. Antimicrobial activity of Portuguese *Asphodelus* root extracts against multidrug-resistant bacteria. Planta Medica (Journal of Medicinal Plant and Natural Product Research), 2023, DOI: 10.1055/s-0043-1774074
2. Malmir M., Lima K., Camões S., Duarte M.P., Miranda, J. Caniça M., Silva Lima B., Silva O. *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root extracts pre-clinical safety assessments. Planta Medica (Journal of Medicinal Plant and Natural Product Research), 2023, DOI: 10.1055/s-0043-1774181
3. Malmir M., Lima K., Duarte M.P., Serrano R., Moreira da Silva I., Caniça M., Silva Lima B., Silva O. Phytochemical, pre-clinical safety, and efficacy evaluation of two Portuguese *Asphodelus* leaf extracts. Planta Medica (Journal of Medicinal Plant and Natural Product Research), 2022, 88 (15): 1517, DOI: 10.1055/s-0042-1759201

Presentations at Conferences and Seminars

Oral communications:

1. Malmir M. Pharmacological potential of medicinal plants from the genus *Asphodelus* L. Conference integrated in the scope of the doctoral program, March 12th, 2018, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.
2. Malmir M., Serrano R., Silva Lima B., Silva O. Portuguese *Asphodelus* medicinal plants as anti-infective agents. 9th iMed.ULisboa postgraduate student and 2nd i3DU meeting, July 13th–14th, 2017, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

Poster communication:

1. Malmir M., Lima K., Camões S., Duarte M.P., Miranda J., Caniça M., Silva Lima B., Silva O. *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root extracts pre-clinical safety assessments. 71st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). July 2nd–5th, 2023, Dublin, Ireland.

2. Malmir M., Lima K., Manageiro V., Moreira da Silva I., Caniça M., Silva Lima B., Silva O. Antimicrobial activity of Portuguese *Asphodelus* root extracts against multidrug-resistant bacteria. 71st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). July 2nd–5th, 2023, Dublin, Ireland.

3. Malmir M., Lima K., Duarte M.P., Serrano R., Moreira da Silva I., Caniça M., Silva Lima B., Silva O. Phytochemical, pre-clinical safety, and efficacy evaluation of two Portuguese *Asphodelus* leaf extracts. 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). August 28th–31st, 2022, Thessaloniki, Greece.

4. Malmir M., Lima K., Manageiro V., Duarte A.P., Serrano R., Moreira da Silva I., Silva Lima B., Caniça M., Silva O. Bioactive constituents of two Portuguese *Asphodelus* leaf extracts. 13th iMed.ULisboa postgraduate student, July 4th–5th, 2022, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

5. Malmir M., Serrano R., Lima K., Caniça M., Silva Lima B., Silva O. Development of quality control parameters of two Portuguese *Asphodelus* root tubers as medicines. Encontro Ciência 2022–Encontro com a Ciência e Tecnologia em Portugal, May 16th–18th, 2022, Lisbon, Portugal.

6. Malmir M., Ferreira E., Manageiro V., Caniça M., Silva O. *In vitro* antibacterial activity of two medicinal Portuguese *Asphodelus* species. 32nd European Congress of Clinical Microbiology & Infectious Diseases (ECCMID), April 23rd–26th, 2022, Lisbon, Portugal.

7. Malmir M., Malú Q., Serrano R., Silva O. Botanical characterization of two Portuguese medicinal *Asphodelus* species, 6th International Congress of Aromatic and Medicinal Plants (CIPAM), May 29th–June 1st 2016, Coimbra, Portugal.
8. Malmir M., Malú Q., Serrano R., Silva O. Botanical characterization of two Portuguese medicinal *Asphodelus* species. 8th iMed.ULisboa Postgraduate Students and 1st i3DU Meeting, July 14th and 15th, 2016, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal.

ABSTRACT

Introduction: *Asphodelus bento-rainhae* subsp. *bento-rainhae*, leaf (AbL) and root (AbR), and *Asphodelus macrocarpus* subsp. *macrocarpus*, leaf (AmL) and root (AmR), have been used traditionally in Portugal for the treatment of skin inflammatory and infectious diseases. *A. bento-rainhae* is an endemic species growing in the Central region of Portugal (Serra da Gardunha), coexisting within the same geographical area as *A. macrocarpus* and is classified as "vulnerable" according to the International Union for the Conservation of Nature (IUCN), Red List of Threatened Species.

The present PhD project aimed to contribute to the validation of the use of *A. bento-rainhae* (both leaf and root) and *A. macrocarpus* (both leaf and root) as anti-infectious agents through quality studies, the establishment of monographic criteria for botanical and chemical identification, dosage, efficacy, and preclinical safety studies. Identifying natural products with antimicrobial activity obtained from these medicinal plants, particularly against bacterial strains responsible for skin infections and resistant to major antibiotics, was also a significant goal of this work.

Methods: Quality studies of AbL, AbR, AmL and AmR, as medicinal plants, involved establishing botanical characteristics by macroscopic, and microscopic identification using optical and scanning electron microscopy techniques and chemical identification by establishing the chromatographic profile of secondary metabolites using thin-layer chromatography (TLC), ultra-high-performance liquid chromatography coupled with a diode array detector and mass spectrometry (UPLC-UV/DAD-ESI/MS), common techniques for isolation, purification, and structural identification of natural products as well as quantification techniques by colorimetric methods and high-performance liquid chromatography coupled with a diode array detector (HPLC-UV/DAD). The evaluation of botanical identification parameters was performed exclusively on the root (fragmented and powdered), which is the mostly used plant part in traditional medicine.

To assess the impact of seasonality on the phytochemical characteristics of the medicinal plants under study, leaf samples collected in two different seasons, Spring (AbLa and AmLa) and Summer (AbLb and AmLb), and root samples collected in Spring (AbRa and AmRa) and Autumn (AbRb and AmRb) were evaluated. Chemical studies were conducted on 70% hydroethanolic and 96% ethanolic extracts from each medicinal plant.

The assessment of antimicrobial activity was conducted by agar microdilution method, using clinical and reference strains (ATCC) of multidrug-resistant Gram-positive and Gram-negative bacterial species, particularly related to skin infections. First, the antimicrobial activity of the 70% hydroethanolic extracts of each medicinal plant and their subsequent liquid-liquid (L-L) partition fractions, obtained by increasing polarity solvents, diethyl ether (AbRb-1, AmRb-1, AbLa-1 and AmLa-1), ethyl acetate (AbRb-2, AmRb-2, AbLa-2 and AmLa-2), and aqueous (AbRb-3, AmRb-3, AbLa-3 and AmLa-3) fractions was evaluated.

The marker constituents of the L-L fractions with the highest antimicrobial activity, when not identifiable by the used dereplication technique, were isolated by column chromatography and identified by structural identification techniques. The content of the constituents of the 70% hydroethanolic extracts was determined by colorimetry, targeting the dosage of the major classes of secondary metabolites present, and the quantification of the major marker compounds of each plant, by LC-UV/DAD.

In addition, considering the major identified chemical classes, the antioxidant activity of different extracts and some isolated fractions and/or constituents was evaluated by colorimetry, using the ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity tests.

Preclinical safety studies were conducted in vitro, in cellular models by MTT and by the Ames test.

Results and discussion: Botanical characterization of the root of both species, showed the presence of an epidermis containing multiple layers (velamen) and a cortex with thin-walled idioblasts (134 ± 2.9 µm in AbR and 150 ± 27.6 µm in AmR), containing raphide crystals of calcium oxalate.

The main morphological difference identified between the roots of the two species was the size of the tubers (3.5x1.0 cm in AbR and 8.7x1.4 cm in AmR). In the microscopic evaluation, differences were observed in the distribution and characteristics of vascular bundles (metaxylem and protoxylem), with the metaxylem vessels in *A. macrocarpus* (± 80 µm in diameter) surrounded by several protoxylems, while in *A. bento-rainhae* (± 70 µm in diameter), they are individually separated.

Colorimetric quantification of the major classes of secondary metabolites present in the leaf and root of both species showed that terpenoids (111.72±22.77, 165.47±26.54, 172.11±19.20, and 154.36±20.53 mg of oleanolic acid equivalents/g dry extract in AbLa, AmLa, AbRb, AmRb, respectively) and condensed tannins (180.96±10.98, 132.60±2.73, 128.64±14.05, 132.60±2.73 mg of catechin equivalents/g dry extract in AbLa, AmLa, AbRb, AmRb, respectively) were the major constituents. Additionally, anthracene derivatives (1.16±0.13, 0.55±0.07, 3.38±0.26, and 2.68±0.19 mg of rhein equivalents/g dry extract in AbLa, AmLa, AbRb, AmRb, respectively) were also detected.

Considering the seasonality of sample collection, the 70% hydroethanolic extracts of the root of both species, harvested during Autumn – November, (AbRb and AmRb), showed a higher content of major constituents. The 70% hydroethanolic extracts of the leaf of both species collected during Spring – May, (AbLa and AmLa), showed a higher content of major constituents. For this reason, these four extracts were selected for further chemical and biological studies.

Bioguided fractionation of the 70% hydroethanolic root extracts of both species showed that the diethyl ether fractions (AbRb-1, AmRb-1) were the most active against all the tested Gram (+) microorganisms (MIC: 16 to 1000 µg/mL), with AbRb-1 being relatively more active than AmRb-1. These fractions were found to contain anthracene derivatives as marker constituents, and five compounds namely, 7'-(chrysophanol-4-yl)-chrysophanol-10'-*C*-beta-*D*-xylopyranosyl-anthrone, 10,7'-bichrysophanol, chrysophanol, 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone and asphodelin were identified. Chrysophanol, identified as the major constituent, was quantified in the 70% and 96% hydroethanolic extracts, in both AbRb (10 and 36 µg/mL, respectively) and AmRb (7 and 23 µg/mL, respectively), and it was

particularly active against *S. epidermidis* (MIC 3.2 to 100 μg/mL). Additionally, six other minor constituents were also identified in this part of the plant, including chlorogenic acid, vanillic acid, caffeic acid, ferulic acid, isochlorogenic acid, and *β*-sitosterol.

From the total leaf extracts of both species (AbLa and AmLa), twelve marker compounds were identified, including neochlorogenic acid, chlorogenic acid, caffeic acid, isoorientin, *p*-coumaric acid, isovitexin, ferulic acid, luteolin, aloe-emodin, diosmetin, chrysophanol, and *β*-sitosterol. Among these, isoorientin and chlorogenic acid were the major compounds. Isoorientin was quantified in the 70% hydroethanolic extracts (184 μg/mL and 112 μg/mL in AbLa and AmLa, respectively) and in the 96% hydroethanolic extracts (107 μg/mL and 30 μg/mL in AbLa and AmLa, respectively). Chlorogenic acid was quantified in the 70% hydroethanolic extracts (66 μg/mL and 90 μg/mL in AbLa and AmLa, respectively) and in the 96% % hydroethanolic extracts (42 μg/mL and 27 μg/mL in AbLa and AmLa, respectively).

Bioguided fractionation of the 70% hydroethanolic leaf extracts of both species showed the diethyl ether fractions (AbLa-1, AmLa-1) as the most active against all the tested Gram (+) microorganisms (MIC: 62 to 1000 μg/mL), with AbLa-1 being relatively more active than AmLa-1. Aloe-emodin was identified as the major constituent of this fraction, and its content was quantified in the 70% and 96% hydroethanolic extracts, in both AbLa (13 and 15 μg/mL, respectively) and AmLa (7 and 8 μg/mL, respectively), showing particularly high activity against *S. epidermidis* (MIC 0.8 to 1.6 μg/mL).

Overall, the 96% hydroethanolic extracts were more active than the 70% extracts and also had the highest content of anthracene derivatives, compounds potentially related to antimicrobial activity.

The antioxidant potential was evaluated in all total extracts and fractions, with leaf extracts showing higher potential than root extracts. The ethyl acetate fraction was the most active (IC_{50} of 800 and 1200 μg/mL in AbLa-2 and AmLa-2, respectively) and had the highest content of phenolic acids and flavonoid derivatives. However, the antioxidant potential of these extracts was lower than that of ascorbic acid (IC_{50} of 83 μg/mL), used as a reference substance.

Toxicity was evaluated in all 70% hydroethanolic extracts and the 96% hydroethanolic extract of AbRb, which exhibited the highest antimicrobial activity. No cytotoxicity was observed up to 125 µg/mL in root extracts and up to 1000 µg/mL in leaf extracts against HepG2 cells and up to 125 µg/mL for AbRb in the 96% extract against HaCaT cells. Genotoxicity was not observed in any of the tested extracts up to a concentration of 5.0 mg/plate (Ames test).

Conclusion: The results of this study provided criteria for quality and differentiation between the four medicinal plants and the two species under study and knowledge of their antimicrobial potential.

The work confirmed the antimicrobial activity of these plants, primarily attributed to the presence of anthracene derivatives. These plants, when used in the form of a tincture at 96%, may have therapeutic relevance for the topical treatment of skin infections, confirming their use in traditional Portuguese medicine.

Keywords*:* anthracene derivatives, antimicrobial activity, antioxidant activity, *Asphodelus bento-rainhae*, *Asphodelus macrocarpus*, ethnopharmacology, preclinical safety, quality control, skin diseases, *Staphylococcus epidermidis*, traditional plant-based preparations

RESUMO

Introdução: *Asphodelus bento-rainhae* subsp. *bento-rainhae*, folha (AbL) e raiz (AbR), e *Asphodelus macrocarpus* subsp. *macrocarpus*, folha (AmL) e raiz (AmR), são usados na medicina tradicional portuguesa para o tratamento de doenças inflamatórias de pele, de presumível etiologia infeciosa.

Asphodelus bento-rainhae é uma espécie endémica da região Centro de Portugal (Serra da Gardunha), coexistindo no mesmo espaço geográfico com *Asphodelus macrocarpus*, e está classificada como "vulnerável" pela "International Union for the Conservation of Nature (IUCN), Red List of Threatened Species".

O presente trabalho de doutoramento teve como objetivo principal contribuir para a validação do uso de *Asphodelus bento-rainhae*, folha e raiz*,* e *Asphodelus macrocarpus* folha e raiz*,* enquanto agentes anti-infeciosos através da realização de estudos: de qualidade, pelo estabelecimento de critérios monográficos para identificação botânica e química, e doseamento; de eficácia, e de segurança pré-clínicas. A identificação de produtos naturais com atividade antimicrobiana obtidos a partir destas plantas medicinais, ativos *in vitro*, em especial contra estirpes bacterianas responsáveis por infeções cutâneas e resistentes aos principais antibióticos foi também um objetivo maior deste trabalho.

Metodologia: Os estudos de qualidade de *Asphodelus bento-rainhae*, raiz (AbR), e folha (AbL) e de *Asphodelus macrocarpus*, raiz (AmR) e folha (AmL), enquanto plantas medicinais, envolveram o estabelecimento dos caracteres de identificação botânica, macro e microscópica, usando técnicas de microscopia ótica e eletrónica de varrimento, e de identificação química através do estabelecimento do perfil cromatográfico em metabolitos secundários por cromatografia em camada fina (TLC), cromatografia líquida de ultra alta resolução acoplada a um detetor de matriz de díodos e um detetor de massa (UPLC-UV/DAD-ESI/MS), técnicas usuais de isolamento, purificação e identificação estrutural de produtos naturais, e técnicas de quantificação colorimétricas e de cromatografia líquida de alta resolução acoplada a

um detetor de matriz de díodos (HPLC-UV/DAD). A avaliação dos parâmetros de identificação botânica foi realizada apenas sobre a raiz (fragmentada e pulverizada) de ambas as espécies, uma vez que é a parte da planta com maior utilização tradicional.

Para avaliar o impacto da sazonalidade nas características fitoquímicas das plantas medicinais em estudo, foram avaliadas amostras de folha colhidas em duas épocas do ano distintas - Primavera (AbLa e AmLa) e Verão (AbLb e AmLb) e amostras de raiz, colhidas na Primavera (AbRa e AmRa) e Outono (AbRb e AmRb). Os estudos químicos foram efetuados em extratos hidroetanólicos a 70% e extratos etanólicos a 96%, preparados a partir de cada uma das plantas medicinais.

A avaliação da atividade antimicrobiana foi efetuada através do método de microdiluição em agar, usando estirpes clínicas e estirpes de referência (ATCC), de espécies bacterianas multirresistentes, Gram-positivas e Gram-negativas, em especial relacionadas com infeções cutâneas. Primeiro, foi avaliada a atividade antimicrobiana dos extratos hidroetanólicos a 70% de cada planta medicinal e de frações de partilha líquido-líquido (L-L) obtidas a partir destes: fração etérica (AbRb-1, AmRb-1, AbLa-1 e AmLa-1), fração de acetato de etilo (AbRb-2, AmRb-2, AbLa-2 e AmLa-2) e fração aquosa (AbRb-3, AmRb-3, AbLa-3 e AmLa-3). Os constituintes marcadores das frações L-L de maior atividade antimicrobiana, quando não passíveis de identificação pela técnica de desreplicação utilizada, foram isolados por cromatografia em coluna e identificados por técnicas identificação estrutural. O teor dos constituintes dos extratos hidroetanólicos a 70% foi determinado por colorimetria, visando o doseamento das principais classes de metabolitos secundários presentes e por quantificação dos constituintes maioritários de cada planta, por LC-UV/DAD.

Em adição, e atendendo às classes químicas maioritárias identificadas, foi avaliada a atividade antioxidante dos diferentes extratos e de algumas frações e/ou constituintes isolados, por colorimetria, usando os testes de capacidade antioxidante por redução do ião férrico (FRAP) e do 2,2-difenil-1-picryl-hidrazilhidrato (DPPH).

Os estudos de segurança pré-clínica foram efetuados estudos *in* vitro em modelos celulares, e teste de Ames.

Resultados e discussão: A nível botânico, foi observada na raiz de ambas as espécies a presença de uma epiderme contendo múltiplas camadas (velame) e de um córtex com idioblastos de parede fina (134 ± 2.9 µm em AbR e 150 ± 27.6 µm em AmR), contendo cristais de oxalato de cálcio em forma ráfide. A principal diferença morfológica identificada entre as raízes das duas espécies foi a dimensão dos tubérculos (3.5x1.0 cm em AbR e 8.7x1.4 cm em AmR). Na avaliação microscópica, observaram-se diferenças ao nível da distribuição e características dos feixes vasculares (metaxilema e protoxilema), sendo os vasos de metaxilema em *A. macrocarpus* (± 80 µm de diâmetro) rodeados por vários protoxilemas, enquanto que em A. bento-rainhae (± 70 µm de diâmetro) estes se encontram isolados.

O doseamento colorimétrico das principais classes de metabolitos secundários presentes na folha e raiz de ambas as espécies mostrou serem os terpenoides (111.72±22.77, 165.47±26.54, 172.11±19.20 e 154.36±20.53 de mg de equivalentes de ácido oleanólico / g extrato seco em AbLa, AmLa, AbRb, AmRb, respetivamente) e os taninos condensados (180.96±10.98, 132.60±2.73, 128.64±14.05, 132.60±2.73 de mg de equivalentes catequina/ g extrato seco em AbLa, AmLa, AbRb, AmRb, respetivamente), os constituintes maioritários, tendo sido, no entanto, também detetados derivados de antracénicos (1.16±0.13, 0.55±0.07, 3.38±0.26 e 2.68±0.19 mg de equivalentes reína/ g extrato seco em AbLa, AmLa, AbRb, AmRb, respetivamente).

Considerando a sazonalidade da colheita das amostras, os extratos hidroetanólicos a 70% da raiz de ambas as espécies (AbRb e AmRb) colhida durante a segunda colheita (Novembro) mostraram maior teor em constituintes maioritários. Os extratos hidroetanólicos a 70% da folha de ambas as espécies (AbLa e AmLa) colhida durante a primeira colheita (Maio) mostraram maior teor em constituintes maioritários. Por esta razão, estes quatro extratos foram selecionados para continuação dos estudos químicos e biológicos.

O fracionamento bioguiado dos extratos hidroetanólicos a 70% de raiz de ambas as espécies mostrou serem as frações etér-dietílicas (AbRb-1, AmRb-1), as de maior atividade contra todos os microorganismos Gram (+) testados (MIC: 16 a 1000 µg/mL),

sendo AbRb-1 relativamente mais ativa do que AmRb-1. Estas frações mostraram conter derivados de antracénicos como constituintes marcadores, nomeadamente, 7'-(crisofanol-4-yl)-crisofanol-10'-*C*-beta-*D*-xilopiranosil-antrona, 10,7'-bicrisofanol, crisofanol, 10-(crisofanol-7'-yl)-10-hidroxicrisofanol-9-antrona e a asfodelina. O crisofanol, identificado como constituinte maioritário, tendo o seu teor sido quantificado nos extratos hidroetanólicos a 70% e a 96%, quer em AbRb (10 e 36 µg/mL, respetivamente) quer em AmRb (7 e 23 µg/mL, respetivamente), é especialmente ativa contra *S. epidermidis* (MIC 3.2 a 100 µg/mL).

Adicionalmente foram também identificados mais seis constituintes minoritários nesta parte da planta, nomeadamente o ácido clorogénico, ácido vaníico, ácido cafeico, ácido ferúlico, ácido isoclorogénico e β-sitosterol.

A partir dos extratos totais da folha de ambas as espécies (AbLa e AmLa) foram identificados 12 compostos marcadores, nomeadamente o ácido neoclorogénico, ácido clorogénico, ácido cafeico, isoorientina, ácido *p*-comárico, isovitexina, ácido ferúlico, luteolina, aloe-emodina, diosmetina, crisofanol e o β-sitosterol. Destes, os compostos maioritários foram a isoorientina e o ácido clorogénico. A isoorientina foi quantificada nos extratos hidroetanólicos a 70% (184 µg/mL e 112 µg/mL em AbLa e AmLa, respectivamente) e a 96% (107 µg/mL e 30 µg/mL em AbLa e AmLa, respectivamente). O ácido clorogénico foi quantificado nos extratos hidroetanólicos a 70% (66 µg/mL e 90 µg/mL em AbLa e AmLa, respectivamente) e a 96% (42 µg/mL e 27 µg/mL em AbLa e AmLa, respectivamente).

O fracionamento bioguiado por serem também as frações de maior conteúdo em derivados antracénicos (frações éter dietílicas AbLa-1, AmLa-1), as de maior atividade contra todos os microorganismos Gram (+) testados (MIC: 62 a 1000 µg/mL), sendo AbLa-1 relativamente mais ativa que AmLa-1. A aloe-emodina foi identificada como constituinte maioritário desta fração, tendo o seu teor sido quantificado nos extratos hidroetanólicos a 70% e a 96%, quer em AbLa (13 e 15 µg/mL, respetivamente) quer em AmLa (7 e 8 µg/mL, respetivamente), especialmente ativa contra *S. epidermidis* (MIC 0.8 a 1.6 µg/mL). De um modo geral, os extratos hidroetanólicos a 96% foram

mais ativos que os a 70% e são também os mais ricos em derivados antracénicos, compostos potencialmente relacionados com a atividade antimicrobiana.

O potencial antioxidante foi avaliado em todos os extratos totais e frações, tendo-se verificado que era superior nos extratos de folha em relação aos da raiz, sendo a fração de acetato de etilo a mais ativa (IC_{50} de 800 e 1200 µg/mL, em AbLa-2 e AmLa-2, respetivamente) e a que tem maior teor em ácidos fenólicos e derivados de flavonoides. Ainda assim o potencial antioxidante destes extratos foi inferior à observada pelo padrão do ácido ascórbico (IC_{50} de 83 µg/mL), usado como substância de referência.

A toxicidade foi avaliada em todos os extratos hidroetanólicos a 70% e no extrato hidroetanólico de AbRb a 96%, o extrato testado com maior atividade antimicrobiana. Não se observou citotoxicidade até 125 µg/mL nos extratos de raiz e até 1000 µg/mL nos extratos de folha contra células HepG2 e até 125 µg/mL para AbRb a 96%, contra células HaCaT. Também não se observou genotoxicidade em nenhum dos extratos testados até uma concentração de 5.0mg/placa (teste de Ames)

Conclusão: Os resultados obtidos deste trabalho permitiram a obtenção dos critérios de qualidade e diferenciadores entre as quatro plantas medicinais e as duas espécies em estudo e o conhecimento do seu potencial antimicrobiano.

O trabalho efetuado permitiu confirmar a atividade antimicrobiana destas plantas, e que esta se deve sobretudo à existência de compostos derivados de antracénicos. Estas plantas, se usadas sob a forma de tintura, a 96%, poderão ter utilidade de interesse terapêutico para o tratamento tópico de infeções cutâneas, confirmando o seu uso na medicina tradicional portuguesa.

Palavras-chave: *Asphodelus bento-rainhae*, *Asphodelus macrocarpus,* atividade antimicrobiana, atividade antioxidante, controle de qualidade, derivados de antracénicos, doenças de pele, Etnofarmacologia, preparação tradicional à base de plantas, segurança pré-clínica, *Staphylococcus epidermidis*

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ABBREVIATIONS

AbLa	*A. bento-rainhae* leaf first collection
AbLb	*A. bento-rainhae* leaf second collection
AbRa	*A. bento-rainhae* root first collection
AbRb	*A. bento-rainhae* root second collection
ABS	absorbance
AcOEt	ethyl acetate
AcOH	Acetic acid
AmLa	*A. macrocarpus* leaf first collection
AmLb	*A. macrocarpus* leaf second collection
AMR	Antimicrobial resistance
AmRa	*A. macrocarpus* root first collection
AmRb	*A. macrocarpus* root second collection
APG	Angiosperm Phylogeny Group
n-BuOH	*n*-butanol
CV	coefficient of variation
D&D	drug discovery and development
DMSO- d6	dimethyl sulfoxide deuterated
DPPH	1,1-diphenyl-2-picrylhydrazine
EDQM	European Directorate for the Quality of Medicines & HealthCare
ESBL	Extended spectrum β-lactamase
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
Et_2O	Diethyl ether
EtOH	ethanol
ESI/MS	Electrospray Ionization Mass Spectrometry
FFUL	Faculty of pharmacy, Universidade de Lisboa
FQR	fluoroquinolone-resistant

FRAP	ferric reducing antioxidant power
GC-MS	Gas Chromatography-Mass Spectrometry
HaCaT	Cultured Human Keratinocyte cell line
HepG2	Hepatoblastoma cell line
HPLC	High-Performance Liquid Chromatography
INSA	National Institute of Health Dr. Ricardo Jorge
IC_{50}	half maximal inhibitory concentration
iMed.ULisboa	Research Institute for Medicines
IP %	percentage of inhibition
IUCN	International Union for the Conservation of Nature
LM	light microscopy
Max	maximum
MDR	multidrug-resistant
MeOH	methanol
MRSA	methicillin-resistant *Staphylococcus aureus*
MTT	methylthiazolyldiphenyl-tetrazolium bromide
NP/PEG	natural product-polyethylene glycol
p.a.	pro-analysis
R_f	retardation factor
t_R	retention time
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
SiG60	silica gel 60 F254
TLC	Thin Layer Chromatography
UPLC	Ultra-High-Performance Liquid Chromatography
UV/DAD	Ultraviolet-Diode Array Detector
VRE	vancomycin-resistant Enterococcus
WCSP	World Checklist of Selected Plant Families
WHO	World Health Organization

Chapter 1

General introduction and objectives

1.1. Antimicrobial resistance

The average life expectancy was significantly lower in the pre-antibiotic era, partly due to the high mortality as a result of infectious diseases. The unveiling of penicillin in 1928 marked a significant milestone in the history of medicine, signifying the dawn of the “antibiotic era” [1]. The global adoption of penicillin began in 1945, ushering in the era where antimicrobial agents became indispensable to modern healthcare. Following the introduction of penicillin, a multitude of additional antimicrobials were discovered, particularly from the 1950s to the 1970s. During this prolific period, more than half of the antimicrobial classes currently in use were isolated, earning it the moniker "the golden era" in antibiotic discovery [2,3].

However, the widespread use of antimicrobials in healthcare and agriculture has significantly accelerated the process of bacterial selection, leading to the dissemination of antimicrobial resistance throughout human society [4]. Despite the discovery of a variety of antimicrobial classes with different targets, no antimicrobial could avoid the development of resistance in hospitals (Fig. 1.1).

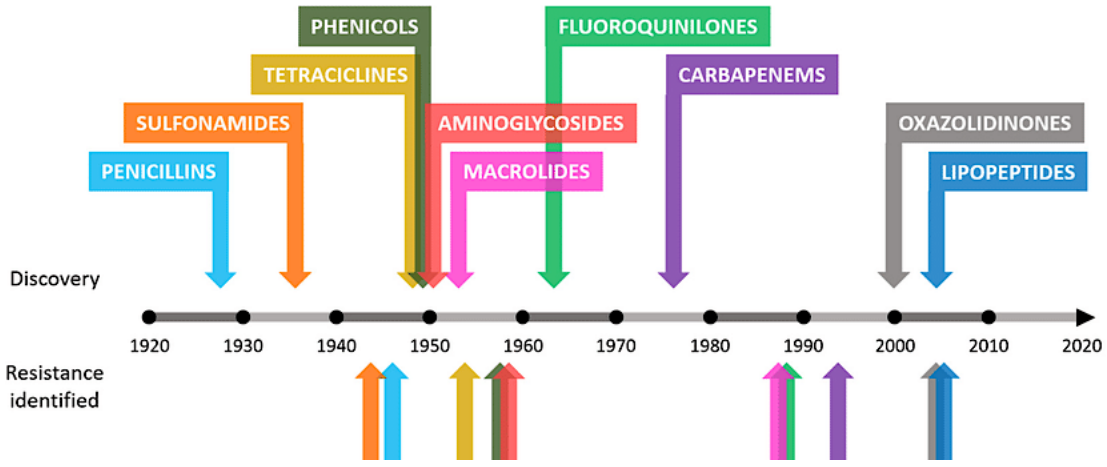


Figure 1.1. Timeline showing some of the key antibiotic discoveries and reports of the emergence of antibiotic resistance strains [5].

Antimicrobial resistance (AMR) has now emerged as a chronic public health problem globally, with the forecast of 10 million deaths per year globally by 2050 (Fig.1.2). AMR occurs when viruses, bacteria, fungi, and parasites do not respond to antimicrobial treatments in humans and animals, thus allowing the survival of the microorganism within the host. The prominent cause contributing to the current crisis remains to be the overuse and misuse of antimicrobials, particularly the inappropriate usage of antibiotics, increasing the global burden of antimicrobial resistance [6]

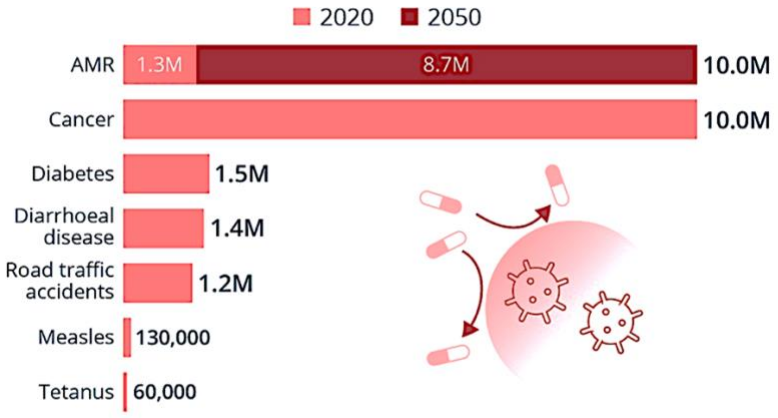


Figure 1.2. Predicted mortality from AMR compared with common causes of current deaths [7].

AMR is widely referred to as the "Silent Pandemic" and is a problem where urgent action is needed immediately and should be managed more effectively and not be considered as a future situation [8]. Europe, Portugal included, is not immune to this problem and the major resistance overall issues (Fig. 1.3.) being related to the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) specially to Methicillin-resistant *S. aureus* (MRSA), extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*, fluoroquinolone-resistant (FQR) Gram-negative bacteria, multidrug-resistant (MDR) *P. aeruginosa*, and the emerging vancomycin-resistant *Enterococcus* (VRE) [9].

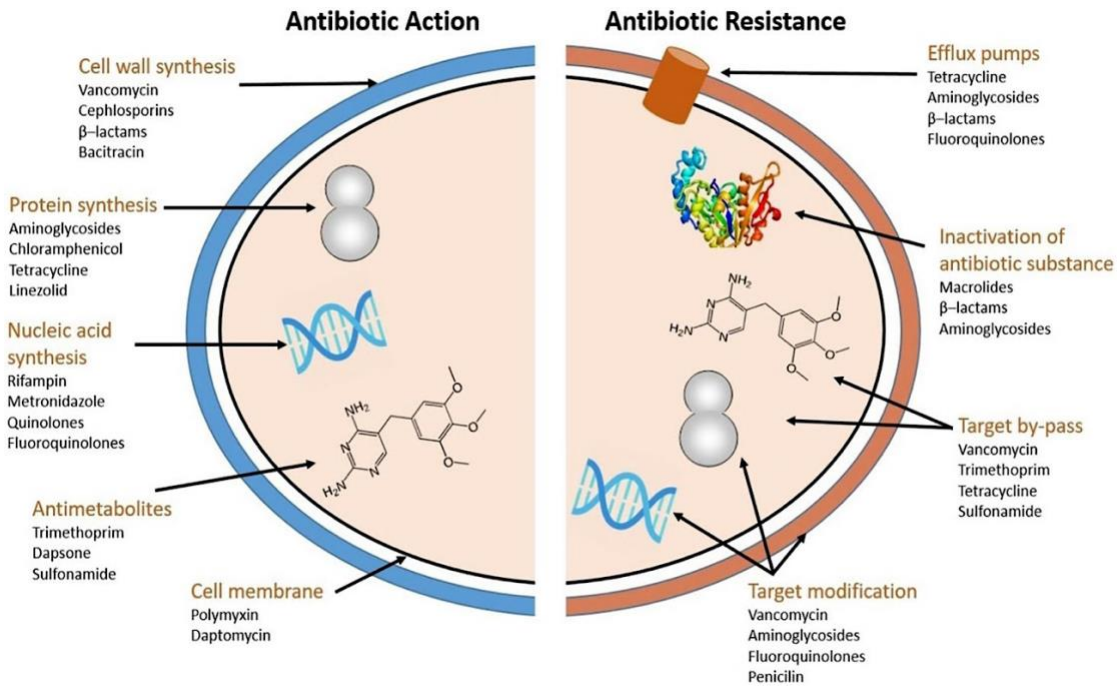


Figure 1.3. Antibiotics action and resistance mechanisms [10]. Examples of antimicrobials of each class are listed.

The emergence of these new pathogens, resistant to antimicrobials, has contributed to worldwide agencies linked to human and animal health to unite efforts in the discovery and development (D&D) of new antimicrobial drugs [11,12]. Among several important and effective approaches to discovering novel antibacterial and antifungal compounds, traditional screening of natural products is still considered a valuables and indispensable method. One of the key advantages of traditional natural product screening for which academia has made important contributions in the past few decades is its rich history of success in identifying bioactive molecules that have been used to develop essential antibiotics and antifungal agents (e.g., penicillin, streptomycin) [13].

1.2. Medicinal plants and natural products as antimicrobials

Only a small fraction of marine, fungal and plant resources have been chemically and pharmacologically investigated, although nature still offers a high potential for drug

lead discovery notably among anti-infective compounds. In fact, in the last decades the majority of the new antibacterial drugs were of natural source and among them, plants were mainly used [14].

The phytochemicals are numerous in structure, generally have few side effects and, as a result, they acquired extra attention in the pharmaceutical industry to enhance the biological activity of existing antibiotics or as a potential source of novel antimicrobial agents active against a variety of pathogens, including MDR bacteria [15]. Moreover, β-lactams, tetracycline, macrolides, glycopeptides, lincosamides, sulfonamides, oxazolidinones, and fluoroquinolones are representative classes of antibiotics of the modern era, of which six represent naturally occurring compounds, and only the latter three are obtained entirely by chemical synthesis [16]

In general, natural-product antibiotics hold a privileged position in this field as they are the products of millions of years of evolution. Through natural selection, they interact with cellular targets with high selectivity and efficiency and avoid drug resistance. Also, unlike numerous synthetic molecules, they are products of evolution that have inherent physicochemical properties necessary to penetrate bacterial cells. Moreover, molecular selectivity is a hallmark of a large number of naturally occurring antibiotics, resulting in relatively small widespread toxicity [17].

In this context, the ethnomedical information provide a bridge between traditional knowledge and modern scientific research leading to the identification of novel antimicrobial agents and potentially offer new solutions to combat infectious diseases [18].

1.3. *Asphodelus* L.

Ethnomedical information on several *Asphodelus* L. species [19], supported by preliminary activity studies indicates a strong antibacterial potential, namely against MRSA and *P. aeruginosa* and also a considerable antifungal activity (e.g. *Cryptococcus neoformans*), due to the presence of secondary metabolites such as anthraquinones, flavonoids and arylcoumarins [20,21].

1.4. *Asphodelus* L. in Portugal

According to Flora Iberica, there are 12 species of *Asphodelus* in the Iberian Peninsula (a total of 18, counting with subspecies and varieties, beside 5 hybrids) namely, *Asphodelus aestivus* Brotero, *Asphodelus albus* Miller subsp. *albus*; subsp. c*arpetanus* Z. Díaz & Valdés; subsp. *delphinensis* (Grenier & Godron) Z. Díaz & Valdés; subsp. *occidentalis* (Jordan) Z. Díaz & Valdés], *Asphodelus ayardii* Jahandiez & Maire, *Asphodelus bento-rainhae* P. Silva subsp. *bento-rainhae*; subsp. *salmanticus* Z. Díaz & Valdés, *Asphodelus cerasifer* J. Gay, *Asphodelus fistulosus* Linnaeus, *Asphodelus lusitanicus* Coutinho, *Asphodelus macrocarpus* Parlatore subsp. *macrocarpus*; subsp. *rubescens* Z. Díaz & Valdés, *Asphodelus ramosus* Linnaeus subsp. *distalis* Z. Díaz & Valdés; subsp. *ramosus*, *Asphodelus roseus* Humbert & Maire, *Asphodelus serotinus* Wolley-Dod and *Asphodelus tenuifolius* Cavanilles [22].

The Portuguese flora exhibits a considerable abundance of *Asphodelus* species, subspecies and varieties compared to the rest of Europe and the Mediterranean Basin. [23]. According to World Checklist of Selected Plant Families (WCSP), In Portugal (Continental, Açores and Madeira islands), *Asphodelus* genus is represented by 8 species (a total of 12, counting with subspecies and varieties) namely, *A. aestivus* Brot., *A. bento-rainhae* P. Silva subsp. *bento-rainhae*, *A. fistulosus* L. subsp. *fistulosus*; subsp. *madeirensis* Simon, *A. lusitanicus* Cout. var. *lusitanicus*; var. *ovoideus* (Merino) Z. Díaz & Valdés, *A. macrocarpus* Parl. subsp. *macrocarpus,* var. *arrondeaui* (J. Lloyd) Z. Díaz & Valdés, *A. ramosus* L. subsp. *distalis* Z. Díaz & Valdés; subsp. *ramosus*, *A. serotinus* Wolley-Dod. and *A. tenuifolius* Cav. together with more than 80 homo- and heterotypic synonym names [23]. However, in the Flora of Portugal checklist (Checklist da Flora de Portugal), there is an extra record of *A. albus* Mill. subsp. *villarsii* (Verl. ex Billot) I. Richardson & Smythies and absence of *A. fistulosus* L. subsp. *madeirensis* Simon and *A. ramosus* L. subsp. *ramosus* [24].

Among the Portuguese *Asphodelus*, *A. bento-rainhae* subsp. *bento-rainhae*, is an endemic species and can only be found in the Gardunha mountain range “Serra da

Gardunha," located in the Central Region of Portugal, covering the counties of "Fundão" and "Castelo Branco" (Figs. 1.4 and 1.6), coexisting with *A. macrocarpus* subsp. *macrocarpus* in the same geographical area (W 07º 29´ 44´´, N 40º 07´ 01´´) (Figs. 1.5 and 1.7) [25]. *A. bento-rainhae* It is also described as a "Vulnerable" species on International Union for the Conservation of Nature (IUCN) Red List of Threatened Species [26].

Figure 1.4. Global geographical distribution of *A. bento-rainhae* subsp. *bento-rainhae* [27].

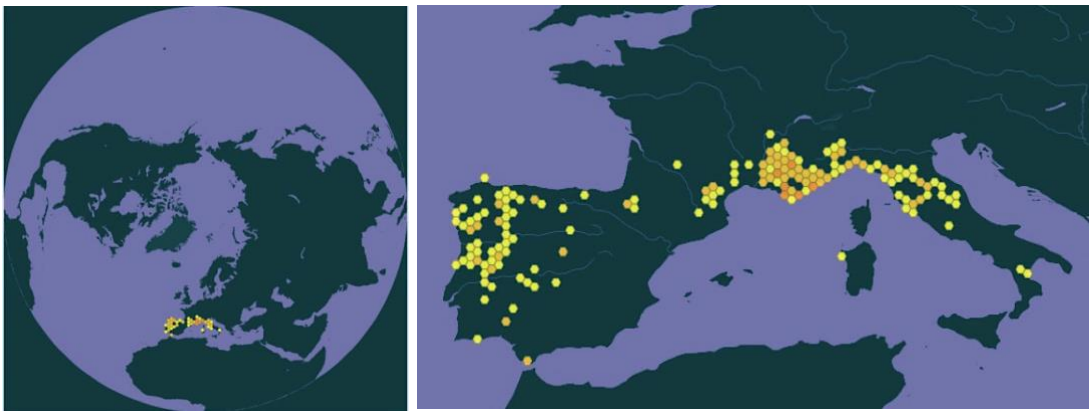
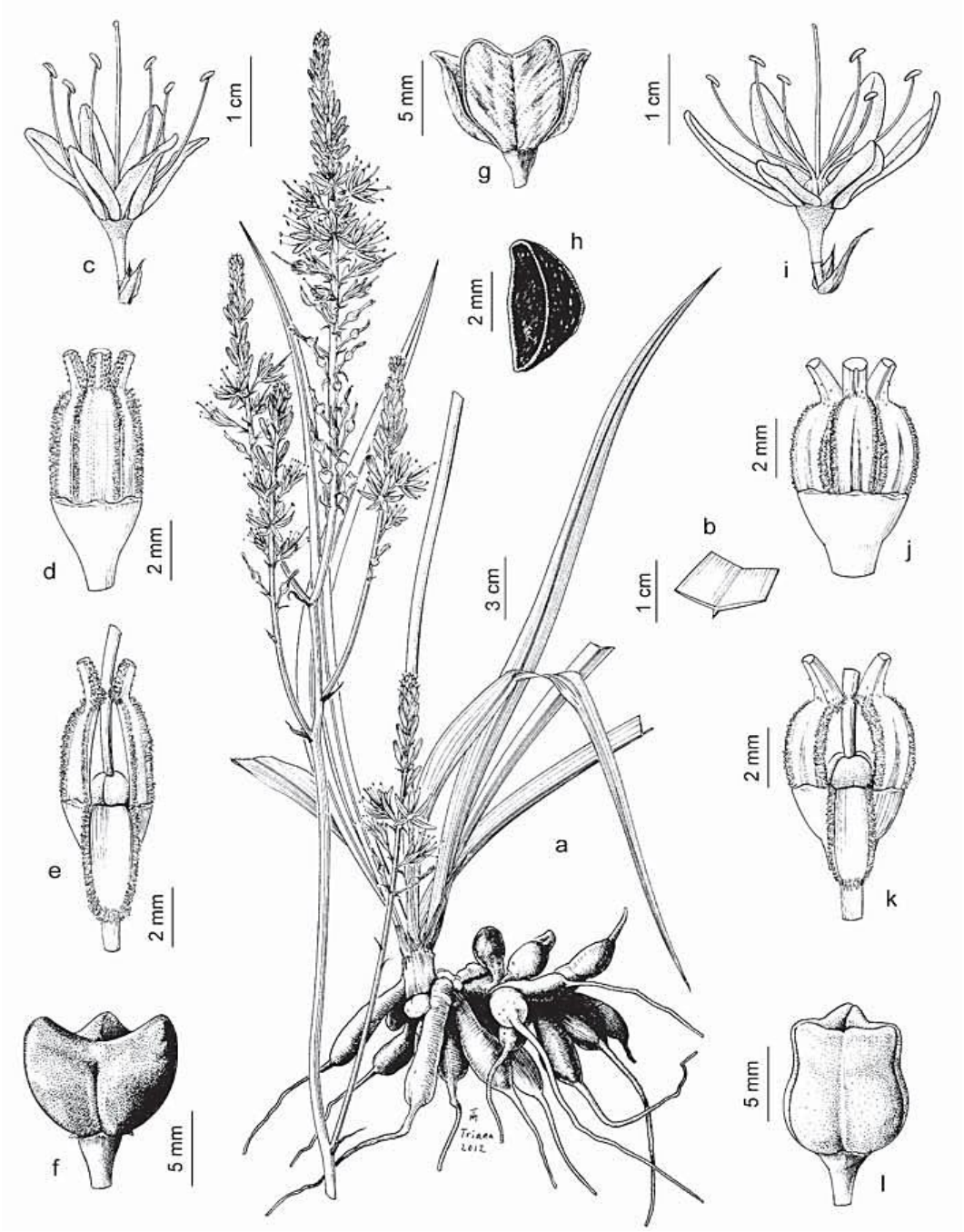


Figure 1.5. Global geographical distribution of *A. macrocarpus* subsp. *macrocarpus* [27].Yellow: Lower occurrence density ; Orange to red: higher occurrence density

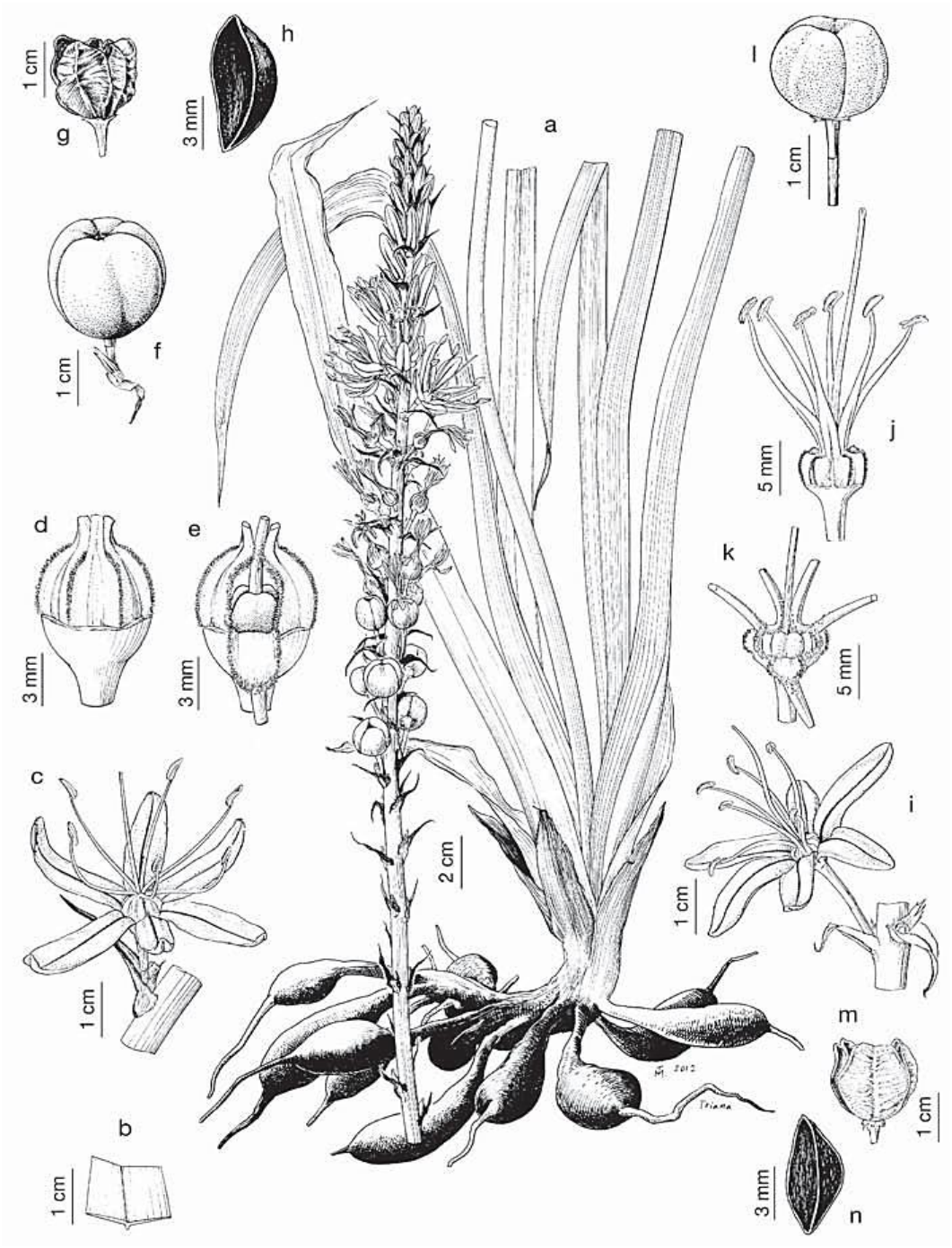
Moreover, *Asphodelus* species have traditionally been used in Portugal for the treatment of inflammatory and infectious skin conditions and are commonly referred to as "abrótea". To our knowledge, the term "abrótea" is derived from "Abrotonum" (Ancient Greek: Ἀβρότονον) which in certain Portuguese translations, it is also

associated with the terms "hanging" or "suspended," likely depicting the arrangement of the aerial parts of these plants.



Abbreviations: a) habitat; b) fragment of a leaf, with cross section; c) flower; d) basal part of the stamens; e) ovary and basal part of the stamens open; f) fresh capsule; g) open capsule; h) seed; i-k) A. *bento-rainhae* subsp. *salmanticus* [22].

Figure 1.6. *Asphodelus bento-rainhae* subsp. *bento-rainhae.*



Abbreviations: a) habitat; b) fragment of a leaf, with cross section; c) flower; d) basal part of the stamens; e) ovary and basal part of the stamens open; f) fresh capsule; g) open capsule; h) seed; i–n) *A. cerasifer* [22].

Figure 1.7. *Asphodelus macrocarpus* subsp. *macrocarpus.*

1.5. Objectives of the thesis

This work began with the recognition that, despite the existence of ethnomedical knowledge supported by preliminary pharmacological activity studies on *Asphodelus*, there is still a lack of information related to the Portuguese species. In addition, to the best of our knowledge, few scientific studies have been documented on *Asphodelus bento-rainhae* and *Asphodelus macrocarpus*. Thus, the present PhD project aimed:

1) to establish the metabolomic profile of *Asphodelus bento-rainhae* subsp. *bento-rainhae*, a Portuguese endemism of relevant interest due to conservation concerns, and *Asphodelus macrocarpus* subsp. *macrocarpus,* it´s co-existent Iberian species, with no significant biological or chemical studies.

2) to characterize the antimicrobial activity of the *A. macrocarpus* and *A. bento-rainhae* root tuber and leaf extracts and contribute to the validation of their traditional medicinal properties .

3) to identify marker lead-molecules with antibacterial/ antifungal activities from the selected medicinal plant parts and to determine their effectiveness against antimicrobial/fungal resistant pathogens.

4) to ascertain the relation between the marker secondary metabolites with antimicrobial/ fungal activities and their chemical structures.

5) to evaluate the toxicity and genotoxicity of the most active extracts and their main marker metabolites with antimicrobial/fungal activities, in order to assess their safety for future therapeutic applications.

6) to establish the principal botanical and chemical monographic quality requirements to enable the future utilization of these medicinal plants as herbal traditional medicines.

Chapter 2

Literature Review

Main part of this chapter was published as follows:

A Comprehensive Review on the Medicinal Plants from the Genus *Asphodelus*.

Maryam Malmir, Rita Serrano, Manuela Caniça, Beatriz Silva-Lima, and Olga Silva.

Plants *2018, 7 (1), 20.*

doi:10.3390/plants7010020.

Impact Factor: 4.658

In this chapter the bibliographic update on the genus *Asphodelus*, concerning botanical, chemical, pharmacological, and toxicological aspects was carried out and briefly summarized.

The focus of this literature review was on various aspects of *Asphodelus* species, including the taxonomic classifications, exploring their traditional uses in different cultures (ethnobotany), chemical composition and biological activity.

In the first part, the review delved into the botanical classification, nomenclature, and identification of the different species within the *Asphodelus* genus, highlighting their morphological characteristics, geographic distribution, and taxonomic challenges or controversies.

The chapter then explored the ethnobotanical aspects of *Asphodelus* species. It examined their traditional uses by various cultures throughout history, including information on cultural significance, rituals, and medicinal applications. Various parts of the plants and their methods of preparation or administration were also discussed.

The review further investigated the chemical composition of *Asphodelus* species. It explored the phytochemical profiles of these plants, including the presence of secondary metabolites such as anthracene derivatives, flavonoids, terpenoids, and phenolic acids. This section also highlighted the type of the extracts used for chemical analysis and reported the identified compounds.

Finally, the chapter addressed the previously reported pharmacological and biological activities of *Asphodelus* species such as antimicrobial, anti-inflammatory, antioxidant, anticancer, or other relevant activities. It could also discuss the mechanisms of action or potential therapeutic applications based on the described properties.

By organizing the present chapter into several subsections, the authors aimed to provide a comprehensive and updated overview of *Asphodelus* species, to grasp the existing knowledge and research gaps associated with them.

2.1. Abstract

Plant-based systems continue to play an essential role in healthcare, and their use by different cultures has been extensively documented. *Asphodelus* L. (*Asphodelaceae*) is a genus of 18 species and of a total of 27 species, sub-species, and varieties, distributed along the Mediterranean basin, and has been traditionally used for treating several diseases particularly associated with inflammatory and infectious skin disorders. The present study aimed to provide a general review of the available literature on ethnomedical, phytochemical, and biological data related to the genus *Asphodelus* as a potential source of new compounds with biological activity. Considering phytochemical studies, 1,8-dihydroxyanthracene derivatives, flavonoids, phenolic acids, and triterpenoids were the main classes of compounds identified in roots, leaf and seeds which were correlated with their biological activities as anti-microbial, anti-fungal, anti-parasitic, cytotoxic, anti-inflammatory or antioxidant agents.

Keywords: anthracene derivatives; antimicrobial; *Asphodelus*; ethnomedicine; skin diseases

2.2. Introduction

2.2.1. Taxonomic position of the genus *Asphodelus* L.

The genus *Asphodelus* Linnaeus belongs to family *Asphodelaceae* Jussieu and is native to temperate Europe, the Mediterranean, Africa, the Middle East, and the Indian Subcontinent and now naturalized in other places (New Zealand, Australia, Mexico, southwestern United States, etc.) [23]. It reaches its maximum diversity in the West of the Mediterranean, particularly in the Iberian Peninsula and in North-West Africa [22].

The family consists of three subfamilies: *Asphodeloideae* Burnett (including 13 genera), *Hemerocallidoideae* Lindley (including 19 genera) and *Xanthorrhoeoideae*

M.W. Chase (with only one genus). This botanical family, now called *Asphodelaceae*, has had a complex history; its circumscription and placement in an order have varied widely. In the Cronquist system of 1981, members of the *Asphodelaceae* were placed in the order Liliales Perleb [28,29].

Cronquist had difficulty classifying the less obviously delineated lilioid monocots; consequently, he placed taxa from both the modern orders Asparagales Link and Liliales into a single family, *Liliaceae* Jussieu [30]. The decision to group three formerly separate families, *Asphodelaceae*, *Hemerocallidaceae* and *Xanthorrhoeaceae*, into a single family first occurred in 2003 as an option in the II Angiosperm Phylogeny Group (APG) classification for the orders and families of flowering plants. The name used for the broader family was then *Xanthorrhoeaceae* Dumortier [31], and the earlier references to this family were related only to subfamily *Xanthorrhoeoideae*.

These changes were a consequence of improvements in molecular and morphological analysis and also a reflection of the increased emphasis on placing families within an appropriate order [18, 20, 21]. Later in 2009, the APG III classification dropped the option of keeping the three families separate, using only the expanded family, still under the name *Xanthorrhoeaceae* [32]. Anticipating a decision to conserve the name *Asphodelaceae* over *Xanthorrhoeaceae*, the APG IV classification of 2016 used *Asphodelaceae* as the name for the expanded family [34].

According to the WCSP, there are 32 accepted names with more than 150 homo- and heterotypic synonyms for all species, subspecies and varieties of the genus *Asphodelus* L. namely, *Asphodelus acaulis* Desfontaines, *Asphodelus aestivus* Brotero, *Asphodelus albus* Miller (subsp. *albus*; subsp. *carpetanus* Z. Díaz & Valdés; subsp. *delphinensis* (Grenier & Godron) Z. Díaz & Valdés; subsp. *occidentalis* (Jordan) Z. Díaz & Valdés), *Asphodelus ayardii* Jahandiez & Maire, *Asphodelus bakeri* Breistroffer, *Asphodelus bento-rainhae* P. Silva (subsp. *bento-rainhae* ; subsp. *salmanticus* Z. Díaz & Valdés), *Asphodelus cerasifer* J. Gay, *Asphodelus fistulosus* Linnaeus (subsp. *fistulosus*; subsp. *madeirensis* Simon), *Asphodelus gracilis* Braun-Blanquet & Maire,

Asphodelus lusitanicus Coutinho (var. *lusitanicus*; var. *ovoideus* (Merino) Z. Díaz & Valdés), *Asphodelus macrocarpus* Parlatore (subsp. *macrocarpus*; subsp. *rubescens* Z. Díaz & Valdés; var. *arrondeaui* (J. Lloyd) Z. Díaz & Valdés), *Asphodelus ramosus* Linnaeus (subsp. *distalis* Z. Díaz & Valdés; subsp. *ramosus*); *Asphodelus refractus* Boissier, *Asphodelus roseus* Humbert & Maire, *Asphodelus serotinus* Wolley-Dod, *Asphodelus tenuifolius* Cavanilles, and *Asphodelus viscidulus* Boissier [23]. However, on the Missouri Botanical Garden database (Tropicos), more two accepted names (*Asphodelus cerasifer* Gay and *Asphodelus microcarpus* Salzmann & Viviani) were recorded [35].

Considering all the above-mentioned data, 18 species (a total of 27, counting with sub-species and varieties) must be considered for the *Asphodelus* genus worldwide. Among all the species, *A. aestivus* and *A. fistulosus* are inscribed as “Least Concern” and *A. bento-rainhae* as “Vulnerable” species on International Union for the Conservation of Nature (IUCN) Red List of Threatened Species [26].

2.2.2. Botanical characteristics

Botanical and systematic descriptions of *Asphodelus* L. genus have been discussed by several taxonomists in various flora and publications. The plants are hardy herbaceous perennials with narrow tufted radical leaf and an elongated stem bearing a spike of white or yellow flower. Many have a small rhizomatous crown and thick, fleshy root [22].

2.2.3. Ethnobotany

Different ethnomedical uses were described to *Asphodelus* species. Different parts of the plant including leaf, fruit, seed, flower, and root are used as traditional herbal medicines, alone or in mixtures to treat various ailments.

Root tubers of *Asphodelus* species have shown to be the most common plant part traditionally used for the treatment of skin-related disorders and infections. In the Iberian Peninsula, the cut root tubers were used for the treatment of skin eczema

(e.g., psoriasis) and their ashes were used against the alopecia [22]. In Portugal, fresh juice or tincture of the root tubers of *A. bento-rainhae* and *A. macrocarpus* have been traditionally used for the treatment of scabies, dermatophytosis and warts. Leaves also, have been widely used in traditional medicine for treatment of ulcers, urinary and inflammatory disorders [36]. In North African countries and the Iberian Peninsula, leaves and stems decoctions of *Asphodelus* species were also used for the treatment of withering and paralysis [22,37].

Beside the medicinal uses, *Asphodelus* L. species, are among the beneficial dietary herbal medicines, consumed in large quantities in the culinary (e.g., soups, pastries, etc.) of several countries and various cultures. Root tubers have been used as daily food, after being moistened and fried beforehand to eliminate the astringent compounds [22]. Leaves of *A. aestivus* are also commonly consumed cooked as a vegetable dish in Turkey, where is known as "çiriş otu" [38]. In Puglia, on the southeast coast of Italy, burrata cheese is always wrapped in *Asphodelus ramosus* L. leaves to indicate the freshness of the cheese before it dries out [39].

Moreover, the alcohol obtained by fermentation of the root tubers is used as fuel in the Iberian Peninsula [22] and the local people of Iran, Turkey and Egypt use the root tuber of *A. aestivus* and *A. microcarpus* to produce a strong glue used by shoemakers and cobblers [37,40], and yellow and brown dyes to dye wool [22].

2.3. Materials and Methods

Ethnobotanical data was collected by our team in Portugal and relevant literature was reviewed until September 2023, by probing scientific databases (PubMed, Scopus, Google Scholar, b-on, Web of knowledge) and other web sources such as records from WCSP, IUCN, APG and the Missouri Botanical Garden database. Various keywords were used during the bibliographic research including: ASPHODELUS SPECIES; TRADITIONAL USES; ETHNOMEDICINAL EVIDENCE; BIOLOGICAL ACTIVITIES; ISOLATED MOLECULES; PHYTOCHEMISTRY. Information was gathered and summarized in table form where appropriate.

2.4. Results and Discussions

2.4.1. Ethnomedical Studies

Table 2.1. summarized the ethnomedicinal data about the *Asphodelus* species including specific information on the plant parts as well as the geographical region where the plant is used.

Table 2.1. *Asphodelus* species ethnomedical uses.

Species	Part Used	Country	Traditional Uses/Application	Ref.
A. aestivus	L, R	Turkey	Peptic ulcers	[41]
	R	Turkey	Hemorrhoids, burns, wounds, and nephritis	[42]
	NI	Cyprus, Spain	Skin diseases	[19]
A. fistulosus	NI	Egypt, Libya	Fungal infections	[43]
A. luteus *	WP	Palestine	Dermatomucosal infections	[44]
A. microcarpus	FR, L, R	Egypt	Earache, withering and paralysis	[37,40]
	R	Palestine	Dermatomucosal infections	[44]
	R	Egypt	Ectodermal parasites, jaundice, microbial infections, and psoriasis	[21,45,46]
	NI	Algeria	Earache, eczema, colds, and rheumatism	[47]
A. ramosus	R	North-Africa	Inflammatory disorders	[48]
	NI	Turkey	Anti-tumoral, diuretic and emmenagogue	[49]
A. tenuifolius	L	India	Diuretic, inflammatory disorders and ulcers	[50]
	L, SE	Egypt	Diuretic	[51]
	R, SE	India	Antipyretic, diuretic, colds and hemorrhoids, inflammatory disorders, rheumatic pain, ulcers, and wounds	[52,53]
	SE	Pakistan	ulcers and inflammatory disorders	[54]
	WP	India	Diuretic, inflammatory disorders, bite of bees and wasps, ulcers	[55,56]
	NI	Pakistan	Diuretic	[57]

Abbreviations: SE: Seed; L: Leaf; WP: Whole plant; FR: Fruit; R: Root; NI: Not indicated, * *Asphodelus luteus* L. synonym of *Asphodeline lutea* was formerly included in the family *Asphodelaceae.*

Ethnomedical records showed that among the 18 species of the genus *Asphodelus*, only 5 species namely *A. aestivus*, *A. fistulosus*, *A. microcarpus*, *A. ramosus* and *A. tenuifolius* have been documented for their traditional uses.

Most commonly, these species were used as anti-inflammatory and anti-infective agents. In particular, *A. aestivus*, *A. fistulosus* and *A. microcarpus* were reported to be used in dermatomucosal infections in various countries including Cyprus, Egypt, Libya, Palestine, and Spain [19,43–46]. *A. microcarpus*, *A. ramosus* and *A. tenuifolius* were generally indicted as anti-inflammatory agents specifically for the treatment of psoriasis, eczema, and rheumatism [21,47,48,50,52–55]. *A. aestivus* and *A. tenuifolius* are also used for ulcer treatment in Turkey, India, and Pakistan [49,53–55]. *A. ramosus* and *A. tenuifolius* have frequently been reported as diuretics among the inhabitants of Egypt, India, Pakistan, and Turkey [49–53,55,57].

2.4.2. Previously Reported Phytochemical Studies

The principal chemical studies and identified compounds of the genus *Asphodelu*s are presented in Table 2.2.

The results revealed the presence of different groups of compounds namely anthraquinones (either in the free or in the glycoside form), phenolic acid derivatives, flavonoids, and triterpenoids from *A. acaulis*, *A. albus*, *A. aestivus*, *A. cerasifer*, *A. fistulosus*, *A. microcarpus*, *A. ramosus*, and *A. tenuifolius*.

Roots were mainly reported to have anthraquinone derivatives such as chrysophanol and aloe-emodin, triterpenoids, and naphthalene derivatives, while aerial parts mostly exhibited the presence of flavonoids such as luteolin, isovitexin and isoorientin, phenolic acids, and few anthraquinones. Fatty acids, namely myristic, palmitic, oleic, linoleic, and linolenic, were also found in seeds and roots. Only *A. aestivus* and *A. microcarpus* were studied for essential oil characterization of flowers [47,58].

Table 2.2. *Asphodelus* species secondary metabolites.

Species	Part Used	Class	Name of Compounds	Ref.
A. acaulis	L	Flavonoid	Luteolin; apigenin	[59]
	R	Anthraquinone	Chrysophanol; asphodelin; 10,7′-bichrysophanol	[60]
A. aestivus	FL	*n*-alkene	Hexadecanoic acid (35.6%), pentacosane (17.4%), tricosane (13.4%), heptacosane (8.4%), heneicosane (4.5%), phytol (4.5%), tetracosane (3%), hexacosane (2%), hexahydrofarnesyl acetone (1.7%), tetradecanoic acid (1.4%), docosane (1.3%), nonadecane (1%)	[58]
	L	Amino acid Anthraquinone Flavonoid Phenolic acid	Adenosine; tryptophan; phenylalanine Aloe-emodin; aloe-emodin acetate; chrysophanol 1-*O*-gentiobioside Isovitexin; isoorientin; isoorientin 4′-*O*-*β* glucopyranoside; 6″-*O*-(malonyl)-isoorientin; 6″-*O*-[(*S*)-3-hydroxy-3-methylglutaroyl]-isoorientin Chlorogenic acid	[61]
	SE	Fatty acid	Butyric acid; nervonic acid	[62]
A. albus	L	Anthraquinone	Aloe-emodin; chrysophanol	[59,63]
		Flavonoid	Luteolin	[59]
	R	Anthraquinone	Chrysophanol; asphodelin; 10,7′-bichrysophanol	[60]
		Fatty acid	Myristic (5.3%); palmitic (18.5%); stearic (2.1%); oleic (13.5%); linoleic (44.1%); linolenic (9.9%); arachidic (2.7%); behenic (1.2%); lignoceric (2.1%) acids	[64]
		Triterpenoid	*β*-sitosterol; *β*-amyrin; campesterol; stigmasterol; fucosterol	
A. albus var. *delphinensis*	R	Anthraquinone	Asphodelin; microcorpin; aloe-emodin; chrysophanol	[65]
A. cerasifer	L	Anthraquinone	Aloe-emodin	[59]
		Flavonoids	Isoorientin; luteolin; luteolin 7-glucoside	[59,66]
	R	Anthraquinone	Asphodelin; microcorpin; aloe-emodin; chrysophanol	[64]

Table 2.2. *Cont.*

Species	Part Used	Class	Name of Compounds	Ref.
** A. delphinensis*	L	Flavonoid	Isoorientin; luteolin; luteolin 7-glucoside	[66]
A. fistulosus	AP	Anthraquinone	Asphodelin; asphodelin 10′-anthrone; aloesaponarin II; aloe-emodin; chrysophanol; desoxyerythrolaccin	[43]
		Flavonoids	Chrysoeriol; luteolin	
	L	Anthraquinone	Dianhydrorugulosin; aloe-emodin; chrysophanol; 1,8 hydroxy-dianthraquinone	[67]
	R	Anthraquinone	Chrysophanol; asphodelin; 10,7′-Bichrysophanol	[60]
	SE	Anthraquinone	Dianhydrorugulosin; aloe-emodin; chrysophanol; 1,8 hydroxy dianthraquinone	[67]
		Carbohydrate	Sucrose; raffinose; stachyose	[68]
		Fatty acid	Myristic (0.5%); palmitic (5.7%); stearic (3.6%); oleic (33.1%); linoleic (54.9%)	[68,69]
		Triterpenoid	*β*-sitosterol; *β*-amyrin	[68]
*** A. luteus*	L	Anthraquinone	Aloe-emodin	[59]
**** A. mauritii*	L	Anthraquinone Flavonoid	Aloe-emodin; chrysophanol Luteolin	[59]
A. microcarpus	FL	Terpenoid	Germacrene D (78.3%); germacrene B (3.9%); *α*- -elemene (3.8%); caryophyllene (3.3%)	[47]
		Flavonoid	Luteolin; luteolin-6-*C*-glucoside; luteolin-*O*-hexoside; luteolin-7-*O*-glucoside; luteolin-*O*-acetylglucoside; luteolin-*O*-deoxyhesosylhexoside; methyl-luteolin, naringenin; apigenin	[70]
		Phenolic acid	3-*O* caffeoylquinic acid; 5-*O* caffeoylquinic acid	
	L	Anthraquinone	Chrysophanol, 10 (chrysophanol-7-yl)-10-Hydroxychrysophanol-9-antrone, asphodoside C, Dianhydrorugulosin; aloe-emodin	[67,71]

Table 2.2. *Cont.*

Species	Part Used	Class	Name of Compounds	Ref.
A. microcarpus	L	Flavonoid	Luteolin-6-*C*-glucoside; luteolin-6-*C*-acetylglucoside; luteolin-*C*-glucoside; luteolin, isoorientin	[66,72]
		Phenolic acid	5-*O*-caffeoylquinic acid; chicoric acid; cumaril exosa malic acid	[72]
	R	Anthraquinone	Dianhydrorugulosin; aloe-emodin; chrysophanol; asphodelin; microcarpin, 8-methoxychrysophanol; emodin; 10-(chrysophanol-7′-yl)-10-hydroxychrysophanol-9-anthrone; aloesaponol-III-8-methyl ether; ramosin; aestivin, asphodosides A-E, chrysophanol dianthraquinone; 5,5′-bichrysophanol; chrysophanol-8-mono-*β*-*D*-glucoside; Methyl-1,4,5-trihydroxy-7-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylate; 6-methoxychrysophanol	[21,67, 73–77]
		Arylcoumarin	Asphodelin A 4′-*O*-*β*-*D*-glucoside; asphodelin A	[45]
		Carbohydrate	Raffinose; sucrose; glucose; fructose	[78]
		Fatty acid	Palmitic; stearic; oleic; linoleic; linolenic; arachidic; behenic; lignoceric; myristic acids	[78,79]
		Naphthalene derivative	2-acetyl-1,8-dimethoxy-3 methylnaphthalene; 1,6-dimethoxy-3-methyl-2-naphthoic acid	[21]
		Mucilage	Composed of glucose; galactose; arabinose	[78]
		Triterpenoid	*β*-sitosterol-*β*-*D*-glucoside, fucosterol	[37,78]
	SE	Anthraquinone	Aloe-emodin; chrysophanol; chrysophanol-8-mono-*β*-*D*-glucoside	[67]
		Carbohydrate Fatty acid Triterpenoid	Sucrose; raffinose; stachyose; melibiose Myristic; palmitic; stearic; oleic; linoleic acids *β*-sitosterol; *β*-amyrin	[68]
A. ramosus	FL	Flavonoid Phenolic acid	Luteolin Caffeic acid; chlorogenic acid; *p*-hydroxy-benzoic acids	[80]
	L	Flavonoid	Luteolin; 7-*O*-glucosyl luteolin; 7-*O*-glucosyl apigenin; isoorientin; isoswertiajaponin (7-methyl orientin); isocytisoside (4′-methyl vitexin)	[49]

Table 2.2. *Cont.*

Species	Part Used	Class	Name of Compounds	Ref.
A. ramosus	R	Anthraquinone	Ramosin; (-)-10′-*C*-[*β*-*D*-xylopyranosyl]; (-)-10′-*C*-[*β*-*D*-glucopyranosyl-(1-4)-*β*-*D*-glucopyranosyl]-1,1′,8,8,10,10′-hexa hydroxy -3,3′- dimethyl-10,7′ bianthracene-9,9′-dione; 10′-deoxy-10-epi-ramosin; 10-(chrysophanol-7′-yl)-10-hydroxychrysophanol-9-anthrone; 7′-(Chrysophanol-4-yl)-chrysophanol-10′anthrone10′-*C*-*α*-rhamnopyranosyl; -*C*-*β*-xylopyranosyl; -*C*-*β*-antiaropyranosyl; -*C*-*α*-arabinopyranosyl; -*C*-*β*-quinovoopyranosyl	[81–83]
	WP	Flavonoid Phenolic acid	Naringin, quercetin, kaempferol Gallic acid, chlorogenic acid, vanillic acid, caffeic acid	[84]
A. tenuifolius	AP	Flavonoid	Luteolin; luteolin-7-*O*-*β*-*D* glycopyranoside; apigenin, chrysoeriol	[51]
		Anthraquinone	Asphodelin(1) and (2)	[85]
	R	Naphthalene derivative Triterpenoid	1,8-dimethylnaphthalene; 2-acetyl-8-methoxy-3-methyl-1-naphthol; 2-acetyl-1,8-dimethoxy-3-methylnaphthalene *β*-sitosterol; stigmasterol	[86]
	SE	Ester	1-*O*-17methylstearylmyoinositol	[87]
		Fatty acid	Myristic (3.96%); palmitic (13.84%); oleic (15.60%); linoleic (62.62%); linolenic (2.60%)	[88,89]
	WP	Amino acid Carbohydrate	Cystine; serine; glycine; proline; alanine, glycine; serine; alanine and valine in the form of protein *D*-glucose; lactose; *D*-glucuronic acid; *D*-arabinose; *D*-fructose, *D*-ribose	[90]
		Chromone	2-hentriacontyl-5,7-dihydroxy-8-methyl-4*H*-1-benzopyran-4-one	[57]
		Triterpenoid	Asphorodin; asphorin A; asphorin B; *β*-sitosterol; *β*-amyrin	[54,55,57]

Abbreviations: AP: Aerial Parts; FL: Flower; FR: Fruit; L: Leaf; R: Root; SE: Seed; WP: Whole plant; NI: Not indicated; * The accepted name is *Asphodelus albus* subsp. *delphinensis* (Gren. & Godr.). ** *Asphodelus luteus* L. (synonym of *Asphodeline lutea*) was formerly included in the family *Asphodelaceae*. *** The accepted name is *Asphodelus macrocarpus* subsp. *rubescens.*

2.4.3. Previously Reported *In vitro/In vivo* Biological Activities

In vitro and *in vivo* biological studies concerning *Asphodelus* extracts are presented in Table 2.3 and those reported from identified pure compounds are shown in Table 2.4. In some of the studies, no data were obtained concerning the tested doses and/or inhibitory values.

The ethanol and aqueous extracts of *A. aestivus* leaf showed moderate *in vitro* anti-fungal activity against *Aspergillus niger* [42], and whole plant ethanol extracts exhibited weak activity against *Staphylococcus aureus* with minimum inhibitory concentration (MIC) of 42 mg/mL and of 60 mg/mL against *Klebsiella pneumoniae* [91]. Both leaf and root extracts showed strong antioxidant activity [38,92]. The root extract also showed significant anti-inflammatory properties, specifically anti-ulcer activity which is one of the documented treatment in Turkish traditional medicine [41]. Root and leaf extracts of *A. aestivus* showed antitumoral activity against human cancer cells (lung and prostate) through DNA damage [92,93].

The aerial parts extract of *A. luteus* showed *in vitro* strong anti-fungal activity against *Trichophyton violaceum* (MIC = 18 µg/mL), *Microsporum canis* (MIC = 25 µg/mL), and *Trichophyton mentagrophytes* (MIC = 30 µg/mL) supporting their traditional use in dermatomucosal infections [44] and weak activity against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (MIC = 1.25–2.5 mg/mL) [94]. Moreover, the methanol root extract showed moderate antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH; IC_{50} = 0.54 mg/mL) [84]. The aerial parts and root extracts of *A. microcarpus* showed moderate antioxidant [70,84] and moderate to weak cytotoxic activities against several cancer cell lines [71,72,95].

The ethanol extract of *A. microcarpus* leaves also demonstrated strong antiviral activity against *Ebola virus* (EBOV) in the concentration of 0.1–0.3 µg/mL [72]. Although the leaf extract seems to have stronger antimicrobial activity in comparison with the root tuber extract, in general, both of them exhibited moderate to weak

antimicrobial/antifungal activities [46,71,72,94]. However, compounds isolated from root tuber extract such as asphodelin A (an anthraquinone) showed potent *in vitro* antimicrobial / antifungal activity against *S. aureus* (MIC = 16 µg/mL), *Escherichia coli* (MIC = 4 µg/mL), *Pseudomonas aeruginosa* (MIC = 8 µg/mL), *Candida albicans* (MIC = 64 µg/mL) [45] and *Botrytis cinerea* (MIC = 128 µg/mL) and asphodoside B (an anthraquinone) against MRSA (IC_{50} = 1.62 µg/mL) [74]. Other isolated compounds from root extracts showed different biological activities. For instance, ramosin showed potent cytotoxic activity against leukemia cell lines [21], aestivin showed potent antimalarial activity against chloroquine-sensitive and resistant strains of *Plasmodium falciparum* with IC_{50} of 0.8–0.7 µg/mL [21] and 3,4-dihydroxy-methyl benzoate exhibited anti-parasitic activity against *Leishmania donovani* promastigotes with IC_{50} of 33.2 µg/mL [77].

Root extracts of *A. ramosus* showed positive *in vivo* anti-inflammatory activity, confirming the traditional uses of the plant in inflammatory disorders [48].

Several root, seed, aerial parts, fruit, and leaf extracts of *A. tenuifolius* showed strong *in vitro* anti-microbial/antifungal activities against *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. aureus*, *Proteus mirabilis*, *C. albicans*, *Aspergillus fumigatus, Vibrio cholerae*, *Salmonella typhi*, and *Candida glabrata*, among other pathogens [50,51,53,56,96,97]. Of note, there is no ethnomedical report of antimicrobial use of *A. tenuifolius.* The whole plant extract of this species showed *in vivo* hypotensive and diuretic activities in normotensive rats [98]. Also the root extract showed anti-oxidant activity (DPPH test, IC_{50} = 2.006 µg/mL) [52] and asphorodin (a triterpenoid), isolated from the whole plant extract, exhibited a potent inhibition of lipoxygenase enzyme, (IC_{50} = 18.1 µM) [54], which may have an important role as an anti-inflammatory agent. The biological properties of *A. tenuifolius* extracts proved their ethnomedical values mostly as anti-inflammatory and diuretic agents [50–57].

Table 2.3. *Asphodelu*s genus biological studies.

Species	Part	Extract	Test/Assay	Result	Ref.
A. aestivus	L	Aqueous, ethanol	*In vitro* anti-fungal activity (*A. niger*)—agar well diffusion method (zone of inhibition in cm^{-1})	Ethanol extract (0.25 and 0.5 mg/mL) showed higher activity than aqueous extract (0.25 and 0.5 mg/mL) and similar activity for concentrations of 1 mg/mL. Both extracts were less active than fluconazole (100 µ/mL).	[42]
			In vitro antioxidant activity—*β*-carotene bleaching effect, metal chelating, total antioxidant activity, DPPH, ABTS, DMPD, NO, $O^{2-}$and O^{2-} and OH radical scavenging activity	Aqueous extract presented higher activity in metal chelating and radical scavenging assays (DPPH, $IC_{50\ aqueous}$ = 4.58 mg/mL and $IC_{50\ Ethanol}$ = 9.54 mg/mL, superoxide, hydroxyl, DMPD) Ethanol extract presented higher activity in *β*-carotene bleaching effect and total antioxidant activity. Aqueous and ethanolic extracts presented similar radical scavenging activity in ABTS and NO assays. Both extracts presented significantly inferior results when compared to reference substances.	
		Acetone, methanol	*In vitro* antioxidant activity—*β*-carotene, reducing power assay, DPPH, ABTS, O^{2-} inhibition of linoleic acid peroxidation	Reducing power and total antioxidant activity were higher in acetone extract; free radical and superoxide radical scavenging activity were higher in methanol extract (DPPH, $IC_{50\ methanol}$ = 0.16 mg/mL and $IC_{50\ acetone}$ = 0.50 mg/mL). Acetone extract presented higher activity in reducing power and total antioxidant activity (inhibition of linoleic acid peroxidation) Methanol extract presented higher activity in superoxide radical scavenging and free radical scavenging activity (*β*-carotene, ABTS and DPPH, $IC_{50\ methanol}$ =0.16 mg/mL, $IC_{50acetone}$ = 0.50 mg/mL).	[38]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. aestivus	L R	Dichloromethane *n*-hexane	*In vitro* cytotoxic activity—MTT assay against human lung cell cancer (A549) and prostate cell cancer (PC3)	Root: Dichloromethane: A549(IC_{50} = 16 µg/mL); PC3 (IC_{50} = 19 µg/mL). *n*-Hexane: PC3 (IC_{50} = 80 µg/mL). Leaf: Dichloromethane: A549 (IC_{50} = 90 µg/mL).	[93]
	R	Aqueous (decoction)	*In vivo* anti-inflammatory—Ethanol induced gastric ulcer model in rats	Decoction gave significant protection against the lesions.	[41]
	R	Aqueous (infusion & decoction) diethyl ether, ethyl acetate, methanol	*In vitro* antioxidant activity—DPPH assay *In vitro* cytotoxic & apoptotic activity—MCF-7 breast cancer cells-trypan blue exclusion assay, comet assay, Hoechst 33258, propidium iodide double staining	Diethyl ether (IC_{50} = 22.46 µg/mL) have a higher scavenging activity than ethyl acetate (IC_{50} = 188.90 µg/mL), both have lower activity than reference substance, rutin (7.77 µg/mL). Methanol and aqueous extract had no scavenging activity. Methanol and aqueous extracts exhibited strong cytotoxic activities. All extracts showed significant DNA damaging and apoptotic activities.	[92]
	SE	Petroleum ether	*In vitro* antimicrobial/fungal activity—broth microdilution method	Active against *S. aureus* (MIC = 512 µg/mL), *Enterococcus faecalis* (MIC = 512 µg/mL), *K. pneumoniae* (MIC = 512 µg/mL) and *C. albicans* (MIC = 512 µg/mL). Not active against *Bacillus cereus*, *Staphylococcus epidermidis, E. coli*, *P. aeruginosa*, *S. typhimurium*, *Salmonella enterica, Candida krusei* and *Candida parapsilosis.*	[62]
	WP	*n*-Butanol, ethanol	*In vitro* anti-microbial/fungal activity—well and disk diffusion method	Active against *S. aureus* (MIC: 42 mg/mL), *K. pneumoniae* (MIC: 60 mg/mL), *E. coli* (MIC: 90 mg/mL), *C. albicans* (MIC: 90 mg/mL).	[91]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. aestivus	WP	Aqueous	*In vitro* antioxidant activity —DPPH assay	Inhibition % = 62.5.	[99]
A. fistulosus	NI	NI	*In vitro* anti-microbial/fungal activity	Positive to *S. aureus* and no activity against *E. coli, Proteus vulgaris, Salmonella sp., P. aeruginosa, C. albicans.*	[100]
	SE	Ethanol Petroleum ether Dichloromethane methanol	*In vitro* antioxidant —DPPH — O^{2-}	Dichloromethane fraction followed by methanolic fraction exhibited the highest DPPH (70 and 65%) and superoxide scavenging (60 and 58%) activities.	[101]
			In vitro antimicrobial/fungal —Disc Diffusion Method	Ethanolic extract: active against *S. epidermidis*; *E. faecalis*; *Streptococcus pneumoniae*; *E. coli*; *K. pneumoniae*; *P. merabilis*; *P. aeruginosa* and *C. albicans* with Inhibition diameter: (12, 14, 12, 16, 14,10,10 and 18 mm, respectively). Petroleum ether fraction: active against *S. epidermidis*; *E. faecalis*; *Streptococcus pneumoniae*; *E. coli*; *K. pneumoniae*; *P. merabilis* and *C. albicans* with Inhibition diameter: (10, 16, 18, 16, 16, 12 and 16 mm, respectively). Dichloromethane fraction: active against *S. epidermidis*; *S. aureus*; *E. faecalis*; *Streptococcus pneumoniae*; *E. coli*; *K. pneumoniae*; *P. merabilis*; *P. aeruginosa* and *C. albicans* with Inhibition diameter: (14, 12, 18, 10, 12, 14, 14, 10 and 14 mm, respectively). Methanol fraction: active against *S. epidermidis*; *S. aureus*; *E. faecalis*; *Streptococcus pneumoniae*; *E. coli*; *K. pneumoniae* and *C. albicans* with Inhibition diameter: (12, 10, 10, 12, 12, 15 and 12 mm, respectively).	

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. fistulosus			*In vitro* anti protozoal activity (antiplasmodial, antileishmanial, antitrypanosomal	Dichloromethane fraction: active against *T. cruzi*, *T. bruci*, *L. infantum* and *P. falciparum*, IC_{50}: (20.8, 58.9, 39.8, 45.9 µg/m, respectively). Methanol fraction: active against *T. bruci* and *P. falciparum*, IC_{50}: (79.6 and 11.4 µg/m, respectively). Ethanolic extract: active against *T. cruzi*, *T. bruci*, *L. infantum* and *P. falciparum*, IC_{50}: (63.5, 43.8, 50.4, 51.2 µg/m, respectively). Petroleum ether fraction: active against *T. cruzi*, *T. bruci*, *L. infantum* and *P. falciparum*, IC_{50}: (58.3, 25.2, 44.2, 68.2 µg/m, respectively) . Dichloromethane fraction: active against *T. cruzi*, *T. bruci*, *L. infantum* and *P. falciparum*, IC_{50}: (20.8, 58.9, 39.8, 45.9 µg/m, respectively). Methanol fraction: active against *T. bruci* and *P. falciparum*, IC_{50}: (79.6 and 11.4 µg/m, respectively).	[101]
A. luteus *	AP	Aqueous	*In vitro* anti-fungal activity—Agar dilution method	Activity against *T. violaceum* (MIC = 18 µg/mL), *M. canis* (MIC = 25 µg/mL) and *T. mentagrophytes* (MIC = 30 µg/mL).	[44]
	AP R	Methanol, petroleum Ether	*In vitro* anti-microbial activity—agar diffusion test; tetrazolium microplate assay (MIC)	Against MRSA isolates Methanol extract: MIC(AP) = 1.25–2.5 mg/mL. MIC(R) = 0.65–1.25 mg/mL. Petroleum ether extract: (R) extract had higher activity than (AP) extract.	[94]
	R	Methanol	*In vitro* antioxidant activity—DPPH assay	IC_{50} (methanol)= 0.54 mg/mL, IC_{50} (reference, BHT) = 0.017 mg/mL.	[84]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. microcarpus	AP	Aqueous	*In vitro* anti-fungal activity—Agar dilution method	Against *T. violaceum* (MIC = 25 µg/mL) and no activity against *M. canis* and *T. mentagrophytes.*	[44]
	AP R	Methanol	*In vitro* anti-microbial activity—agar diffusion test; tetrazolium microplate assay (MIC)	Against MRSA isolates MIC(AP) = 1.25–5 mg/mL. MIC(R) = 1.25–2.5 mg/mL.	[94]
	AP L FL R	Methanol	*In vivo* anti-inflammatory activity—carrageenan induced rat paw edema and xylene induced ear edema in mice	Oral administration of the AP and R extract produced significant ($p < 0.05$) anti-edematogenic effect with a dose of 500 mg/kg in the carrageenan induced paw edema after 6 h (58.04%, 58.75%, respectively). AP, L, FL, and R extracts at 100, 300 and 500 mg/kg, exhibited significant ($p<0.05$) inhibition of xylene induced ear edema.	[102]
	FL L R	Aqueous, ethanol, methanol	*In vitro* antimelanogenic activity—tyrosinase inhibition (mushroom tyrosinase assay and mouse melanoma cells viability), kojic acid as positive control *In vitro* antioxidant activity—DPPH (reference—Trolox)	Antimelanogenic activity Ethanol extract (F) had the highest tyrosinase inhibition activity in mushroom assay and melanoma cell assay. Antioxidant activity Ethanol extract (F): IC_{50} = 28.4 µg/mL. ethanol extract (L): IC_{50} = 55.9 µg/mL. Trolox: IC_{50} = 3.2 µg/mL.	[70]
	L	Methanol	peripheral and central analgesic activities—Koster and Tail Flick	Dose of 50 mg/kg, showed a significant inhibitory effect on the number of abdominal cramps which exceeded of aspirin, used as a reference at the dose of 200 mg/kg. About the test Tail flick, methanolic extract didn't have a significant inhibitory effect compared to morphine at the dose of 5mg/kg, on the pain caused by the heat of the light beam.	[103]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. microcarpus	L	Ethanol	*In vitro* antimicrobial/fungal activity—micro broth dilution method	Active against *Bacillus clausii* (MIC = 250 µg/mL), *S. aureus* (MIC = 250 µg/mL), *Staphylococcus haemolyticus* (MIC = 250 µg/mL) and *E. coli* (MIC = 500 µg/mL).No activity against *Streptococcus* spp. and yeasts.	[72]
			In vitro antiviral activity (IFN-β induction)—luciferase reporter gene assay	Antiviral activity Active against EBOV in concentration of 0.1–3 µg/mL.	
			In vitro cytotoxicity-Cell viability of A549 cells, positive control (camptothecin)	Cytotoxicity IC_{50} (extract) > 100 µg/mL. IC_{50} (camptothecin) = 0.54 µg/mL.	
	L	Methanol	*In vitro* antimicrobial/fungal—two-fold serial dilution technique	Antimicrobial activity Active against *S. aureus* (MIC = 78 µg/mL), *Bacillus subtilis* (MIC = 156 µg/mL), *Salmonella* spp. (MIC = 313 µg/mL), *E. coli* (MIC = 125 µg/mL), *Aspergillus flavus* (MIC = 125 µg/mL), *C. albicans* (MIC = 78 µg/mL).	[71]
			In vitro antiviral activity—CPE inhibition assay against HSV-1 and HAV-10	Antiviral activity Moderate activity against Hepatitis A *virus* (HAV-10) and no activity against Herpes Simplex Virus (HSV-1).	
			In vitro cytotoxicity—viability assay against human tumor cell lines of the lung (A-549), colon (HCT-116), breast (MCF-7) and prostate (PC3). cisplatin as standard	Cytotoxicity Highest activity against human lung carcinoma cells (A-549), IC_{50} = 29.3 µg/mL.	
	R	Methanol	*In vitro* anti-microbial—Disk diffusion assay	Positive against *S. aureus* with14 mm of inhibition diameter .	[104]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. microcarpus	R	Methanol	*In vitro* antioxidant activity—DPPH assay	IC_{50} (methanol) = 0.30 mg/mL, IC_{50} (reference, BHT) = 0.017 mg/mL.	[84]
	R	Methanol	*In vitro* anti-microbial—Disk diffusion assay	No activity against *S. aureus*, *B. subtilis* and *E. coli.*	[46]
	WP	Aqueous, ethanol	*In vitro* antioxidant activity—DPPH assay *In vitro* cytotoxic activity—Trypan blue technique for Ehrlich Ascites Carcinoma Cells (EACC)	Ethanol extract (100 µg/mL) with moderate activity (inhibition percentage—60.3%) higher than aqueous extract (100 µg/mL, inhibition percentage—49.5%). Weak anti-cancer activity of both extracts.	[95]
A. ramosus	R	Aqueous, chloroform, ethanol, methanol	*In vivo* anti-inflammatory—Arachidonic acid test (mouse ear oedema) Carrageenan test (sub-plantar oedema)	Arachidonic acid test: positive activity from chloroform and ethanol extracts. Carrageenan test: no activity was observed.	[48]
	WP	Aqueous, methanol, methanol 50%	*In vitro* antioxidant activity—DPPH assay at 35°C and 65°C	Aqueous extract at 65°C had the highest inhibition percentage.	[105]
A. tenuifolius	AP	Butanol, ethyl acetate, methylene-chloride	*In vitro* anti-microbial/fungal activity—disc diffusion method	All extracts showed antimicrobial activity, the methylene-chloride as the most active against *S. aureus* (MIC = 1.6 mg/mL*), E. faecalis* (MIC = 1.0 mg/mL), *E. coli* (MIC = 1.8 mg/mL) and *P. aeruginosa* (MIC = 0.15 mg/mL) All extracts showed antifungal activity against *C. albicans, C. parapsilosis, C. glabrata, C. krusei.*	[51]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
	AP	Aqueous 10% and 20%	*In vitro* antifungal activity—poisoned food technique	Aqueous extracts showed positive activity at both concentrations of 10% and 20% for the Fusarium mycelia growth inhibition (speed decreased to 0.28 mm/h at 20% concentration of extract). The extract strongly inhibited *Fusarium graminearum* mycelium growth, with inhibition percentages of 56.89 and 60.34% at the concentrations 10 and 20%, respectively.	[106]
A. tenuifolius	FR	Acetone, aqueous, benzene, chloroform, methanol, petroleum ether	*In vitro* anti-microbial/fungal activity —Kirby-bauer disc diffusion method	Significant activity against *S. aureus (*acetone, MIC = 125 µg/mL); *S. epidermidis* (acetone, MIC = 125 µg/mL; chloroform and methanol, MIC = 250 µg/mL)*; P. vulgaris* (methanol, MIC = 250 µg/mL; chloroform, MIC = 125 µg/mL), *P. mirabilis (*benzene, MIC = 125 µg/mL; acetone and methanol, MIC = 250 µg/mL; chloroform, MIC = 500 µg/mL*) E. coli* (acetone, chloroform and methanol, MIC = 125 µg/mL*); K. pneumoniae* (acetone and methanol, MIC = 125 µg/mL; chloroform and benzene, MIC = 500 µg/mL)*; P. aeruginosa* (acetone, MIC = 250 µg/mL; chloroform, MIC = 500 µg/mL)*; C. albicans* (acetone, MIC = 125 µg/mL)*; A. fumigatus (*benzene and chloroform, MIC = 250 µg/mL; acetone, MIC = 500 µg/mL).	[53]
	L	Methanol (80%)	*In vitro* antioxidant activity—FRAP, DPPH and ABTS assays.	Mean FRAP value of 0.69 mmol/g, DPPH IC_{50} of 1.72 mg/ml and ABTS of 0.36 mg/ml.	[107]
	L	Acetone, methanol	*In vitro* anti-microbial/fungal activity—agar disc diffusion method	Methanol extract was positive against *S. aureus*, *B. cereus*, *Citrobacter freundii*, *Candida tropicalis* and acetone extract was positive against *K. pneumoniae*, *C. tropicalis* and *Cryptococcus luteolus.*	[50]

Table 2.3. *Cont.*

Species	Part	Extract	Test/Assay	Result	Ref.
A. tenuifolius	R	Methanol	*In vitro* antioxidant activity—DPPH, $ABTS^{+}$, OH, O_2^{-}, $ONOO^{-}$ assays, Oxidative DNA damage	Positive activity, DPPH (IC_{50} = 2.006 µg/mL), ABTS·+ (IC_{50} = 156.94 µg/mL), OH (IC_{50} = 50.13 µg/mL), O_2^{-} (IC_{50} = 425.92 µg/mL) and ONOO- (IC_{50} = 3.390 µg/mL), oxidative DNA damage: 1.85 µg/mL of extract prevented DNA damage.	[52]
	R	Benzene, chloroform, ethyl acetate, methanol, petroleum ether	*In vitro* anti-microbial/fungal activity—Disc diffusion method	All extracts were active against *B. subtilis*, *P. vulgaris*, *P. aeruginosa*, *Trichophyton rubrum*, *E. coli*, *K. pneumoniae*, *Shigella sonnei*, *S. aureus, C. albicans, A. niger* and *A. flavus.*	[96]
	SE	Aqueous, ethanol, methanol, petroleum ether	*In vitro* anti-microbial/fungal activity—modified Kirby-bauer disc diffusion method	Petroleum ether: no antibacterial activity. Ethanol: activity against *P. aeruginosa*, *Vibrio cholerae* and *S. aureus* (MIC = 16 µg/mL); *P. mirabilis, S. typhi, Shigella flexneri* and *Serratia marcescens* (MIC = 32 µg/mL) Methanol: activity against *S. aureus* (MIC = 16 µg/mL); *V. cholerae, P. aeruginosa, S. typhi, S. flexneri* and *S. marcescens* (MIC = 16 µg/mL) Aqueous: activity against *V. cholerae, S. aureus, S. typhi* and *S. flexneri* (MIC = 32 µg/mL); *P. aeruginosa* and *P. mirabilis* (MIC = 16 µg/mL) No antifungal activity against *C. albicans* and *A. niger.*	[56]
	WP	Methanol	*In vitro* antimicrobial/fungal activity—disk diffusion method *In vitro* anti-parasitic activity—trophozoites growth inhibition assay	Good activity against *E. coli* and moderate activity against *S. aureus, S. typhi, K. pneumoniae, P. aeruginosa, C. albicans* and *A. niger* Active against *Giardia lamblia* (IC_{50} = 219.82 µg/mL) and *Entamoeba histolytica* (IC_{50} = 344.62 µg/mL).	[97]

Table 2.3. *Cont.*

A. tenuifolius	WP	Aqueous	*In vivo* hypotensive activity—blood pressure (BP) measure after parenteral administration of aqueous extracts in rats. Acetylcholine and verapamil as positive controls in co administration with atropine	Hypotensive activity The extract decreased blood pressure in normotensive rats (35.2% decrease with 30 mg/Kg), similar to Verapamil. The response was independent from atropine effect	[98]
			In vivo diuretic activity—measure of rat urine output and urinary electrolytes. After 6 hr. administration. Saline solution and furosemide as controls	Diuretic activity: significant increase in urinary volume and electrolytes excretion with 300 and 500 mg/Kg	

Abbreviations: AP: Aerial Parts; FL: Flower; FR: Fruit; L: Leaf; R: Root; SE: Seed; WP: Whole plant; NI: Not indicated; * *Asphodelus luteus* L.—synonym of *Asphodeline lutea* was formerly included in the family *Asphodelaceae*. ABTS+: 2,2'-azinobis-(3-ethylbenzothiazole-6-sulphonate) radical cation, DMPD: N, N-dimethyl-p-phenylenediamine dihydrochloride, DPPH: 2,2-diphenyl-1-picrylhydrazyl radical, NO: nitric oxide radical, O^{2-}: superoxide anion radical, ·OH: hydroxyl radical, $ONOO^-$:Peroxynitrite radicals, EBOV: Ebola virus.

Table 2.4. *Asphodelus* genus metabolites biological studies.

Species	Pure Compounds	Test/Assay	Result	Ref.
A. microcarpus	Asphodelin A 4'-*O*-*β*-*D*-glucoside (1), asphodelin A (2)	*In vitro* antimicrobial/fungal activity—micro dilution assay	Positive activity against *S. aureus* (MIC_1 = 128 µg/mL, MIC_2 = 16 µg/mL), *E. coli* (MIC_1 = 128 µg/mL, MIC_2 = 4 µg/mL), *P. aeruginosa* (MIC_1 = 256 µg/mL, MIC_2 = 8 µg/mL), *C. albicans* (MIC_1 = 512 µg/mL, MIC_2 = 64 µg/mL) and *B. cinerea* (MIC_1 = 1024 µg/mL, MIC_2 = 128 µg/mL	[45]
	3-Methyl anthralin, chrysophanol, aloe-emodin	Psoriasis	Positive (patent)	[108,109]
	1,6-Dimethoxy-3-methyl-2-naphthoic acid (1), asphodelin (2), chrysophanol (3), 8-methoxychrysophanol (4), emodin (5), 2-acetyl-1,8-dimethoxy-3-methylnaphthalene (6), 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone (7), aloesaponol-III-8-methyl ether (8), ramosin (9), aestivin (10)	*In vivo* anti-parasitic activity *In vitro* cytotoxic activity -human acute leukemia HL60 cells/human chronic leukemia 562 cells *In vitro* antimalarial activity -chloroquine sensitive and resistant strains of *Plasmodium falciparum* *In vitro* anti-microbial/fungal activity	Compounds 3 and 4 showed moderate to weak against a culture of *L. donovani* promastigotes (IC_{50} = 14.3 and 35.1 µg/mL, respectively). Compounds 7 and 9 exhibited a potent cytotoxic activity against leukemia HL60 and K562 cell lines. Compound 10 showed potent antimalarial activities against both chloroquine-sensitive and resistant strains of *P. falciparum* (IC_{50} = 0.8–0.7 µg/mL) without showing any cytotoxicity to mammalian cells. Compound 4 exhibited moderate antifungal activity against *Cryptococcus neoformans* (IC_{50} = 15.0 µg/mL), compounds 5, 7 and 10 showed good to potent activity against methicillin resistant *S. aureus* (MRSA) (IC_{50} = 6.6, 9.4 µg/mL and 1.4 µg/mL respectively). Compounds 5, 8 and 9 displayed good activity against *S. aureus* (IC_{50} = 3.2, 7.3 and 8.5 µg/mL, respectively)	[21]

Table 2.4. *Cont.*

Species	Pure Compounds	Test/Assay	Result	Ref.
A. microcarpus	Methyl-1,4,5-trihydroxy-7-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylate (1), (1*R*) 3,10-dimethoxy-5-methyl-1*H*-1,4 epoxybenzo[*h*]isochromene (2), 3,4-dihydroxy-methyl benzoate (3), 3,4-dihydroxybenzoic acid (4), 6 methoxychrysophanol (6)	*In vitro* anti-parasitic activity	Compound 3 showed activity against a culture of *L. donovani* promastigotes (IC_{50} = 33.2 µg/mL).	[77]
		In vitro anti-microbial/activity	Compound 1 showed a potent activity against methicillin resistant *S. aureus* (MRSA) and *S. aureus* (IC_{50}: 1.5 and 1.2 µg/mL, respectively)	
	Asphodosides A; B; C; D and E	*In vitro* anti-microbial activity	Compounds B, C and D showed activity against methicillin resistant *S. aureus* (MRSA) (IC_{50}: 1.62, 7.0 and 9.0 µg/mL, respectively). activity against *S. aureus* (non-MRSA), IC_{50} = 1.0, 3.4 and 2.2 µg/mL, respectively	[74]
A. tenuifolius	Asphorodin	*In vitro* anti-inflammatory -inhibition of lipoxigenase enzyme	Potent inhibitory activity (IC_{50} = 18.1 µM), standard: baicalein (22.6 µM)	[54]

Chapter 3

A. bento-rainhae and *A. macrocarpus* Root Tubers

In Chapter **3.** which is divided into two main subsections **3.1.** and **3.2.**, the focus was on the botanical and chemical monographic quality parameters and pre-clinical safety assessment, along with the biological and phytochemical analysis and identification of the main marker secondary metabolites of both *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root tubers extracts.

In the first section, the plant material used in the study was described. It included information about the species, variety, and the source of the plant material. This subsection also covered the collection of the plant material and provided details about the location, time, and method of collection, as well as any specific criteria for sample selections and preparation procedures and steps involved in cleaning, drying, and storing the plant material before further analysis. Furthermore, it discussed the botanical and chemical monographic quality parameters, needed for the proper characterization of dried root tubers of both species.

In the next section, the chemical procedures employed to extract and isolate marker compounds from the plant material, following a bioguided antimicrobial study, were explained. This included providing details about the selection of solvents, extraction techniques, assays or tests performed to assess the crude extracts effects, purification processes, and identification of active marker compounds. Once the compounds were detected and/or isolated, they were characterized with the aid of hyphenated techniques used to identify and determine their chemical structures .

For the pre-clinical safety assessments, *in vitro* toxicity (cytotoxicity, genotoxicity/ mutagenicity) was employed and details of the cell culture techniques, such as cell line selection, cell culture media, and incubation conditions were briefly explained.

Finally, both subsections briefly discussed the statistical methods that were used to analyze the data obtained from phytochemical and *in vitro* biological experiments. They also included an analysis of the obtained results, highlighting key findings, interpretations, and insights.

The obtained findings were presented in a concise and organized manner, using tables, figures, or charts as appropriate. They were also accompanied by statistical measures of significance, such as *p*-values or confidence intervals, to support their validity. These results were further interpreted in the context of the research objectives, discussing their implications and furthermore, were compared to previous studies or existing literature to establish connections or divergences in the findings.

Chapter 3.1

A. bento-rainhae and *A. macrocarpus* Root Tubers —

Monographic Quality Parameters and Genotoxicity Assessment

Main part of this chapter was published as follows with minor modifications:

Monographic Quality Parameters and Genotoxicity Assessment of *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* Root Tubers as Herbal Medicines.

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3.1.1. Abstract

Root tubers of *Asphodelus bento-rainhae* subsp. *bento-rainhae* (AbR), an endemic species with relevant interest due to conservation concerns, and *Asphodelus macrocarpus* subsp*. macrocarpus* (AmR) have been traditionally used for culinary and medicinal purposes, mainly associated with skin infection and inflammation. The present study aims to establish the quality control criteria for the proper characterization of dried root tubers of both species as herbal substances, together with their preclinical safety assessments. Botanical identification using macroscopic and microscopic techniques and phytochemical evaluation/quantification of the main classes of marker secondary metabolites, including phenolic compounds (flavonoid, anthraquinone, condensed and hydrolysable tannin) and terpenoids were performed. Additionally, *in vitro* genotoxicity/mutagenicity was evaluated by Ames test. Evident morphological differences in the development of tubercles (3.5 × 1 cm in AbR and 8.7 × 1.4 cm in AmR) and microscopically in the arrangements and characteristics of the vascular cylinder (metaxylem and protoxylems) were found. Anatomical similarities such as multiple-layered epidermis (velamen) and the cortex area with thin-walled idioblasts (134 ± 2.9 µm and 150 ± 27.6 µm) containing raphide crystals (37.2 ± 14.2 µm and 87.7 ± 15.3 µm) were observed between AbR and AmR, respectively. Terpenoids (173.88 ± 29.82 and 180.55 ± 10.57 mg OAE/g dried weight) and condensed tannins (128.64 ± 14.05 and 108.35 ± 20.37 mg CAE/g dried weight) were found to be the main class of marker secondary metabolites of AbR and AmR extracts, respectively. No genotoxicity (up to 5 mg/plate, without metabolic activation) was detected in these medicinal plants' tested extracts. The obtained results will contribute to the knowledge of the value of the Portuguese flora and their future commercial cultivation utilization as raw materials for industrial and pharmaceutical use.

Keywords: *Asphodelus bento-rainhae*; *Asphodelus macrocarpus*; herbal medicines; quality control; root tubers

3.1.2. Introduction

Medicinal plants and herbal medicines provide effective and affordable primary health care for much of the world's population. Therapies involving these agents, with a long history of traditional use in several cultures, still offer great potential in the treatment of various diseases; however, they do not comprise an adequate assessment of their authenticity, quality, and safety. In fact, to date, many of them remain unproven and rarely monitored [114,115].The genus *Asphodelus* L. belonging to the family *Asphodelaceae* is among the most popular source of medicinal plants in the Iberian Peninsula [22]. Root tubers of *Asphodelus* species have shown to be the most common plant part traditionally used for the treatment of skin-related disorders and infections such as wounds, eczema, alopecia, and psoriasis [36]. Besides the medicinal uses, tubers have been used as daily food after being moistened and fried beforehand to eliminate the astringent compounds [22,38]. As already stated by us [36], the local people of Iran, Turkey and Egypt use the root tubers *of A. aestivus* and *A. microcarpus* to produce a strong glue used by shoemakers and cobblers [22,37,40] and as yellow and brown dyes to dye wool [22].

A broad range of *in vitro* and *in vivo* biological activities of *Asphodelus* species root extracts have been documented [36] and found to have antimicrobial [45,46,74,91,94,96,97], antiparasitic [77], antimalarial [21], antitumoral [92,93,95], antioxidant [52,70,84,92,99], anti-inflammatory [41,48,54,109], hypotensive and diuretic [98] activities. They were mainly reported to have anthraquinone derivatives, triterpenoids, and naphthalene derivatives as major secondary metabolites [21,60,64,65,67,73–75,77,116].

According to the Flora Iberica [22], 12 *Asphodelus* species are present in the Iberian region, and based on the information obtained from the World Checklist of Selected Plant Families [23], the Checklist of the Vascular Plants of Portugal (Checklist da Flora de Portugal) [24] and Flora-On [25], only eight, namely, *Asphodelus aestivus* Brotero, *Asphodelus bento-rainhae* P. Silva (subsp. *bento-rainhae*), *Asphodelus fistulosus* Linnaeus (subsp. *fistulosus*; subsp. *madeirensis* Simon), *Asphodelus lusitanicus*

Coutinho (var. *lusitanicus*; var. *ovoideus* (Merino) Z. Díaz and Valdés), *Asphodelus macrocarpus* Parlatore (subsp. *macrocarpus*; var. *arrondeaui* (J. Lloyd) Z. Díaz and Valdés) *Asphodelus ramosus* Linnaeus (subsp. *distalis* Z. Díaz and Valdés; subsp. *ramosus*), *Asphodelus serotinus* Wolley-Dod and *Asphodelus tenuifolius* Cavanilles, exist in Portugal with high distribution within the mainland and Madeira islands.

Among the species mentioned above, *Asphodelus bento-rainhae* subsp. *bento-rainhae* is an endemic species from the Gardunha mountain range "Serra da Gardunha," located in the central region of Portugal, and considered vulnerable on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species [26]. It is located only in this region, covering the counties of Fundão and Castelo Branco [25], coexisting with *Asphodelus macrocarpus* subsp. *macrocarpus* in the same geographical area. They are known by the common Portuguese name abrotea, and their root tubers have been traditionally used for the treatment of scabies, dermatophytosis and warts in Portugal. General botanical and systematic descriptions of these species have been discussed by several taxonomists in various flora publications [22,117–119], describing them as perennial and glabrous herbs with horizontal or oblique rhizomes (with some or any fibrous remains of old leaves) and short, thick, fusiform root tubercles, more abruptly narrowed in the distal part than in the proximal [120,121].

Although there are ethnomedical data supported by botanical, phytochemical, and biological studies of *Asphodelus* species, to the best of our knowledge, no scientific studies have been documented on *Asphodelus bento-rainhae* and *Asphodelus macrocarpus*. Moreover, considering their threatened status and valuable traditional and medicinal properties, their conservation and further commercial cultivation are extremely important. Therefore, the present study was conducted to establish the principal botanical and chemical specifications of their root tubers following the official quality monograph criteria, together with a preclinical safety assessment of both species to allow their future use as herbal substances for human use.

3.1.3. Materials and Methods

3.1.3.1. Reagents

Acetone (CH_3COCH_3), aluminum chloride ($AlCl_3$), 9-aminoacridine hydrochloride monohydrate, ammonium sodium phosphate dibasic tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$), *d*-(+)-biotin ($C_{10}H_{16}N_2O_3S$), dimethyl sulfoxide/DMSO [$(CH_3)_2SO$], gallic acid [$C_6H_2(OH)_3COOH$], glucose monohydrate ($C_6H_{12}O_6.H_2O$), 2-nitrofluorene ($C_{13}H_9NO_2$), *tert*-butyl hydroperoxide/T-BHP [$(CH_3)_3COOH$], and vanillin [4-$(HO)C_6H_3$-3-$(OCH_3)CHO$], were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ferric chloride hexahydrate ($FeCl_3.6H_2O$), hydrochloric acid (HCl), l-histidine monohydrochloride monohydrate ($C_6H_9N_3O_2{\cdot}HCl{\cdot}H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), methanol (CH_3OH), perchloric acid ($HClO_4$), potassium iodate (KIO_3), sodium acetate trihydrate ($CH_3COONa.3H_2O$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), were purchased from Merck (Germany). (+)-Catechin ($C_{15}H_{14}O_6$) and oleanolic acid ($C_{30}H_{48}O_3$) were acquired from Extrasynthese (Genay, France). Citric acid monohydrate [$HOC(COOH)(CH_2COOH)_2{\cdot}H_2O$], di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were purchased from PanReac AppliChem (Barcelona, Spain). Sodium chloride (NaCl) and di-potassium hydrogen phosphate (K_2HPO_4) were from Honeywell Fluka™ (Seelze, Germany). Bacto™ agar from Becton Dickinson and Co (Franklin Lakes, NJ, USA), *n*-butanol [$CH_3(CH_2)_3OH$] from Fisher Scientific™ (Loughborough, UK), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) from MandB laboratory chemicals (London, UK), Folin-Ciocalteu from Biochem chemopharma (Cosne-Cours-sur-Loire, France), glacial acetic acid (CH_3CO_2H) from Chem-Lab NV (Zedelgem, Belgium), sodium azide from J.T. Baker Chemical Company ((Phillipsburg, NJ, USA) and nutrient broth (NB) nº 2 from Oxoid ((Basingstoke, UK) were acquired. In the preparation of all solutions, dilutions, and culture media, ultra-pure water from a Milli-Q water purification system, Millipore (Molsheim, France), was used.

3.1.3.2. Plant Material

3.1.3.2.1. Sample Collection

Root tubers of *A. bento-rainhae* (AbR) and *A. macrocarpus* (AmR) (Fig. 3.1.1), were collected from Serra da Gardunha, Portugal, first at the early flowering stage (AbRa and AmRa) in in Spring (May 2019) and then for the second time, during the root dormancy (AbRb and AmRb) in Autumn (November 2019). The collected samples were identified by Maria Cristina Duarte, scientific curator of the LISC Herbarium and plant collections in the Tropical Botanical Garden and Lisbon Botanical Garden. All samples were dried in a well-ventilated, dark space at room temperature. Corresponding voucher specimens were deposited in the Laboratory of Pharmacognosy, Department of Pharmacy, Pharmacology and Health Technologies, Faculty of Pharmacy, Universidade de Lisboa (voucher specimens' number: OSilva_201901—A. *bento rainhae* and OSilva_201902—*A. macrocarpus*).

Figure 3.1.1. *Asphodelus* species sample collection and authentication.

3.1.3.3. Botanical Identification

3.1.3.3.1. General materials

Ruler, slides, cover-glasses of standard size, a set of botanical dissecting instruments containing needle, scalpel, petri dish, blade, razor blade, paintbrush, filter paper and tweezers were used. The macroscopic and microscopic identification of the selected dried samples was performed according to the standard methods described in European Pharmacopoeia.

3.1.3.3.2. Macroscopic analysis

Macroscopic analysis was based on shape, size, colour, surface characteristics, texture, fracture characteristics and appearance of the cut surface. Samples were directly observed by the naked eye and photographed using an Olympus SZ61 optical stereo microscope (Heerbrugg, Switzerland) coupled with an Olympus ColorView IIIu CCD 5.0MP camera featuring 2576×1932-pixel resolution (Tokyo, Japan). Several macroscopical characteristic were analyzed as follows:

Size: A graduated ruler in millimeters was used for the measurement of the length and width of the samples.

Colour: The untreated samples were directly observed by the naked eye and examined under both diffuse daylight and artificial light source with wavelengths similar to those of daylight.

Surface, texture, and fracture characteristics: The untreated samples were examined using a magnifying lens (6x to 10x). Wet with water or reagents as required, to observe the characteristics of a cut surface. The samples were touched to determine if they were soft or hard; and they were bended and ruptured to obtain information on brittleness and the appearance of the fracture plane.

Odour: A small portion of the sample was placed in the palm of the hand and the air was inhaled slowly and repeatedly over the material. If no distinct odour was perceptible, the sample was crushed between the thumb and index finger or between the palms of the hands using gentle pressure. First, it was determined the strength of the odour (none, weak, distinct, strong) and then the odour sensation (aromatic, fruity, musty, rancid).

3.1.3.3.3. Microscopic analysis

3.1.3.3.1. Light microscopy

The light microscopic analysis was performed using an optical Olympus CX40 (objectives with a magnification of 4×, 10×, 20×, 40× and 100×) upright microscope (York, United Kingdom), coupled with a Leica MC170 HD camera.

The dried plant material was immersed in a Petri box containing distilled water for a few minutes to soften the surfaces and allow sections to be cut. For the anatomical analysis, cross or transverse sections were prepared by cutting or microtome at a right angle to the longitudinal axis of the material. Longitudinal sections were prepared by cutting with a razor blade, either in a radial direction (radial section) or in a tangential direction (tangential section). surface preparations of the leaf as well as transverse sections of lamina and midrib region were prepared manually. All the sections as well as surface preparations were prepared as thinly and evenly as possible. Powdered plant material was obtained using an Analytical Mill A-10 water-cooled laboratory mill (Staufen, Germany). All the samples were cleared and mounted in 60% chloral hydrate aqueous solution and a cover slip placed onto the sample, the whole space under the cover slip was filled with chloral hydrate solution.

3.1.3.3.2. Scanning electron microscopy

Part of the selected plant material was sectioned, dehydrated at 35 ºC for 24 h, and directly mounted on stubs using double-side adhesive tape. Prepared samples were then sputtered with a thin layer of gold in a Polaron E 5350 and observed using a JEOL JSM-T220 scanning electron microscope at 15 kV, with a digital image acquisition integrated system (Peabody, MA, USA).

3.1.3.3.4. Statistical analysis

Preliminary measurements and analysis of morphological and anatomical characteristics of the samples were performed using the Leica application suite (LAS)

software, version 4.8.0. Statistical values have been calculated using the Microsoft Excel software.

3.1.3.4. Preparation of Extracts

After preparing the powder of the dried samples by grinding, extraction was performed using the maceration method (1:10 with a mixture of ethanol/water 70%) under agitation and filtration (3×, 24 h each). Hydroethanolic extracts were concentrated under reduced pressure at a temperature of less than 40 ºC using a rotary evaporator and freeze-dried. The extracts yield (%) was determined according to the formula (weight of freeze-dried extract × 100)/ (weight of the original sample).

3.1.3.5. Chromatographic Conditions

3.1.3.5.1. Thin Layer Chromatography (TLC)

Silica gel 60 $F2_{54}$ and silica gel 60 RP-18 F_{254} precoated plates (Merck®, Darmstadt, Germany) were used in appropriate size according to the methods described in the European pharmacopoeia and number of samples to be analyzed, allowing 60-80 mm for development. Samples (10 µL) were applied with a glass capillary tube as a narrow uniform band of 5 mm. Spots were separated 15 mm from each other and from the side edge of the plate. After development (each plate was eluted with the suitable solvent system (Table. 3.1.1) using CAMAG Twin through chamber (10 x 20 cm), TLC plates were dried, and zones visualized. For detection of compounds, short-UV light (254 $_{nm}$) and long-UV light (366 $_{nm}$) was used to visualize UV absorbing/ quenching and fluorescent compounds before and after spraying with adequate spraying reagents. For this study a CAMAG UV cabinet consists of a CAMAG Dual Wavelength UV Lamp, and a viewing box was used.

Table 3.1.1. TLC developing systems and detection spray reagents.

Systems	Stationary phase	Mobile phase	Detection
S1	SiG60	EtOAc: HCOOH: H_2O (82: 9: 9, v/v/v)	(NP/PEG) /UV:366 $_{nm}$
S2	SiG60	*n*-hexane: EtOAc: AcOH (31 :14: 5, v/v/v)	(NP/PEG) /UV:366 $_{nm}$
S3	SiG60 RP_{18}	H_2O: MeOH (0.5:19.5 v/v)	Potassium hydroxide solution 5%:366 $_{nm}$
S4	SiG60	EtOAc: TOL (1:9 v/v)	Anisaldehyde-sulfuric acid

Abbreviations: EtOAc- ethyl acetate; H_2O-water; HCOOH-formic acid; TOL-toluene; MeOH: methanol

Different spray reagents were used either to visualize non-UV absorbent compounds or to screen certain class of compounds in chromatograms. Anisaldehyde-sulfuric acid was used as general spray reagent for detection of terpenoids and prepared according to Wagner et al., 1996 (reagent no 3) [122]. A solution of 0.5 % anisaldehyde in MeOH: H_2SO_4: AcOH (85:5:10, v/v/v) were sprayed on chromatograms and then the plate heated at 105 ºC for 10 min. Natural product-polyethylene glycol reagent (NP/PEG = NEU) was used for searching the phenolic compounds and sprayed with a solution of diphenylboric acid aminoethyl ester (diphenylboryloxyethylamine) (NP) in 1% methanol, followed by 5% ethanolic solution of polyethylene glycol-400 (PEG) (reagent no 28 in Wagner et al., 1996) [122]. Potassium hydroxide (KOH) was used for searching anthracene derivatives and prepared as 5% ethanolic solution of potassium hydroxide (reagent no 35 in Wagner et al., 1996) [122]. The R_f values measurement of the main compounds detected on each extract as well as the R_f of the different standards used were determined. For each of these compounds, when detected in more than one sample, mean R_f values were calculated and used.

3.1.3.5.2. High-performance liquid chromatography (LC-UV/DAD)

HPLC technique coupled with a photodiode array detector (UV/DAD) was applied for the analysis of samples using a Waters Alliance 2690 Separations Module coupled with a Waters 996 photodiode array detector (Waters®, Milford, MA, USA). An Atlantis T3 column, RP-18 end-capped, particle size 5 µm, 150 × 4.6 mm, connected to a pre-column with the same stationary phase (Waters®, MA, USA) was used. A mixture of water with 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) was used as the mobile phase (Table 3.1.2).

Table 3.1.2. LC-UV/DAD mobile phase (gradient mode).

Time (min)	%H_2O+0.1% HCOOH	% CH_3CN	Flow (mL/min)
0	95	5	1,0
20	71	29	1,0
30	67	33	1,0
35	64	36	1,0
45	50	50	1,0
60	0	100	1,0
65	0	100	1,0
75	95	5	1,0

Abbreviations: CH_3CN- acetonitrile; H_2O - water; HCOOH- formic acid.

Before the analysis, the column was first equilibrated with the mobile phase before injection of samples. Samples, depending on the polarity were dissolved in acetonitrile or ultrapure water and filtered through a polytetrafluoroethylene syringe filter (0.2 µm). A certain amount of filtrate (20 mg/mL of crude extracts, 25 µL) was then injected into the column with a flow rate of 1mL/min. Chromatograms were monitored and registered on Maxplot wavelength (240–650 nm) and the obtained data were analyzed using a Waters Millennium® 32 Chromatography Manager Software (Waters Corporation, Milford, MA, USA). Chromatograms were monitored and registered on *Maxplot* wavelength (240–650 nm) and the obtained data were

analyzed using a Waters Millennium® 32 Chromatography Manager Software (Waters Corporation, Milford, MA, USA).

3.1.3.6. Quantification Assays of the Main Classes of Secondary Metabolites

3.1.3.6.1. Total phenolic content (TPC)

TPC of the crude extracts were determined by a modified Folin-Ciocalteu method [123]. Two milliliters of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) were mixed with 400 µL of the extract and then 1.6 mL of anhydrous sodium carbonate (Na_2CO_3, 75 g/L). After two hours the absorbance of samples and standard solution were measured at 765 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing gallic acid concentration (10-70 µg/mL) with equation of Y= 0.0087x + 0.0264, R^2= 0.994 were used to obtain a standard curve (Fig.3.1.2). Values were obtained in 3 sets of experiments and assessed in triplicate for method validation. Distillated water was used for blanks controls. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample weight (mg GAE/g DW). Data are presented as the mean +/- standard deviation.

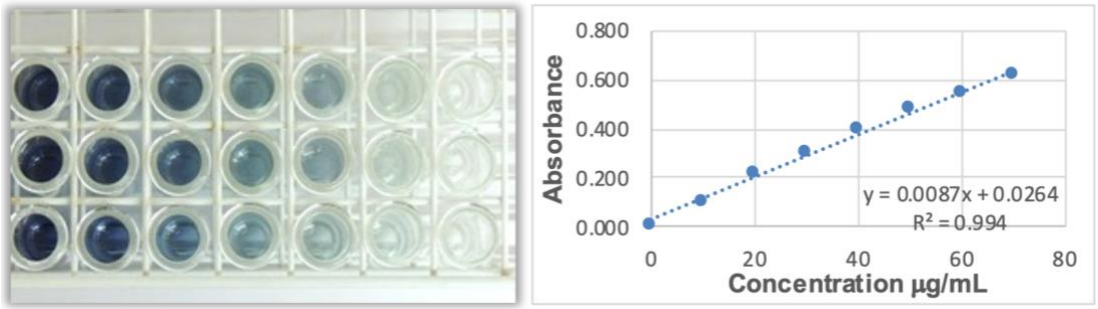

Figure 3.1.2. Colour changes of gallic acid at different concentration levels and its calibration curve as standard in determination of total phenolic content.

3.1.3.6.2. Total flavonoid content (TFC)

TFC was determined by Olivera et al.,2008 [124], with some modifications. To 500 µL of plant extract samples was added 2 mL distilled water and 150 µL 5% $NaNO_2$ and

left to incubate for 5 minutes. After that was added 150 µL 10% $AlCl_3$ and incubate for 6 minutes. Then added 1 ml of 1M NaOH.

Solution was mixed and incubate at 18°C in dark for 20 minutes. Absorbance was measured at 510 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing catechin concentration (50-200 µg/mL) with equation of Y= 0.0039x + 0.027, R^2= 0.993 were used to obtain a standard curve (Fig.3.1.3). Values were obtained in 3 sets of experiments and assessed in triplicate for method validation. Distillated water was used for blanks control. Results were expressed as milligrams of catechin equivalents (CAE) per gram of dried sample weight (mg CAE/g DW). Data are presented as the mean +/- standard deviation.

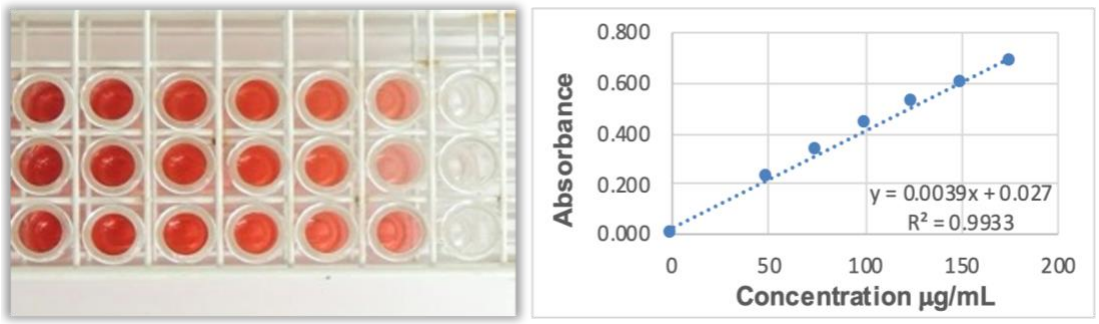

Figure 3.1.3. Colour changes of catechin at different concentration levels and its calibration curve as standard in determination of total flavonoid content.

3.1.3.6.3. Total condensed tannin content (TCTC)

TCTC was determined according to the butanol–HCl assay described by Scalbert et al.,1989 [123]. To 500 µL of extract was added 5 mL of an acid solution of ferrous sulfate (77 mg of $FeSO_4$ x 7 H_2O dissolved in 500 mL of 2:3 HCl / *n*- butanol). The tubes were loosely covered and placed in a water bath at 95°C for 15 minutes. The absorbance was recorded at 530 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing catechin concentration (200-2000 µg/mL) with equation of Y= 0.0002x + 0.0324, R^2= 0.9806, were used to obtain a standard curve (Fig.3.1.4). Values were obtained in 3 sets of experiments and assessed in triplicate for method

validation. For blanks one was used distillated water. Results were expressed as milligrams of catechin equivalents (CAE) per gram of dried sample weight (mg CAE/g DW). Data are presented as the mean +/- standard deviation.

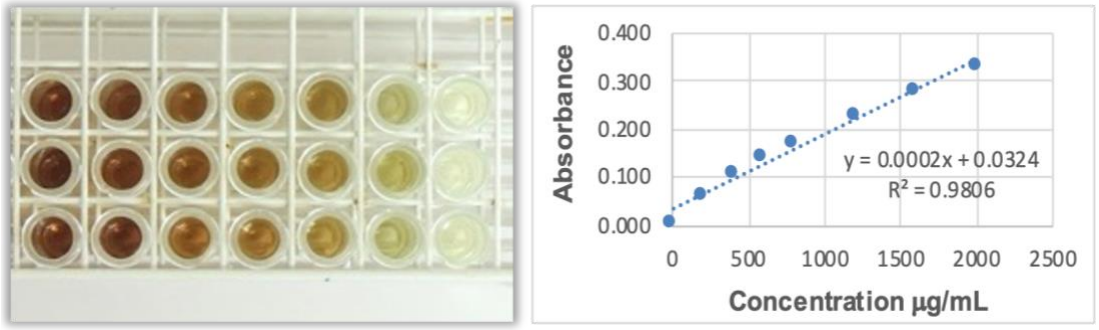

Figure 3.1.4. Colour changes of catechin at different concentration levels and its calibration curve as standard in determination of total condensed tannin content.

3.1.3.6.4. Total hydrolysable tannin content (THTC)

For quantification of THTC, the potassium iodate assay was performed [125]. The reaction was initially performed by adding 1.5 mL of a saturated potassium iodate solution to 3.5 mL of extracts (diluted 1mL of extracts with 2.5 mL of solvent 20% acetone/water), followed by a 40-minute incubation at 0 ºC. Since the red intermediate turns yellow over time, it was important to be consistent in terms of the time and temperature of the reaction.

The absorbance was recorded at 550 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing gallic acid concentration (100-600 µg/mL) with equation of Y= 0.001x + 0.054, R^2= 0.9773, was used to obtain a standard curve (Fig.3.1.5). Values were obtained in 3 sets of experiments and assessed in triplicate for method validation. Distillated water was used for blanks control. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample weight (mg GAE/g DW). Data are presented as the mean +/- standard deviation.

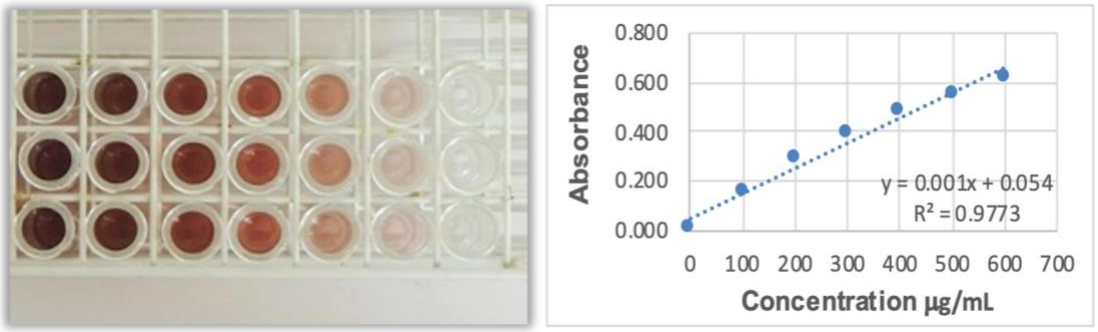

Figure 3.1.5. Colour changes of gallic acid at different concentration levels and its calibration curve as standard in determination of total hydrolysable tannin content.

3.1.3.6.5. Total anthraquinone content (TAC)

According to the method described by Sakulpanich et al., 2008 [126] for determination of TAC, 300 mg of the powdered drug was introduced into a flask. 30 mL of water was added, mixed, and weighed. Then was heated in a water-bath under a reflux condenser for 15 minutes, allowed to cool and centrifuged (4000 rpm, 10 min). 10 mL of supernatant was transferred to a 100 mL round-bottomed flask with a ground-glass neck. Added 20 mL of ferric chloride (10.5 % $FeCl_3.6H_2O$) solution and mixed. The solution was heated under a reflux condenser on a water-bath for 20 minutes, added 1 mL of hydrochloric acid (2M) and heated for a further 20 minutes, shaking frequently. After Cooling, transferred to a separating funnel and was shaken with three quantities, each of 25 mL of ether previously used to rinse the flask. Combined the ether extracts and wash with two quantities, each of 15 mL of water. Filtered the ether extracts through a plug of absorbent cotton into a volumetric flask and diluted to 100 mL with ether. Evaporated 25 mL carefully to dryness on a water-bath and dissolved the residue in 10 mL of a 5 g/L (0.5%) solution of magnesium acetate in methanol. The absorbance was recorded at 515 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing rhein concentration (3-18 µg/mL) with equation of Y= 0.0215x - 0.0016, R^2 = 0. 9983, was used to obtain a standard curve (Fig.3.1.6). Values were obtained in 3 sets of experiments and assessed in triplicate for method validation. Distillated water was used for blank. Results were

expressed as milligrams of rhein equivalents (RhE) per gram of dried sample weight (mg RhE/g DW). Data are presented as the mean +/- standard deviation.

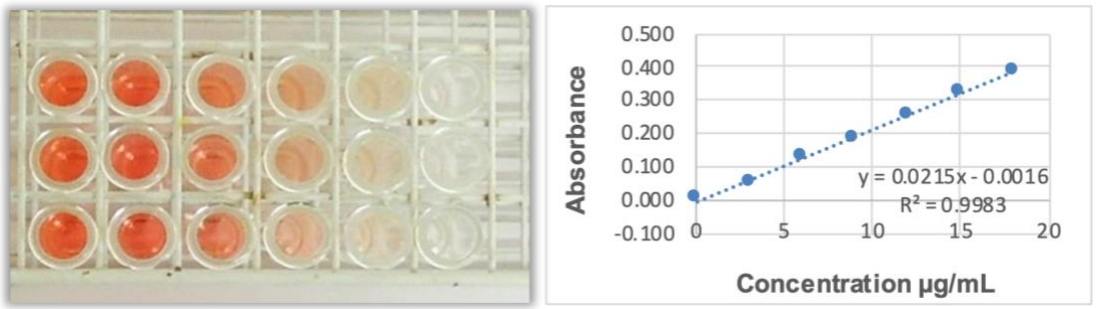

Figure 3.1.6. Colour changes of rhein at different concentration levels and its calibration curve as standard in determination of total anthraquinone content.

3.1.3.6.6. Total terpenoid content (TTC)

TTC was determined following procedure developed by Chang et al., 2012 [127]. Briefly, 100 µL of extract was mixed with vanillin/glacial acetic acid (150 µL, 5% w/v) and perchloric acid solution (500 µL). The sample solutions were heated for 45 minutes at 60 ºC and then cooled in an ice water bath to the ambient temperature. After the addition of glacial acetic acid (2.25 mL), the absorbance was recorded at 548 nm using UV-Vis spectrophotometer (Hitachi, U–2000). Increasing oleanic acid concentration (100-800 µg/mL in methanol) with equation of Y= 0.0012x + 0.0849, R^2= 0. 9939, was used to obtain a standard curve (Fig.3.1.7). Values were obtained in 3 sets of experiments and assessed in triplicate for method validation. For blanks one was used distillated water. Results were expressed as milligrams of oleanic acid equivalents (OAE) per gram of dried sample weight (mg OAE/g DW). Data are presented as the mean +/- standard deviation.

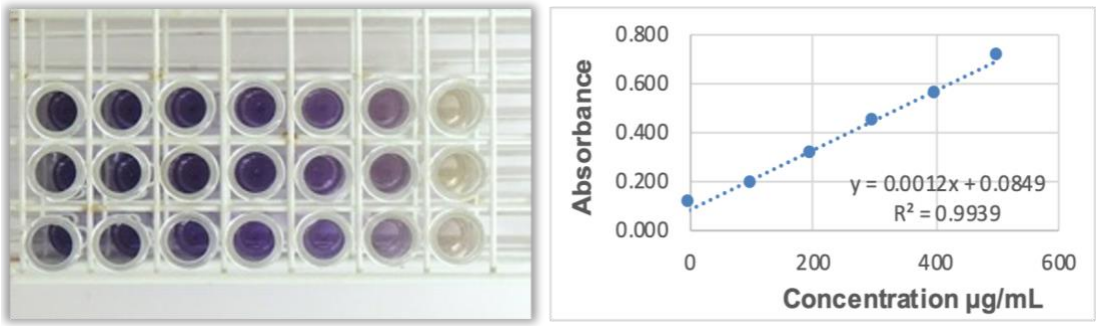

Figure 3.1.7. Colour changes of oleanic acid at different concentration levels and its calibration curve as standard in determination of total terpenoid content.

3.1.3.6.7. Statistical analysis of the obtained quantification data

All the obtained data were analyzed statistically by one-way analysis of variance (ANOVA) with *Asphodelus* species as the source of variance. Once both *Asphodelus* species were also collected in two different seasons, the obtained data were then analyzed by ANOVA with the season as the source of variance. The significant value was set for a *p*-value < 0.05.

3.1.3.7. Genotoxicity/Mutagenicity Evaluation

The assessment of genotoxicity/mutagenicity was performed using the direct plate incorporation method with and without metabolic activation described by Maron and Ames, 1983 [128] following the OECD No. 471 [129] and ICH S2 (R1) guidelines [130]. Five *Salmonella enterica* serovar Typhimurium tester strains (TA98, TA100, TA102, TA1535, and TA1537) were used in this study (with and without metabolic activation) in a direct plate incorporation method. TA100, TA98, TA102 and TA1535 were kindly provided by the Genetic Department of the Nova Medical School of the Universidade Nova de Lisboa (Portugal), having them received from Professor B.N. Ames (Berkeley, CA, USA). TA1537 was obtained from ATCC, NUMBER: 29630™, LOT: 7405375. The strains were inoculated in nutrient broth medium and incubated for 12–16 h, at 37 ºC in the dark, shaking at 210 rpm in an orbital incubator, and kept at 4 ºC util use. S9

mix (10%, v/v rat liver S9, 0.4 M $MgCl_2$, 1.65 M KCl, 1 M glucose-6-phosphate, 0.1 M nicotinamide adenine dinucleotide phosphate, and 0.2 M sodium phosphate buffer, pH 7.4) was freshly prepared and kept on ice during the experiment.

The extracts (25 mg/mL). An amount of 200 µL of extracts dilutions were mixed with 500 µL sodium phosphate buffer (0.1 M, pH 7.4) (assay without metabolic activation) or S9 mix (assay with metabolic activation), 100 µL of the bacterial culture, and 2 mL of melted top-agar, supplemented with 0.05 mM biotin and histidine, at 45 ºC. This mixture was then vortexed and plated on Petri dishes with Vogel-Bonner agar medium, supplemented with 2% glucose. After a 48h incubation at 37 ºC, manual counting of His+ revertant colonies for each concentration was performed. All assays were performed in triplicate. The results were expressed as the mean number of revertant colonies with the standard deviation (mean±SD). The positive controls were sodium azide (SA, 1.5 µg/plate for TA100 and TA1535), 2-nitrofluorene (2-NF, 5 µg/plate for TA98), 9-aminoacridine (9-AA, 100 µg/plate for TA1537), and tert-butyl hydroperoxide (tBHP, 50 µg/plate for TA102) in the assay without metabolic activation, and 2-aminoathracene (2-AA, 2 µg/plate for TA98 and 10 µg/plate for TA102, TA1535 and TA1537) and benzo(*a*)pyrene (BaP, 5 µg/plate for TA100) in the assay with metabolic activation.

3.1.4. Results and Discussion

3.1.4.3. Botanical Characterization

Although macroscopic and microscopic observations of flowers [131–140] and leaves [141] of several *Asphodelus* species have been documented, however, data regarding the anatomy of root tubers are scarce.

Considering our obtained data, macroscopically (Figure.3.1.8, Table 3.1.3), morphological variations were observed between the studied species in accordance with the general botanical description found in the Flora Iberica. The root tubers of *A. bento-rainhae* were short, fusiform, and developed directly on the rhizome, up to 2

cm; however, in *A. macrocarpus* (6-13 cm x1.2-1.7 cm) they developed at a distance of 2-7 cm from the rhizome.

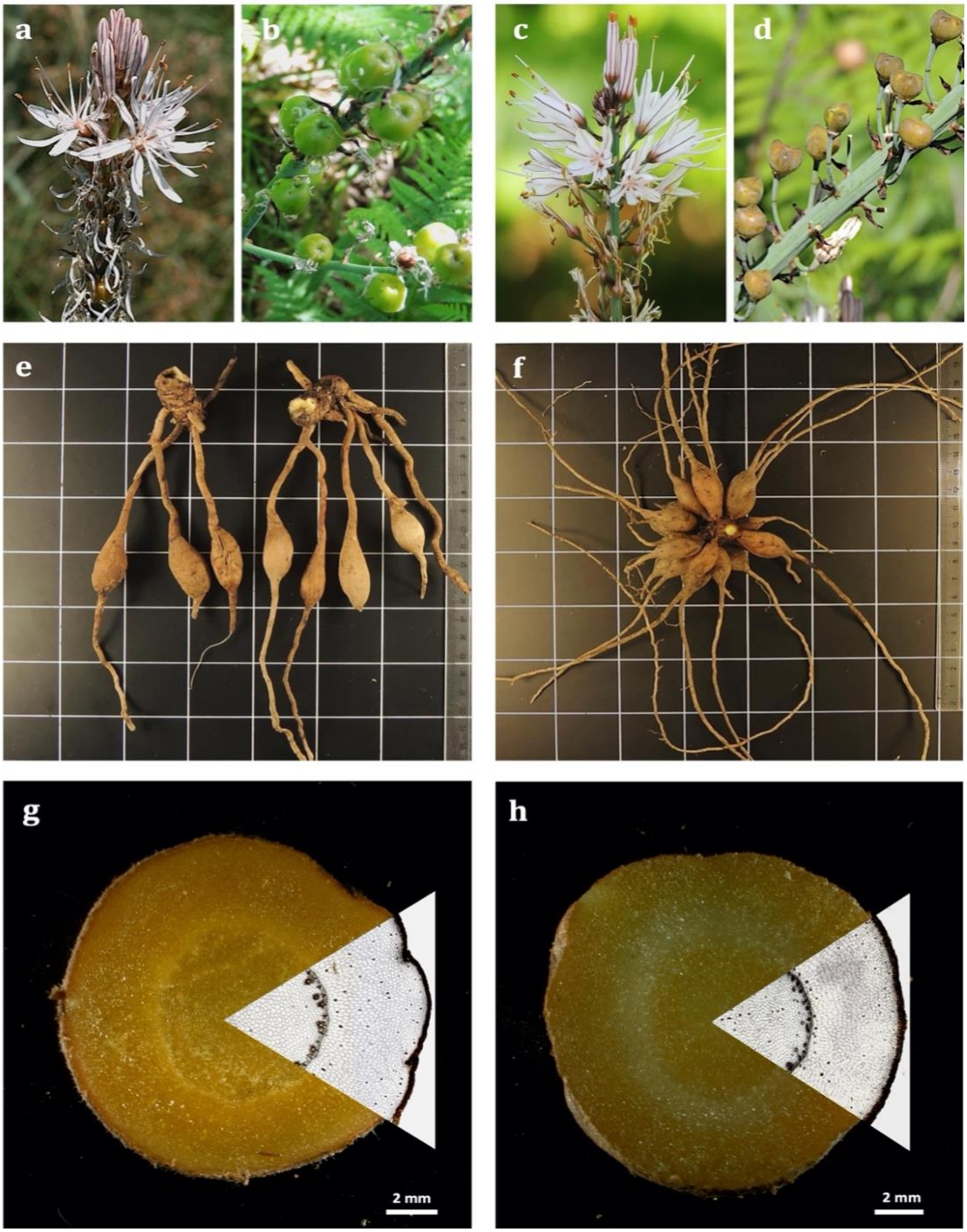

Figure 3.1.8. Macroscopic features of Portuguese *Asphodelus* species.
Images of flowers and fruits of *A. macrocarpus* (a,b) and *A. bento-rainhae* (c,d) growing in their natural habitats, Serra da Gardunha, Fundão. Root tubers in general and cross-section views in *A. macrocarpus* (e,g) and *A. bento-rainhae* (f,h).

Microscopically (Figure 3.1.9, Table 3.1.3), the multiple-layered epidermis (velamen), without cuticle on the outer surface (Figure 3.1.9 a-d, Table 3.1.3) and an average of 4-5 cells wide in both species. In addition, single-celled hairs (Figure 3.1.9 c, d), which are responsible for rapid water uptake, water loss reduction, osmotic and mechanical protection, were observed.

The cortex area (Figure 3.1.9 e, f, Table 3.1.3) between the velamen and central cylinder is up to 24± cells wide in *A. bento-rainhae* and ±37 in *A. macrocarpus*, composed of oil cells and thin-walled idioblasts (58.3–62.5 μm in AbR and 60.7–114.6 μm in AmR). They contain numerous needle-shaped (raphides) crystals of calcium oxalate (20.8–62.5 μm in AbR and 78–114.3 μm in AmR) in their vacuole (Figure 3.1.9 m–r, Table3.1.3).

The uniseriate endodermis cells (Figure 3.1.9 e–h, Table 3.1.3) with Casparian strips, are periclinally orientated with thick walls. The vascular cylinder comprises the uniseriate pericycle, periclinally orientated (Figure 3.1.9e–h), and isodiametric cells.

The root xylem (protoxylem and metaxylem) consists of vessels in short radial rows (Figure 3.1.9 g–j, Table 3.1.3), alternating with broadly elliptical to variable-shaped clusters of phloem cells (Figure 3.1.9 e–h).

The vascular tissue is surrounded by sharply differentiated, somewhat thick-walled, polygonal parenchyma. The parenchymatous pith (Figure 3.1.9 c–h, Table 3.1.3) comprises oval and almost circular, thin-walled cells with triangular, square, and rectangular intercellular spaces.

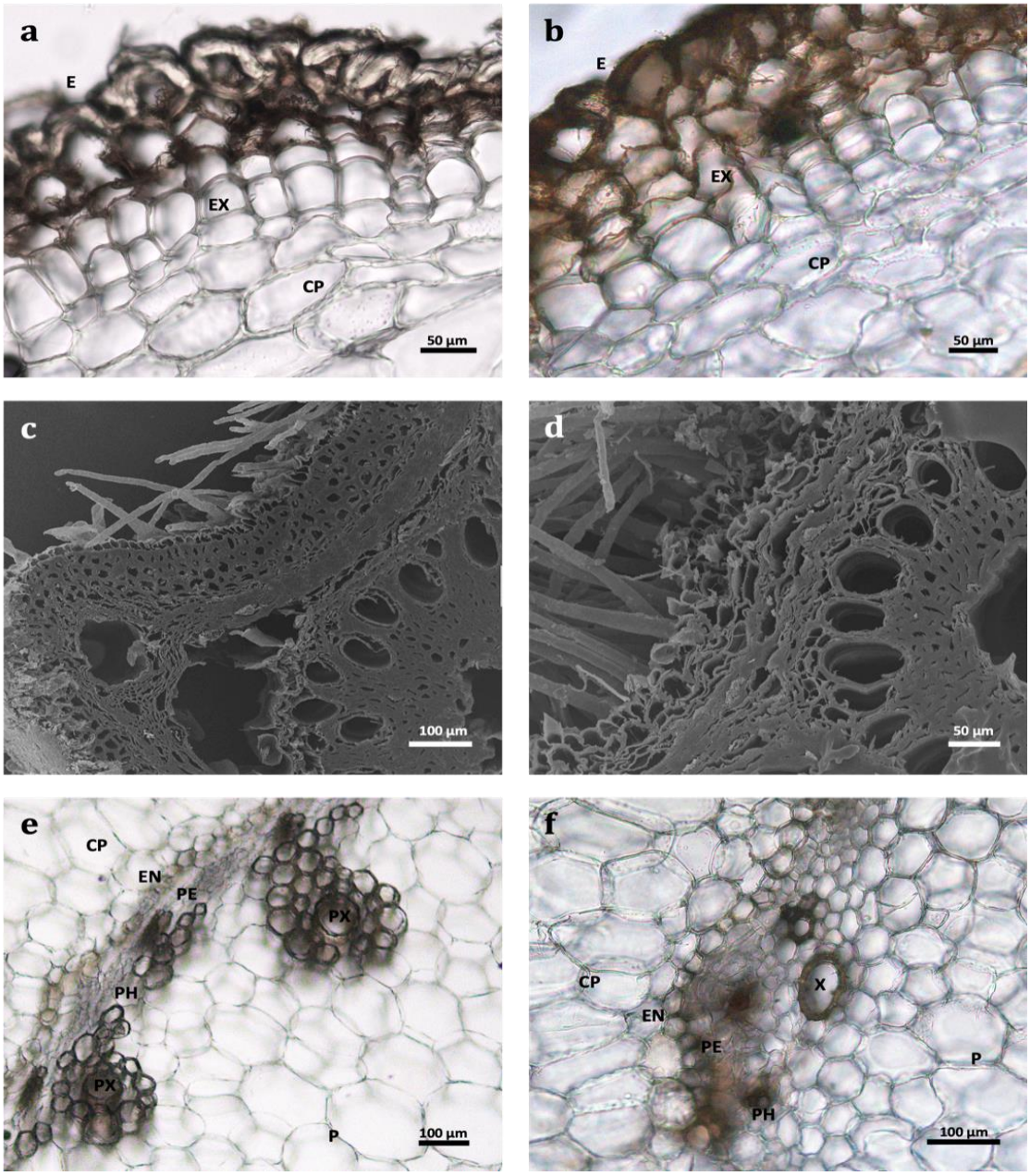

Figure 3.1.9. *Cont.*

Considering the abovementioned common anatomical structures between the two species, a noticeable size difference is evident. The width of idioblast cells and the length of raphides and endodermis cells are larger in *A. macrocarpus* compared to *A. bento-rainhae* (Table 3.1.3). The two species also differ considerably in the arrangements and characteristics of the vascular cylinder (metaxylem and protoxylems).

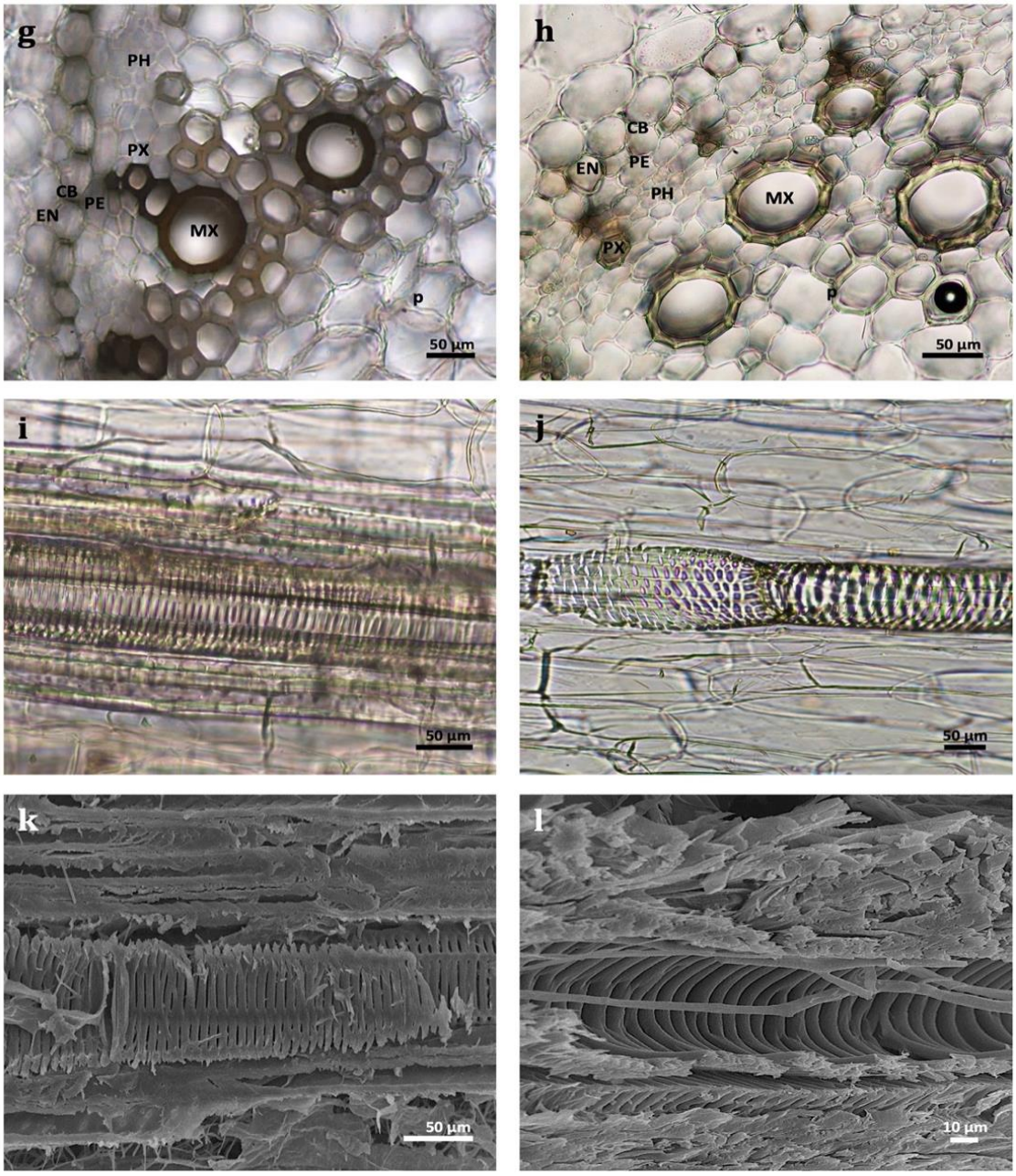

Figure 3.1.9. *Cont.*

Metaxylem (Figure 3.1.9 e–h, Table 3.1.3) vessels in *A. macrocarpus* (±80 µm diameter) were found to be surrounded by several protoxylems; however, in *A. bento-rainhae* (±70 µm diameter), they are individually separated. These anatomical differences can be helpful in differentiating the two species in their dried whole, fragmented, or powdered forms.

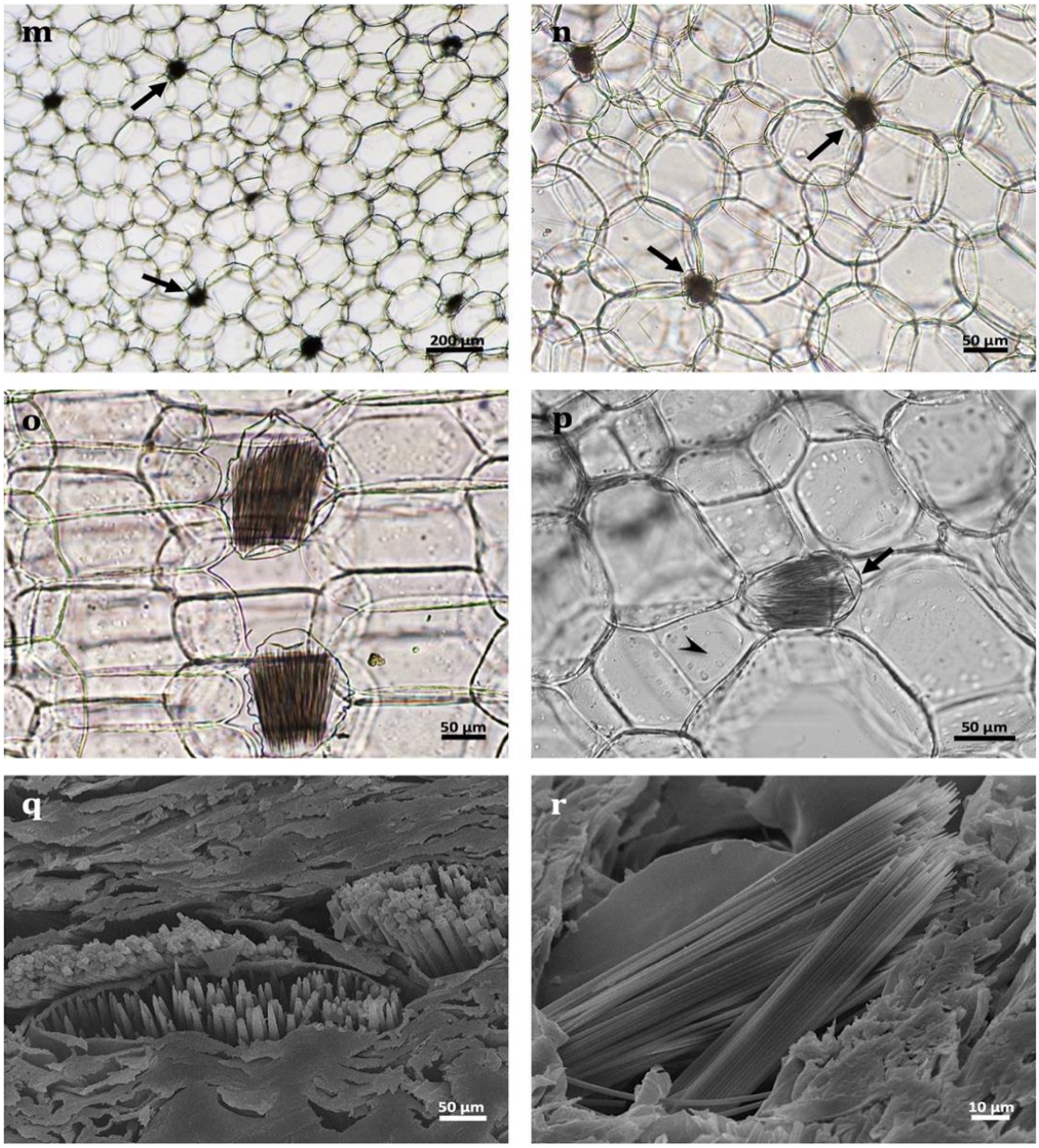

Figure 3.1.9. Microscopic features of Portuguese *Asphodelus* species.

LM (a,b,e,f,g,h) and SEM (c,d) images of velamen with multiple layers (E-epidermis, EX-exodermis cells with suberized walls, CP-cortical parenchyma cells) in *A. macrocarpus* (a,c) and *A. bento-rainhae* (b,d) together with root hairs. Details of cortical parenchyma cells (CP), endodermis (EN), Casparian bands (CB) and vascular cylinder (PE-pericycle; PX—protoxylem; MX-metaxylem; PH—phloem; P-pith cells) in *A. macrocarpus* (c,e,g) and *A. bento-rainhae* (d,f,h) in transversal view. LM (i,j) and SEM (k,l) images of scalariform vessels with lignified secondary cell wall deposition and fibers in longitudinal view in A. macrocarpus (i,k) and *A. bento-rainhae* (j,l). LM (m–p) and SEM (q,r) images of cortex parenchyma showing thin-walled idioblasts with numerous calcium oxalate crystals, type raphides (arrows) in transversal view (m,n,q) and longitudinal view (o,p,r) in *A. macrocarpus* (m,o,q) and *A. bento-rainhae* (n,p,r); Details of parenchyma cells with polysaccharides (p) such as starch grains (arrowhead).

Table 3.1.3. Principal morphological and anatomical features of *A. bento-rainhae* and *A. macrocarpus* root tubers.

Anatomical characteristic	*A. bento-rainhae*			*A. macrocarpus*		
	Min-Max*	Mean	±SD*	Min-Max*	Mean	±SD*
Root length (cm)	2-5	3.5	0.7	6-13	8.7	2.2
Root diameter (cm)	0.7-1.6	1	0.2	1.2-1.7	1.4	0.2
Velamen (numbers of cell layers)	4-5	4	0.25	4-7	5	0.54
Cortex (numbers of cell layers)	17-24	21	3.1	21-37	29	4.7
Idioblast cell width (µm)	58.3-62.5	134	2.9	60.7-114.6	150	27.6
Protoxylem wall thickness (µm)	4.2-5	4.5	0.4	4.1-6	4.6	0.3
Protoxylem diameter(µm)	20.8-25	22.9	2.95	8.33-61	36.1	35.8
Metaxylem wall thickness (µm)	8.3-13.9	10.4	1.7	10.8-14.6	12.4	1.6
Metaxylem diameter (µm)	50-99.6	70.3	13.1	52-101.8	80.7	19.0
Pith cell diameter (µm)	73.2-121.9	93	5.6	94.4-140.7	114	4.6
Raphids length (µm)	20.8-62.5	37.2	14.2	78-114.3	87.7	15.3

Abbreviations: AbR, *A. bento-rainhae* root; AmR, *A. macrocarpus* root; Min, minimum; Max, maximum; SD, standard deviation.

Multiseriate epidermis (velamen), enabling quick gain of transiently available soil water, the large parenchyma water-storing cells, the cortex cells containing soluble sugars, the oil cells containing lipid material of possibly defense character, and the cell idioblasts, which contain raphide crystals, were found among numerous anatomical similarities, between the root tubers of *A. aestivus*, our studied Portuguese *Asphodelus* and several other species from *Liliaceae* and *Orchidaceae*. These characteristics are, in fact, the means of synchronization and adaptation of these plants with the seasonality of the Mediterranean climate, as discussed by Sawidis et al., 2005 [142].

3.1.4.4. Phytochemical Analysis

Thin-layer chromatography (TLC) in this study was used as a rapid, reliable (due to its high sensitivity), and inexpensive technique for monitoring and detection of several samples, which could be analyzed simultaneously with low solvent usage [143,144]. Followed by the TLC method and for a more detailed phytochemical screening of the extracts and their constituents, high-performance liquid chromatography (HPLC) technique coupled to a photodiode detector (UV/DAD) was applied.

The obtained TLC fingerprint (Figure 3.1.10 a–c) confirmed the presence of phenolic acids (bands (a–e) with light to greenish blue colors under 366_{nm} UV light), anthracene derivatives (bands (f–l) with orangish to red color under 366_{nm} UV light) and terpenoids (bands (m–r) with pink to purple color, using an increased temperature under visible light) after spraying with specific revealing reagents in both species.

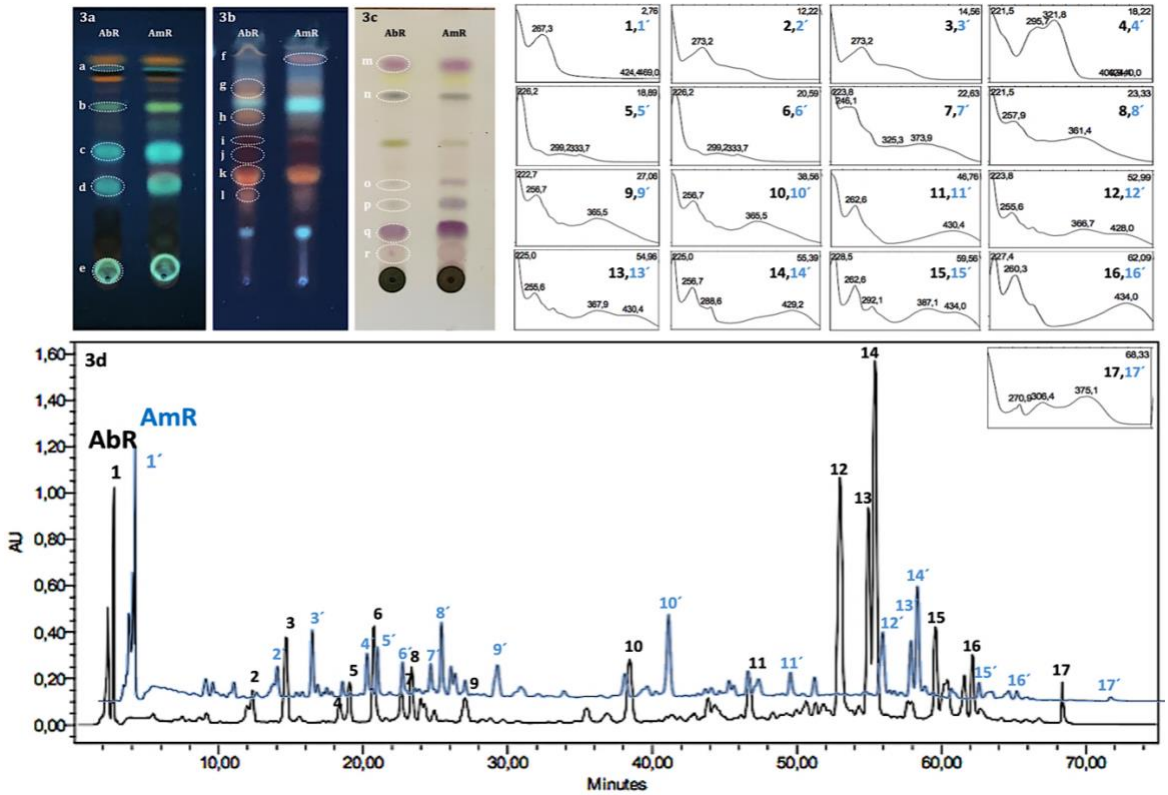

Figure 3.1.10. TLC and LC-UV/DAD *Maxplot* (210–600 nm) chromatographic profiles of secondary metabolites of *A. bento-rainhae* and *A. macrocarpus* root tuber extracts.

Abbreviations: AbR, *A. bento-rainhae* root tuber 70% hydroethanolic extract; AmR, *A. macrocarpus* root tuber 70% hydroethanolic extract. (**a**): Phenolic acid derivatives detection, SiG60, spraying with NP/PEG, solvent system: S1, UV:366_{nm}; (**b**): anthracene derivatives detection, SiG60 RP_{18}, spraying with KOH 5%, solvent system: S2, UV:366_{nm}; (**c**): terpenoids detection, SiG60 spraying with anisaldehyde-sulphuric acid, solvent system: S3, visible light; (**d**): comparative chromatograms of AbR and AmR root tuber 70% hydroethanolic extracts.

The presence of phenolics and coumarins in *A. microcarpus* root extracts using TLC was previously reported by Abuhamdah et al., 2013 [46]. The LC-UV/DAD chromatographic profiles of both species (3.1.10 d) were qualitatively similar in their chemical composition, characterized by the presence of phenolic acids and anthracene derivatives, based on spectral analysis, and compared with the data in the literature. Considering the quantification results of the principal chemical classes of marker secondary metabolites (Table 3.1.4), TTC (173.88 ± 29.82, 180.55 ± 10.57 mg OAE/g dried weight) and TCTC (128.64 ± 14.05, 108.35 ± 20.37 mg CAE/g dried weight) were the main chemical classes of *A. bento-rainhae,* and *A. macrocarpus* extracts, respectively. Noticeably, anthraquinones exhibited the least content among all the other phenolic compounds in both species. However, in the studies related to the chemical composition of other *Asphodelus* species root extracts, they were referred to as the main chemical class responsible for their biological activities [36].

The statistical analysis of our results also showed that the contents of the marker secondary metabolites of AbR and AmR were dependent on the sample collection season. In fact, in AbR extracts, the total TAC, TCTC, and TFC content (3.38 ± 0.26 mg RhE/g DW, 128.64 ± 14.05 and 16.71 ± 1.12 mg CAE/g DW, respectively) were significantly higher in the second season collection in comparison with its first one (*p*-values: 0.011, 0.022 and 0.02, respectively), and for AmR extracts, the content of TAC (3.21 ± 0.21 mg RhE/g DW) was significantly higher in the first season collection than in the second season (*p*-value: 0.023).

Concerning the chemical content of AmR and AbR extracts of the samples collected in the same season, results showed that TFC content (18.90 ± 0.26 mg CAE/g DW) in the first season was significantly higher in AmR when compared to AbR (*p*-value: 0.003).

Table 3.1.4. Quantification of principal chemical classes *of A. bento-rainhae* and *A. macrocarpus* root tuber 70% hydroethanolic extracts.

Assays	AbRa	AbRb	AmRa	AmRb
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TPC				
(mg GAE/ g dried extract)	20.36±4.2	26.45 ±7.52	29.14±9.32	27.35 ±8.13
(mg GAE/ g dried root)	10.94±2.26	13.76±3.91	12.76±4.08	10.12±3.01
TFC				
(mg CAE/ g dried extract)	10.55±1.17	16.71±1.12*	18.90±0.26	17.70±0.24
(mg CAE/ g dried root)	5.67±0.63	8.69±0.58	8.28±0.11	6.55 ±0.09
TTC				
(mg OAE/ g dried extract)	173.88±29.82	172.11±19.20	180.55±10.57	154.36±20.53
(mg OAE/ g dried root)	93.46±16.03	89.50±9.99	79.08±4.63	57.11 ±7.60
TAC				
(mg RhE/ g dried extract)	2.43±0.17	3.38±0.26*	3.21±0.21**	2.68±0.19
(mg RhE/ g dried root)	1.31±0.12	1.67±0.16	1.48±0.14	0.99 ±0.09
TCTC				
(mg CAE/ g dried extract)	93.80±9.39	128.64±14.05*	88.08±7.83	108.35±20.37
(mg CAE/ g dried root)	50.42±20.76	66.89±7.30	38.58±3.43	40.09 ±7.54
THTC				
(mg GAE/ g dried extract)	21.91±7.43	32.73±8.61	25.81±7.25	28.09±6.16
(mg GAE/ g dried root)	11.78±4.91	17.02±4.48	11.31±3.17	10.39±2.28

Abbreviations: AbRa, *A. bento-rainhae* root first collection; AbRb, *A. bento-rainhae* root second collection; AmRa, *A. macrocarpus* root first collection; AmRb, *A. macrocarpus* root second collection; SD, standard deviation; TPC, total phenolic content; TFC, total flavonoid content; TTC, total triterpenoid content; TAC, total anthraquinones content; TCTC, total condensed tannin content; THTC, total hydrolysable tannin content; GAE, gallic acid equivalents; CAE, catechin equivalents; OAE, oleanolic acid equivalents; RhE, rhein equivalents. * Significantly higher content (p-value < 0.05) when compared between different seasons of collection of the same species. ** Significantly higher content (p-value < 0.05) when compared between different species from the same season of collection.

The previously reported TPC and TFC values for other *Asphodelus* root extracts indicated the critical role of solvent selection in the extraction procedure. In fact, *A. microcarpus* ethanolic [70] (39.35 ± 4.2 mg GAE/g of DW) and methanolic [70,84] (15.31 ± 7.8 and 17.90 GAE/g of DW) extracts exhibited significantly higher amounts of total polyphenols in comparison to an aqueous extract [70]. However, Mayouf et al., 2019 [102] reported a significantly elevated amount of total polyphenols (377 ±

0.030 mg GAE/g of DW) in methanolic extracts of this species. Total flavonoids of methanolic extracts of *A. microcarpus* reported by Di Petrillo et al. [70] (3.94 ± 1.05 mg QUE/g of DW) were significantly smaller than the ones reported by Kitaz 2017 [84], (14.69 mg RUE/g of DW).

Dichloromethane and ethyl acetate extracts of the roots of *A. albus* and *A. aestivus* showed high phenolic content (30.74 ± 0.41, 20.21 ± 0.19 mg GAE/g of DW, respectively) and *A. aestivus* presented higher flavonoid content (13.82 ± 0.80, mg RE/g of DW) when compared to *A. albus* [145]. Hydromethanolic extracts of *A. tenuifolius* [97] showed smaller amounts of polyphenols and flavonoids (11.4 ± 0.82 mg GAE/g of DW, 3.2 ± 0.08 QUE/g of DW, respectively) in comparison to our obtained contents of hydroethanolic extracts of both species. The presence of alkaloids, flavonoids, and tannins in *A. tenuifolius* root extracts using a colorimetric test tube was also previously reported by Menghani et al., 2012 without quantification data [96].

Overall, as discussed by Kitaz, 2017 [84], the difference in amounts of secondary metabolites in different *Asphodelus* species, is probably related to geographical, environmental, and climatic factors and conditions, processing methods, and other intrinsic (genetic and development stage) and extrinsic (environmental and handling) factors.

3.1.4.5. Preclinical Safety Assessment

The bacterial reverse mutation test (Ames test) has been commonly employed as an initial screening method to evaluate the genotoxicity potential of herbal substances and preparations. This test is favored for its rapidity, cost-effectiveness, ease of execution, and its proven ability to identify relevant genetic alterations and detect most genotoxic carcinogens for both rodents and humans [146]. The assessment of mutagenicity by Ames test is part of the standard genetic toxicology testing battery required by regulatory agencies for the chemical, cosmetic industry, pharmaceutical,

and agro-industrial fields to enable the marketing of these products [146]. Regulatory acceptance of the Ames test data often requires the performance of the test according to the Organization for Economic Cooperation and Development (OECD) test guideline 471 [129] and ICH, S2R1 [130].

The Ames test has emerged as a crucial tool in genetic toxicology for appraising the safety of chemical compounds. This is primarily attributed to the positive correlation observed between mutagenicity and carcinogenicity [147]. Furthermore, the presence of mutagenic compounds in plant extracts has been raising concerns about the carcinogenic risks resulting from the long-term use of plants as food, medicines, and source of raw materials in the pharmaceutical industry; therefore, genotoxicity studies (e.g., Ames test) are extremely important to assess the preclinical safety of plant extracts/herbal preparations to verify their mutagenic potential for both safety and economic purposes [148–150].

Following the guidelines on genotoxicity, for a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds/substance must be more than twice the number of colonies produced on the solvent control plates (i.e., a ratio above 2.0). In addition, a positive dose-response should be evident for the various concentrations of the tested mutagen [151,152]. Considering the obtained data of the quantification analysis of all the collected samples and their consecutive extracts, the obtained extracts from the second season collection (AbRb, AmRb), exhibited the higher contents of the main classes of secondary metabolite and subsequently were selected for further examination of their safety.

According to our obtained results presented in Table 3.1.5, in the plate assay method without metabolic activation, both AbRb and AmRb extracts did not induce an increase in the number of revertant colonies in any of the tested strains at any tested concentration (250, 625, 1250, 2500, 3750 and 5000 µg of extracts/plate). Moreover, cytotoxicity did not occur, since there was neither a decrease in the number of spontaneous revertants nor a decrease on the background lawn of the plates, in any

of the concentrations tested. Therefore, under the conditions of this study, the extracts of both species did not show mutagenic activity, which is crucial to ensure their safety [128,147,152].

Table 3.1.5. Mutagenicity of *A. bento-rainhae* and *A. macrocarpus* root tuber 70% hydroethanolic extracts in the bacterial reverse mutation test (Ames Test).

AbRb µg/plate	Number of revertant colonies without metabolic activation, mean (n=3) ± standard deviation (SD)				
	TA98	**TA100**	**TA102**	**TA1535**	**TA1537**
250	16±3	174±8	365±21	19±5	10±1
625	21±3	158±3	334±15	20±3	11±2
1250	23±3	164±10	354±16	25±3	10±2
2500	23±2	177±22	363±9	20±1	10±2
3750	20±1	164±2	392±41	19±3	10±2
5000	23±4	183±17	365±20	17±2	10±3
AmRb µg/plate					
250	21±5	177±13	347±9	26±6	9±1
625	18±1	158±8	354±10	23±4	9±2
1250	22±6	179±17	379±30	17±1	11±2
2500	23±5	177±8	397±23	21±1	10±2
3750	21±5	179±13	394±10	19±1	11±2
5000	22±1	166±16	395±29	17±2	9±1
NC	19±2	156±17	320±4	21±3	7±1
PC	2-AA	BaP	2-AA	2-AA	2-AA
	488±30	1048±43	881±26	827±13	1354±5

Abbreviations: AbRb, *A. bento-rainhae* root second collection extract; AmRb, *A. macrocarpus* root second collection extract; NC: negative control /solvent control (DMSO 30%), PC: positive control reference, 2-NF: 2-nitrofluorene, SA: sodium azide, tBHP: *tert*-butyl hydroperoxide, 9-AA: 9-aminoacridine.

Chapter 3.2

A. bento-rainhae and *A. macrocarpus* Root Tubers — Bioguided Identification of Active Antimicrobial Compounds

Main part of this chapter was published as follows with minor modifications:

Bioguided Identification of Active Antimicrobial Compounds from *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* Root Tubers.

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3.2.1. Abstract

Root tubers of *Asphodelus bento-rainhae* subsp. *bento-rainhae* (AbRb), a vulnerable endemic species, and *Asphodelus macrocarpus* subsp. *macrocarpus* (AmRb) have traditionally been used in Portugal to treat inflammatory and infectious skin disorders. The present study aims to evaluate the *in vitro* antimicrobial activity of crude 70% and 96% hydroethanolic extracts of both medicinal plants, specifically against multidrug-resistant skin-related pathogens, to identify the involved marker secondary metabolites and also to assess the pre-clinical toxicity of these medicinal plant extracts. Bioguided fractionation of the 70% hydroethanolic extracts of both species using solvents of increasing polarity, namely diethyl ether (DEE: AbRb-1, AmRb-1), ethyl acetate (AbRb-2, AmRb-2) and aqueous (AbRb-3, AmRb-3) fractions, enabled the identification of the DEE fractions as the most active against all the tested Gram-positive microorganisms (MIC: 16 to 1000 µg/mL). Furthermore, phytochemical analyses using TLC and LC-UV/DAD-ESI/MS techniques revealed the presence of anthracene derivatives as the main constituents of DEE fractions, and five known compounds, namely 7'-(chrysophanol-4-yl)-chrysophanol-10'-*C*-beta-*D*-xylopyranos-yl-anthrone (**p**), 10,7'-bichrysophanol (**q**), chrysophanol (**r**), 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone (**s**) and asphodelin (**t**)**,** were identified as the main marker compounds. All these compounds showed high antimicrobial activity, particularly against *Staphylococcus epidermidis* (MIC: 3.2 to 100 µg/mL). Importantly, no cytotoxicity against HepG2 and HaCaT cells (up to 125 µg/mL) for crude extracts of both species and genotoxicity (up to 5000 µg/mL, with and without metabolic activation) for AbRb 96% hydroethanolic extract was detected using the MTT and Ames tests, respectively. Overall, the obtained results contribute to the concrete validation of the use of these medicinal plants as potential sources of antimicrobial agents in the treatment of skin diseases.

Keywords: antimicrobial activity; anthracene derivatives; *Asphodelus bento- rainhae*; *Asphodelus macrocarpus*; root tubers; toxicity

3.2.2. Introduction

Antimicrobial resistance (AMR) is a growing global healthcare problem due to the loss of efficacy of first-line antibiotics. Many pathogens are developing resistance to multiple drugs, making infections difficult or, in some cases, impossible to treat [153]. In response to the increasing demand for alternative medicines, the screening of natural products has emerged as one of the most successful methods for detecting/ identifying antibacterial agents. Although in recent decades, the majority of new antibacterial drugs were from natural sources [14], only a small fraction of marine, fungal and plant resources have been investigated, and nature still offers a high potential for drug-lead discovery, notably among anti-infective compounds [13].

In this context, ethnomedical knowledge has an important role in plant-derived drug discovery [18,154], and based on this information, the genus *Asphodelus* L. belonging to the family *Asphodelaceae* is referred to as one of the most promising sources of medicinal plants [36]. Root tubers of *Asphodelus* species have been traditionally used for the treatment of skin-related disorders and infections such as wounds, eczema, alopecia and psoriasis [36]. Furthermore, ethnomedical information on several *Asphodelus* species, supported by *in vitro* and *in vivo* biological activity studies, indicates their strong antimicrobial potential [45,46,74,91,94,96,97], particularly against resistant pathogens, due to the presence of secondary metabolites such as anthraquinones, arylcoumarins, terpenoids and naphthalene derivatives [21,60,64,65,67,73–75,77,116].

The Portuguese flora exhibits a considerable abundance of *Asphodelus* species, sub-species and varieties compared to the rest of Europe and the Mediterranean Basin. Besides the above-mentioned medical applications, root tubers are also used as daily food in the Iberian Peninsula, after being moistened and fried to eliminate the astringent compounds [36].

Asphodelus bento-rainhae subsp. *bento-rainhae* P. Silva is a vulnerable [26] endemic species from the Gardunha mountain range [25], located in the central region of Portugal, co-existing with *Asphodelus macrocarpus* subsp. *macrocarpus* Parlatore in

the same geographical area. Both species are commonly known by the Portuguese name “abrotea”, and their root tubers have traditionally been used for the treatment of skin diseases such as scabies, dermatophytosis and warts in Portugal.

The objective of this study is derived from the fact that although there are promising ethnomedical, phytochemical and biological data related to the *Asphodelus* species, to the best of our knowledge, no scientific studies on *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* root tubers have been documented so far. Thus, the present study aims to establish the chemical profiles of the potential antimicrobial constituents together with the pre-clinical safety evaluation and validation of the use of the studied plants as herbal medicines.

3.2.3. Materials and Methods

3.2.3.1. Chemical and Biological Reagents

2-aminoanthracene, 9-aminoacridine hydrochloride monohydrate, ammonium sodium phosphate dibasic tetrahydrate, benzo(*a*)pyrene, chlorogenic acid, chrysophanol, *d*-(+)-biotin, dimethyl sulfoxide/DMSO, glucose monohydrate, glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2-nitrofluorene and *tert*-butyl hydroperoxide/T-BHP were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anisaldehyde, l-histidine monohydrochloride monohydrate, magnesium sulfate heptahydrate, methanol and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Caffeic acid, ferulic acid and vanillic acid were acquired from Extrasynthese (Genay, France). Citric acid monohydrate, di-sodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate were purchased from PanReac AppliChem (Barcelona, Spain). Sodium chloride and di-potassium hydrogen phosphate were from Honeywell Fluka™ (Seelze, Germany). β-sitosterol and 2-aminoethyl diphenylborinate were obtained from Acros organics (Geel, Belgium). Bacto™ agar was acquired from Becton Dickinson & Co (Franklin Lakes, NJ, USA), ethanol was sourced from Carlo Erba Reagents (Val-de-Reuil, France),

glacial acetic acid came from Chem-Lab NV (Zedelgem, Belgium), polyethylene glycol 400/PEG was sourced from VWR Chemicals (Rosny-sous-Bois, France), sulfuric acid was acquired from PanReac AppliChem (Barcelona, Spain), sodium azide came from J.T. Baker Chemical Company (Phillipsburg, NJ, USA) and nutrient broth (NB) Nº 2 was sourced from Oxoid (Basingstoke, UK). Aroclor 1254-induced rat liver S9 was purchased from Trinova Biochem (GmbH, Giessen, Germany). In preparing all solutions, dilutions and culture media, ultra-pure water from a Milli-Q water purification system, Millipore (Molsheim, France), was used.

3.2.3.2. Plant Materials

Root tubers of *A. bento-rainhae* (AbRb) and *A. macrocarpus* (AmRb) were collected from Serra da Gardunha, Portugal, during root dormancy in November 2019. The corresponding voucher specimens were deposited in the Laboratory of Pharmacognosy, Department of Pharmacy, Pharmacology and Health Technologies, Faculty of Pharmacy, Universidade de Lisboa (voucher specimens: OSilva_201901-*A. bento rainhae* and OSilva_201902-*A. macrocarpus*). The collected samples were dried in a well-ventilated dark space at room temperature. The authors' previous monographic study give a more detailed description of both species' botanical identification and sample selections [155].

3.2.3.3. Preparation of Extracts

The collected samples were dried and extracted using the maceration method (with a mixture of ethanol/water 70% and 96%) at room temperature under agitation (3×, 24 h each). Hydroethanolic extracts were concentrated under reduced pressure using a rotary evaporator and freeze-dried. The obtained AbRb and AmRb 70% hydroethanolic extracts were then submitted to liquid–liquid partitioning (L-L), using solvents of increasing polarity (diethyl ether, ethyl acetate and water). Briefly, the extract (30 gr) was dissolved in mild water (300 mL) and poured into a clean

separatory funnel and diethyl ether (300 mL) added to the solution. The funnel stoppered and inverted. The stopcock was slowly opened to release any built-up pressure, and then closed. The separatory funnel was gently shaken to allow intimate mixing of the solutions and effect extraction of the compounds from the organic mixture The separatory funnel Clamped to a retort stand and allow the mixture to separate into two layers. The stopper was removed, and aqueous phase (the lower layer) got separated from the etheric phase (the upper layer) resulting in AbRb-1, AmRb-1 extracts. All the above steps were repeated 3 times and the same was applied to the ethyl acetate solvent and its subsequent extracts (AbRb-2, AmRb-2). Aqueous extracts (AbRb-3, AmRb-3) were obtained by collecting the residue of the previous extractions.

3.2.3.4. Chromatographic Analysis

Thin-layer chromatography (TLC) was performed using Silica gel 60 F_{254} and RP-18 F_{254} pre-coated plates (Merck®, Darmstadt, Germany). Anisaldehyde–sulfuric acid, natural product polyethylene glycol reagent (NP/PEG= NEU) and potassium hydroxide (KOH) 5% ethanolic solution were used as spray reagents for the detection of the secondary marker metabolites such as terpenoids, phenolic acids and anthracene derivatives, respectively [122].

High-performance liquid chromatography (HPLC) was carried out using a Waters Alliance 2690 Separations Module coupled with a Waters 996 autosampler and photodiode array detector (UV/DAD) (Waters Corporation, Milford, MA, USA). Crude extracts/L-L fractions (10 mg/mL, 25µL) were initially solubilized in acetonitrile/water, and standard solutions (1 mg/mL, 10µL) were prepared in acetonitrile and filtered through a polytetrafluoroethylene syringe filter (0.2 µm). An Atlantis RP-18 T3 column (5 µm, 150 × 4.6 mm) was used for the analysis of the injected samples with a flow rate of 1 mL/min. Water with 0.1% (*v/v*) formic acid (solvent A) and acetonitrile (solvent B) were used as the mobile phase, and gradients of 95% A: 5% B to 0% A: 100% B for a total run time of 75 min were used (See in the Table. 3.1.2., sub-section:

3.1.3.5.2). Chromatograms were monitored and registered on *Maxplot* (wavelength 240–650 nm), and the obtained data were analyzed using Waters Millennium® 32 Chromatography Manager Software (Waters Corporation, Milford, MA, USA).

Quantification of major marker metabolites was done by external standardization, using the respective standards, at the wavelengths of maximum absorption of the compounds. Standard curve was constructed with 10 different concentrations, covering the expected concentrations of the samples, each concentration being injected in triplicate. Linearity was evaluated by 3 injections of standard solutions at concentrations varying from 1 to 10 ppm (µg/mL). Limits of Detection (LOD) and Quantification (LOQ) were calculated as the concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Peak retention time and area were evaluated by 3 consecutive injections of the standard solutions.

Mass spectrometry (MS) analysis was conducted using the same HPLC equipment in tandem with a triple quadrupole mass spectrometer (Micromass® Quatro Micro™ API, Waters®, Drinagh, Ireland) using an electrospray ionization source (ESI) operating in both positive and negative mode.

A LiChrospher 100 RP-18 (5 µm) 250x4 mm column with respective pre-column (Merck, Germany) was used. A mixture of water with 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) was used as the mobile phase (Table 3.2.1). Data were acquired and analyzed using MassLynx™ V4.1 software (Waters®, Drinagh, Ireland).

Table 3.2.1. LC-UV/DAD mobile phase (gradient mode) for MS analysis.

Time (min)	%H_2O+0.1% HCOOH	% CH_3CN	Flow (mL/min)
0	95	5	1,0
20	80	20	1,0
60	50	50	1,0
90	0	100	1,0
100	0	100	1,0
101	95	5	1,0
120	95	5	1,0

Abbreviations: H_2O - water; HCOOH- formic acid; CH_3CN- acetonitrile.

3.2.3.5. Isolation and Identification of the Main Marker Compounds

Column chromatographic technique (CC) was used for the secondary fractionation of the extracts (AbRb-1) and isolation of the bioactive compounds. The adsorbent was suspended in the first eluent solvent and this suspension carefully added to the glass column that was previously half filled with the solvent used.

The column was allowed to stand 2-3 hours prior to sample application. Immediately before sample application, the solvent inside the column was reduced to the minimum necessary to keep the adsorbent wet and the samples then applied on the top of the packed column. Sample was dissolved in the minimum volume of the eluent solvent used and the solution carefully applied. In the beginning of elution, small amounts of eluent solvent were added to the column till all the sample was adsorbed to the column and no more dissolved in the eluent. Bigger volumes of eluent were then added to the column. Elution was made at a slow and constant rate of about 1 mL/min.

For the isolation of the main compounds of the most active extract, C_{18}-reversed phase silica gel, 90 A pore size, Sigma-Aldrich (St. Louis, MO, USA) and Sephadex LH20 (25-100) µm, Honeywell Fluka™ (Seelze, Germany) were used. The isolation scheme of the main isolated compounds from the diethyl ether fraction (AbRb-1) is shown in Figure 3.2.1.

1200 mg of the active extract (AbRb-1) was applied to the Sephadex LH-20 column. Several fractions were collected and concentrated through evaporation of the solvent. Then, the TLC control of the fractions was performed on silica gel 60 RP-18 plates using an H_2O: MeOH (0.5:19.5, *v/v*) solvent system and screened under UV_{254} and UV_{366}. Fractions with similar profiles were mixed, and the collected fractions were bulked into six main fractions: AbRb-1a (665 mg), AbRb-1b (60 mg), AbRb-1c (255 mg), AbRb-1d (117 mg), AbRb-1e (71 mg) and AbRb-1f (29 mg).

Compounds **p**, **q**, **r**, **s** and **t** were purified using a C18 reversed-phase silica gel column eluted with MeOH: H_2O (90:10). The identification of compounds was based on co-

chromatographic techniques and the obtained data related to the retention times, ultraviolet absorption and mass spectral characteristics recorded using LC-UV/DAD-ESI/MS, together with their TLC characteristics in comparison to those of standards and published data.

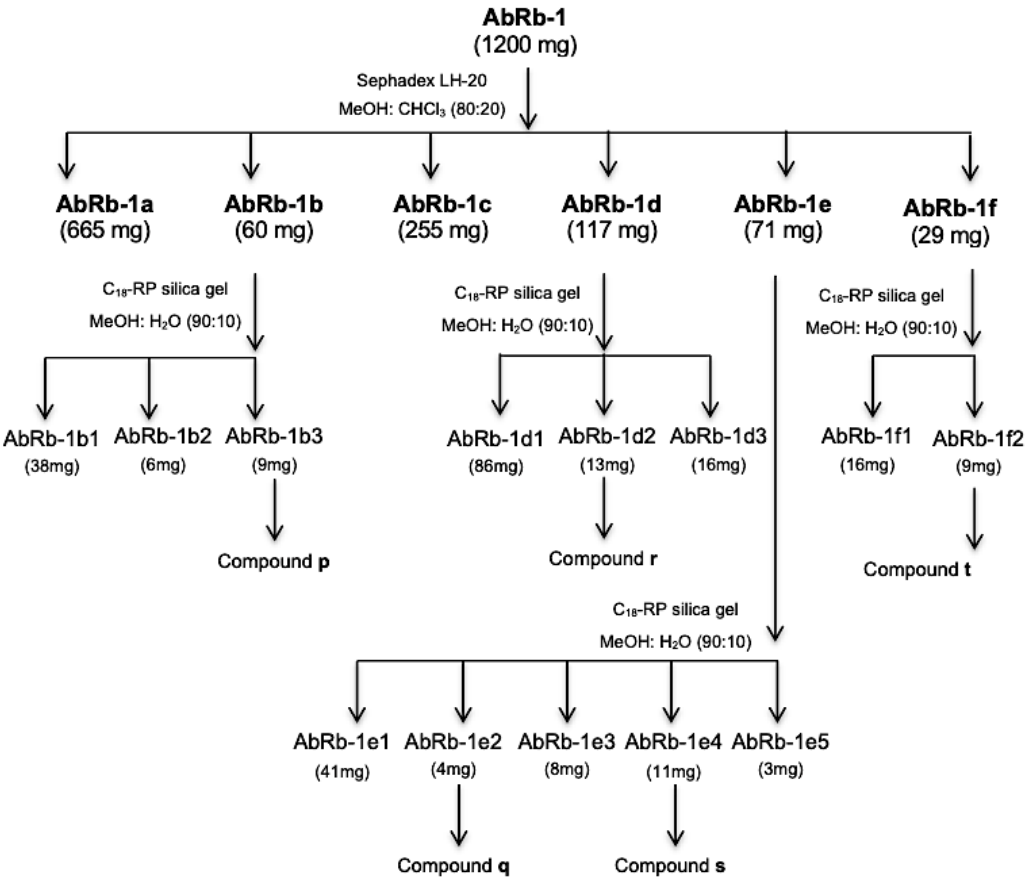

Figure 3.2.1. Isolation scheme of the main constituents of AbRb-1.

3.2.3.6. *In vitro* Antimicrobial Activity

The broth microdilution method was used for an *in vitro* evaluation of the antibacterial potential [156], using 96-well tissue culture plates (VWR®, Radnor, PA, USA) to determine the minimum inhibitory concentrations (MIC) of the tested

samples against twelve reference (ATCC, LGC Standards S.L.U., Barcelona, Spain) and clinical strains (INSA clinical strains collection) of both Gram-positive (*Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*) (Table 3.2.2) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Acinetobacter baumannii*) multidrug-resistant skin-related bacteria.

Table 3.2.2. Composition of the Gram-positive pathogen panel under study.

Bacteria (Gram +)	Demonstration of resistance to the antibiotics											
	CIP	DAP	ERY	FA	FOX	GN	LNZ	OXA	PEN	TEI	TET	VAN
S. aureus ATCC 29213								S	MS			
S. aureus CQINSA4923	R		R	S	R	R	S	R	R	S	S	S
S. aureus INSArefV					R					R		R
S. aureus INSA936		R										
S. aureus INSA896	R			R	R		R					
S. saprophyticus INSA842			R	R								
S. saprophyticus INSA867										R		
S. epidermidis INSA796	R				R		R			R		
S. epidermidis INSA958							R			R		
S. epidermidis INSA960										R		
S. haemolyticus INSA982	R				R					R		
S. haemolyticus INSA984	R	R			R							

Abbreviations: ATCC: American Type Culture Collection, INSA: Instituto Nacional de Saúde clinical strains collection, CIP: ciprofloxacin, DAP: daptomycin, ERY: erythromycin, FA: fusidic acid, FOX: cefoxitin, GEN: gentamicin, LNZ: linezolid, OXA: oxacillin, PEN: penicillin, TEI: teicoplanin, TET: tetracycline, VAN: vancomycin, MS: methicillin-susceptible, R: resistant, S: susceptible.

Samples to be tested were initially prepared in water or DMSO 10%, and serial dilutions (2–2000 µg/mL for crude extracts/fractions and 0.2–200 for pure compound) were performed in a Mueller–Hinton medium and were distributed (50 µL) in each of the microplate wells using a microplate liquid handler (Precision^{TM} BioTek, Winooski,

VT, USA). Inoculums were prepared from a pure bacterial culture on agar, and suspensions with a turbidity of 0.5 for Gram-negative and 0.25 for Gram-positive bacteria on the McFarland scale (Grant Bio™ DEN-1B, Cambridge, UK) were prepared in Mueller–Hinton medium and stored at 4 °C until use. For MIC determination, the prepared suspensions were diluted at a ratio of 1:10, and 50 µL of this dilution was added to all the wells.

To verify the absence of contamination and to check the viability of the inoculum, two controls were included for each tested sample (crude extract, fraction, or compound), one plate in the absence of the extract solution and the other in the presence of the solvent (DMSO). After incubation at 37 ºC for 18 hours, the plates were read in a lighted place and the MIC was determined. As previously described, all experiments were carried out in triplicate to obtain consistent values.

3.2.3.7. *In vitro* Cytotoxicity Evaluation using MTT Assay

In vitro cytotoxicity evaluation was performed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) viability assay [157] on the human liver cell line HepG2 (ATCC, Cat. No. HB-8065, Manassas, VA, USA) and the human spontaneously immortalized keratinocyte cell line HaCaT (CLS, Cat. No. 300493, Eppelheim, Germany).

HepG2 and HaCaT were inoculated at a density of 8.5×10^4 cells/cm^2 in a Alpha Minimum Essential Medium (α-MEM, Sigma-Aldrich®, St. Louis, MO, USA) with 1 mM sodium pyruvate (PAN Biotech, Aidenbach, Germany), 1% non-essential amino acids (NEAA; PAN Biotech) and 10% fetal bovine serum (FBS, Gibco® Thermo Fisher Scientific™ (Waltham, MA, USA), and of 4.0×10^4cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich®) with 4 g/L *D*-(+)-glucose (AppliChem, Darmstadt, Germany) and 10% fetal bovine serum (FBS, Gibco® Thermo Fisher Scientific™ (Waltham, MA, USA), respectively.

Both cell lines were maintained in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. After 48h, medium was replaced by fresh medium with extracts at final concentrations of 25, 50, 75, 100, and 125 µg/mL or pure compound at final concentrations of 2.5, 5, 7.5, 10, and 12.5 µg/mL.

Complete cell culture medium, DMSO 1% and DMSO 20% in *α*-MEM or DMEM were used as a positive, solvent, and negative control, respectively. After 48 h, cells were washed with 100 µL PBS and 200 µL 0.5 mg/mL MTT (Sigma-Aldrich®) was added to the cell culture medium. HepG2 and HaCaT were incubated for 3 h and 2 h, respectively, in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. Next, 200 µL DMSO was used for solubilizing the formed purple crystals prior to measuring absorbance at 570 nm using a microplate spectrophotometer (SPECTROstar Omega; BMG LabTech, Ortengerg, Germany). The results are expressed as a percentage relative to the solvent control. Four wells were used for each sample, and at least two independent experiments were performed.

Data analysis was performed by Chi Square test and graphs were plotted using GraphPad Prism® software (version 9.0.0.121, GraphPad Software, San Diego, CA, USA). The results are presented as mean ± standard deviation with $p < 0.05$ considered as significant.

3.2.3.8. *In vitro* Genotoxicity/Mutagenicity Evaluation using Ames Test

The screening of the genotoxicity potential was performed using a bacterial reverse mutation test (the Ames test) for the detection of genotoxic carcinogens and relevant genetic changes. The technique was conducted following the OECD No. 471 [129] and ICH S2 (R1) [130] guidelines as well as the published reference protocols [128,147].

Salmonella enterica serovar Typhimurium tester strains (TA98, TA100, TA102, TA1535 and TA1537) were used in this study (with and without metabolic activation) in a direct plate incorporation method. TA100, TA98, TA102 and TA1535 were kindly provided by the Genetic Department of the Nova Medical School of the Universidade

Nova de Lisboa (Portugal), having received them from Professor B.N. Ames (Berkeley, CA, USA). TA1537 was obtained from ATCC, NUMBER: 29630™, LOT: 7405375.

S9 mix (10%, *v/v* rat liver S9, 0.4 M $MgCl_2$, 1.65 M KCl, 1 M glucose-6-phosphate, 0.1 M nicotinamide adenine dinucleotide phosphate and 0.2 M sodium phosphate buffer, pH 7.4) was freshly prepared and kept on ice throughout the experiment.

The AbRb 96% hydroethanolic extract (50 mg/mL) was initially dissolved in DMSO, and 100 µL of the extract dilutions was mixed with 500 µL sodium phosphate buffer (0.1 M, pH 7.4) (in the assay without metabolic activation) or S9 mix (in the assay with metabolic activation). Then, 100 µL of the bacterial culture and 2 mL of melted top-agar, supplemented with 0.05 mM biotin and histidine, were added to the mixture. After a 48h incubation at 37 °C, manual counting of His+ revertant colonies for each concentration was performed. The results are expressed as the mean number of revertant colonies with the standard deviation (mean ± SD).

The positive controls were sodium azide (SA, 1.5 µg/plate for TA100 and TA1535), 2-nitrofluorene (2-NF, 5 µg/plate for TA98), 9-aminoacridine (9-AA, 100 µg/plate for TA1537) and tert-butyl hydroperoxide (tBHP, 50 µg/plate for TA102) in the assay without metabolic activation, and 2-aminoathracene (2-AA, 2 µg/plate for TA98 and 10 µg/plate for TA102, TA1535 and TA1537) and benzo(*a*)pyrene (BaP, 5 µg/plate for TA100) in the assay with metabolic activation. All assays were performed in triplicate to obtain consistent values.

3.2.4. Results and Discussion

3.2.4.1. Drug–Extract Ratio (DRE)

The drug–extract ratio was calculated as 1.9: 1 and 5.5: 1 for the *A. bento-rainhae* root tuber (AbRb) and 2.7: 1 and 6.7: 1 for the *A. macrocarpus* root tuber (AmRb) 70% and 96% hydroethanolic extracts, respectively. Considering these results, AbRb exhibited a higher percentage of yield in both hydroethanolic extracts compared to the AmRb

extracts. Moreover, extraction with ethanol 96% noticeably reduced the percentage of yield in both species.

3.2.4.2. Bioguided Phytochemical Analysis

3.2.4.2.1. Phytochemical Screening and Antimicrobial Activity

Hyphenated analytical techniques were applied for the phytochemical dereplication of the samples. Following our previous study's results [155], the chromatographic profiles of AbRb and AmRb extracts showed excellent qualitative similarity in their chemical composition, characterized by the presence of terpenoids, phenolic acids and anthracene derivatives. Therefore, in continuation of the above-mentioned study searching for potent antimicrobial metabolites from these Portuguese *Asphodelus* species, liquid–liquid fractionations of both plant extracts with increasingly polar solvents, namely diethyl ether (AbRb-1, AmRb-1), ethyl acetate (AbRb-2, AmRb-2) and water (AbRb-3, AmRb-3), were performed. Both species' crude extracts and their subsequent L-L fractions were then submitted to *in vitro* antimicrobial evaluation in order to select the most active fractions for further phytochemical identification of their lead secondary metabolites.

The antimicrobial activity of the crude extracts, their derived L-L fractions and isolated compounds were evaluated through determination of the MIC values, an *in vitro* quantitative method of susceptibility testing against both selected Gram-positive and Gram-negative resistant pathogens.

As shown in Table 3.2.3., among all the tested samples, AbRb-1 and AmRb-1 were the only fractions that demonstrated prominent antibacterial activity against all the Gram-positive microorganisms (*Staphylococcus* spp. strains), with MIC values ranging from 16 to 1000 µg/mL. The AbRb-1 fraction exhibited stronger antibacterial activity, with about two-fold higher inhibition potential against *S. saprophyticus* INSA842, *S. epidermidis* INSA958, *S. epidermidis* INSA960, *S. haemolyticus* INSA982 and *S. haemolyticus* INSA984 and about three-fold stronger activity against *S. epidermidis*

INSA796 when compared to the AmRb-1 fraction. No activity (MIC > 2000 µg/mL) was found against Gram-negative microorganisms (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) in the tested range of concentrations (up to 2000 µg/mL).

Table 3.2.3. *In vitro* antimicrobial activity of crude extracts and their subsequent L-L fractions of *A. bento-rainhae* and *A. macrocarpus* root tubers against Gram-positive strains.

Bacteria (Gram +)	MIC (µg/mL)							
	AbRb 70%	**AmRb 70%**	**AbRb-1**	**AmRb-1**	**AbRb-2**	**AmRb-2**	**AbRb-3**	**AmRb-3**
S. aureus ATCC 29213	> 2000	> 2000	250	250	> 2000	> 2000	> 2000	> 2000
S. aureus CQINSA4923	> 2000	> 2000	125	125	> 2000	> 2000	> 2000	> 2000
S. aureus INSA790	> 2000	> 2000	250	250	> 2000	> 2000	> 2000	> 2000
S. aureus INSA936	> 2000	> 2000	250	250	> 2000	> 2000	> 2000	> 2000
S. aureus INSA896	> 2000	> 2000	125	125	> 2000	> 2000	> 2000	> 2000
S. saprophyticus INSA842	> 2000	> 2000	125	250	> 2000	> 2000	> 2000	> 2000
S. saprophyticus INSA867	> 2000	> 2000	500	1000	> 2000	> 2000	> 2000	> 2000
S. epidermidis INSA796	> 2000	> 2000	125	500	> 2000	> 2000	> 2000	> 2000
S. epidermidis INSA958	> 2000	> 2000	250	500	> 2000	> 2000	> 2000	> 2000
S. epidermidis INSA960	> 2000	> 2000	125	250	> 2000	> 2000	> 2000	> 2000
S. haemolyticus INSA982	> 2000	> 2000	16	32	> 2000	> 2000	> 2000	> 2000
S. haemolyticus INSA984	> 2000	> 2000	62	125	> 2000	> 2000	> 2000	> 2000

Abbreviations: AbRb 70%: *A. bento-rainhae* 70% hydroethanolic extract; AmRb: *A. macrocarpus* 70% hydroethanolic extract; AbRb-1, AmRb-1: diethyl ether fractions; AbRb-2, AmRb-2: ethyl acetate fractions; AbRb-3, AmRb-3: aqueous fractions; ATCC, American Type Culture Collection; INSA, Instituto Nacional de Saúde clinical strains collection; MIC, minimum inhibitory concentration.

Considering the previously reported results of the antimicrobial activity of *Asphodelus* spp. root tuber crude extracts and in agreement with the obtained results verified in our species, weak to moderate activities against a similar pathogen panel with MIC values higher than 2000 µg/mL were observed [91]. The methanolic root extracts of *A. luteus* and *A. microcarpus* showed antimicrobial potential against methicillin-resistant *S. aureus* (MRSA), with MIC values of 650 to 1250 and 1250 to 2500 µg/mL, respectively [94]. Screening of *A. microcarpus* tuber methanolic extract using an agar well diffusion assay revealed moderate activity against *S. aureus,* with an inhibition diameter zone of 14 mm [104]. Furthermore, the 80% hydromethanolic whole plant extract of *A. tenuifolius* was also found to be significantly active against *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumonia,* with inhibition diameter zones of 16, 29, 18 and 18 mm, respectively, determined using the disc diffusion method [96,97].

3.2.4.2.2. Isolation, Detection, Tentative Identification and Quantification of the Main Bioactive Marker Compounds

Following the obtained antimicrobial activity results, the AbRb-1 and AmRb-1 fractions were selected for further phytochemical characterization of their constituents. LC-UV/DAD-ESI/MS spectral data (Figure 3.2.2) confirmed the presence of phenolic acids, phenylpropanoids, anthracene derivatives and triterpenoids in both fractions.

Six compounds, namely chlorogenic acid (**b**, LC-UV/DAD: t_R: 11.7 min, λ_{max}: 241sh, 296sh, 326 nm), vanillic acid (**c**, LC-UV/DAD: t_R: 12.4 min, λ_{max}: 261, 292sh nm), caffeic acid (**d**, LC-UV/DAD: t_R: 12.8 min, λ_{max}: 240sh, 296sh, 324 nm), ferulic acid (**g**, LC-UV/DAD: t_R: 18.5 min, λ_{max}: 235sh, 296sh, 323 nm), isochlorogenic acid A (**h**, LC-UV/DAD: t_R: 20.4 min, λ_{max}: 242sh, 296sh, 326 nm) and *β*-sitosterol (**u**, TLC: R_f: 0.4), were identified as minor compounds based on co-chromatography techniques using authentic standards.

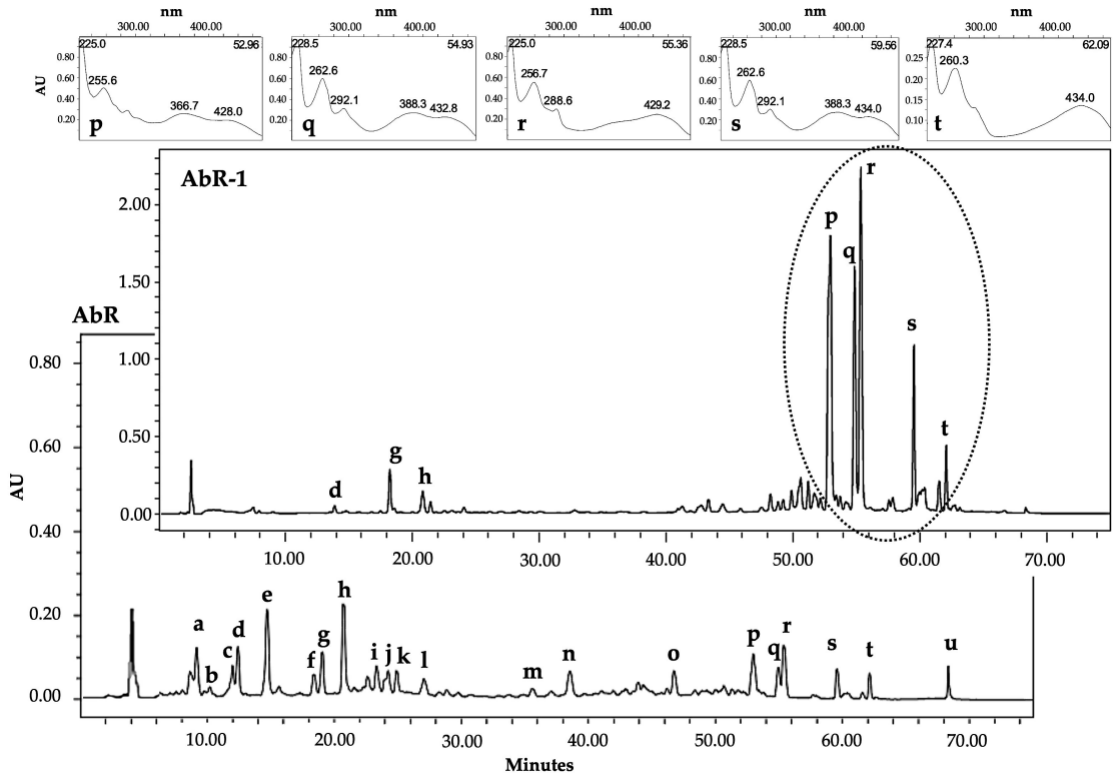

Figure 3.2.2. Comparative LC-UV/DAD *Maxplot* (210–600 nm) chromatographic profiles of *A. bento-rainhae* root tuber extract and its highly active diethyl ether L-L fraction.
Abbreviations: AbR, *A. bento-rainhae* root tuber extract; AbR-1, AbR diethyl ether fraction; The five main isolated compounds (**p** to **t**) and their UV spectra.

In order to separate, purify and identify the major marker constituents of both extracts and their active fractions, further phytochemical studies were conducted. The AbR-1 fraction was submitted to the column chromatography technique using reversed-phase Silica gel 90 C_{18} and Sephadex LH-20. The procedure resulted in the isolation of five anthraquinones in pure form (Figure 3.2.3).

Compound **p** was identified as 7'-(chrysophanol-4-yl)-chrysophanol-10'-*C*-beta-*D*-xylopyranosyl-anthrone, also known as 10'R)-1,1',8,8'-tetrahydroxy-10'-beta-*D*-xylopyranosyl-3,3'-dimethyl-4,7'-bianthracene-9,9',10(10'H)-trione, with empirical formula $C_{35}H_{28}O_{11}$ and PubChem CID:102153707, found as a reddish amorphous powder, with UV: λ_{max} (CH_3CN) 255, 288sh, 366, 428 nm and mass of 625 $[M + H]^+$ and fragments of 493 and 475 (*m/z*).

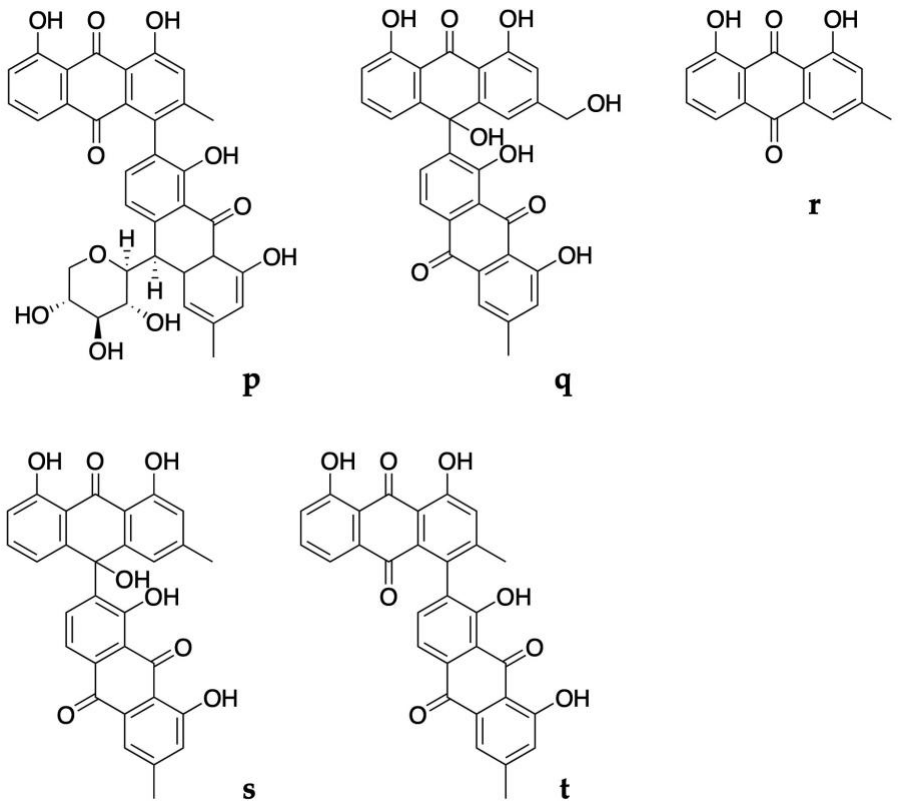

Figure 3.2.3. Main marker compounds of *A. bento-rainhae* and *A. macrocarpus* root tuber extracts. **Abbreviations:** **p**, 7′-(chrysophanol-4-yl)-chrysophanol-10′-*C*-beta-*D*-xylopyranosyl-anthrone; **q**, 10,7′-bichrysophanol; **r**, chrysophanol; **s**, 10-(chrysophanol-7′-yl)-10-hydroxychrysophanol-9-anthrone; **t**, asphodelin.

Compound **q** was identified as 10,7′-bichrysophanol (chrysalodin), with empirical formula $C_{30}H_{20}O_9$ and PubChem CID:13940829, found as orange crystals, with UV: λ_{max} (CH_3CN) 262, 292sh, 388, 432 nm, mass of 523 $[M-H]^-$ and prominent fragments of 269 and 253 (*m/z*). Compound **r** was identified as chrysophanol (chrysophanic acid), with empirical formula $C_{15}H_{10}O_4$ and PubChem CID:10208, found as orange crystals, with UV: λ_{max} (CH_3CN) 256, 288sh, 429 nm and mass of 253 $[M-H]^-$ (*m/z*). Compound **s** was identified as 10-(chrysophanol-7′-yl)-10-hydroxychrysophanol-9-anthrone, with empirical formula $C_{30}H_{20}O_8$ and PubChem CID:14584824 found as orange crystals, with UV: λ_{max}(CH_3CN) 262, 292sh, 388, 434 nm and mass of 507 $[M-H]^-$ and prominent fragments of 490 and 253 (*m/z*). Compound **t** was identified as asphodelin (4,7′-bichrysophanol), with empirical formula $C_{30}H_{18}O_8$ and PubChem CID: 182665, found

as dark red crystals with UV: λ_{max} (CH_3CN) 260, 290sh, 434 nm and mass of 505 $[M-H]^-$ and fragments of 488, 460 and 253 (*m/z*).

Chrysophanol, the major marker compound of the diethyl ether fraction of both species was quantified as 10 and 36 µg/mL for *A. bento-rainhae* root tuber 70% and 96% hydroethanolic extracts, respectively and 7 and 23 µg/mL for *A. macrocarpus* root tuber 70% and 96% hydroethanolic extracts, respectively, using a calibration curve with equation of Y= 137898x + 129785, R^2= 0.9924. The limit of detection was 1.25 µg/mL, and the limit of quantification was 3.35 µg/mL.

All the isolated compounds have been previously identified in *Asphodelus* species. For instance, chlorogenic acid, caffeic acid and vanillic acid were reported from an *Asphodelus ramosus* L. whole plant extract [84]. Chlorogenic acid was detected in the leaf extract of *Asphodelus aestivus* Brot. [61] and caffeic acid was reported from the flower extract of *A. ramosus* [80]. 7'-(chrysophanol-4-yl)-chrysophanol-10'-*C*-beta-*D*-xylopyranosyl-anthrone was also reported from the root tuber extract of *A. ramosus* [83]. 10,7'-bichrysophanol was identified as one of the main anthracene derivatives of *A. acaulis*, *A. albus* and *A. fistulosus* root extracts [60]. This compound was also reported from seed extracts of *A. microcarpus* [158].

Chrysophanol was found to be the most common anthracene derivative of the *Asphodelus* species, reported from *A. acaulis*, *A. albus*, *A. fistulosus* [60] and *A. microcarpus* [21] root and *A. albus* [59,63], *A. fistulosus* [43,67], *A. macrocarpus* subsp. *rubescens* [59] and *A. microcarpus* aerial parts extracts [67,71]. 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone was recorded from *A. microcarpus* [21] and *A. ramosus* [82] root and leaf [71] extracts. Asphodelin was found in *A. acaulis*, *A. albus* and *A. fistulosus* root extracts [60]. This compound was also detected in *A. microcarpus* root [21,73] and seed [158] extracts.

Since anthracene derivatives are considered the main secondary metabolites of *Asphodelus* species, the detected and identified compounds could effectively be used for the chemotaxonomic classification of both *A. bento-rainhae* and *A. macrocarpus* species [56,60]. *β*-sitosterol, a common phytosterol, was identified in root extracts of

A. albus, *A. microcarpus* and *A. tenuifolius* [37,64,78,86] and seeds extracts of *A. fistulosus* and *A. microcarpus* [68].

3.2.4.2.3. Antimicrobial Activity of the Major Marker Compounds and 96% Hydroethanolic Extracts of Both *Asphodelus* Root Tubers

The results for the antimicrobial activity of the five main isolated marker compounds of both diethyl ether L-L fractions (AbRb-1, AmRb-1) are presented in Table 3.2.4. Additionally, considering the chemical class and polarity of these compounds and in order to verify whether the activity of the total extract is relevant to the major or minor constituents, a less polar hydroethanolic extract (96%) of both species was also prepared and tested.

The AbRb 96% hydroethanolic extract was found to be the most active crude extract with higher contents of chrysophanol derivatives as its main marker metabolites in comparison to AmRb 96% and both species' 70% hydroethanolic extracts. This was also confirmed by the obtained results of chrysophanol quantification which is in accordance with fundamental role of these compounds in the antimicrobial activity exhibited by these medicinal plants.

All the tested compounds were found to be active against all the tested Gram-positive strains, particularly against *Staphylococcus epidermidis,* with MIC values between 3.2 and 100 μg/mL (Table 3.2.3). Among these strains, teicoplanin- and linezolid-resistant *S. epidermidis* INSA958 showed the highest susceptibility to all the tested compounds. Moreover, chrysophanol, the major marker compound of both species, showed remarkable activity (MIC: 3.2 μg/mL) against this strain, which is often found on the human skin and mucous membrane; however, according to hospital surveillance reports, the bacterium is a common cause of nosocomial wound infections. Similar to the obtained results of the tested crude extracts, no activity regarding these compounds was found against the tested Gram-negative microorganisms in the tested range of concentrations (up to 200 μg/mL).

Table 3.2.4. *In vitro* antimicrobial activity of 96% hydroethanolic extracts and the isolated marker compounds (**p** to **t**) of *A. bento-rainhae* and *A. macrocarpus* root tuber extracts against Gram-positive strains.

Bacteria (Gram +)	MIC (µg/mL)						
	AbRb 96%	AmRb 96%	p	q	r	s	t
S. aureus ATCC 29213	125	1000	25	100	100	100	200
S. aureus CQINSA4923	125	2000	100	100	200	100	100
S. aureus INSA790	500	> 2000	100	200	200	200	200
S. aureus INSA936	250	> 2000	100	200	200	200	200
S. aureus INSA896	250	> 2000	100	200	200	200	200
S. saprophyticus INSA842	500	> 2000	200	200	200	200	200
S. saprophyticus INSA867	1000	> 2000	200	200	200	200	200
S. epidermidis INSA796	500	> 2000	25	100	50	100	50
S. epidermidis INSA958	1000	2000	12.5	12.5	3.2	12.5	100
S. epidermidis INSA960	250	> 2000	12.5	100	100	100	100
S. haemolyticus INSA982	125	2000	6.25	200	100	200	100
S. haemolyticus INSA984	250	> 2000	6.25	200	200	200	200

Abbreviations: AbRb 96%, *A. bento-rainhae* root tuber 96% hydroethanolic extract; AmRb 96%, *A. macrocarpus* root tuber 96% hydroethanolic extract; ATCC, American Type Culture Collection; INSA, Instituto Nacional de Saúde clinical strains collection; MIC, minimum inhibitory concentration. **p**, 7´-(chrysophanol-4-yl)-chrysophanol-10´-*C*-beta-*D*-xylopyranosyl-anthrone; **q**, 10,7'-bichrysophanol; **r**, chrysophanol; **s**, 10-(chrysophanol-7'-yl)-10-hydroxychrysophanol-9-anthrone; **t**, asphodelin.

To the best of our knowledge, no data related to the resistant strains employed in this study have been reported; however, chrysophanol and its derivatives have been previously reported to have potential antibacterial activity against other *S. aureus* strains (MIC values of 90 to 190 µg/mL) [159]. So far, there has not been enough research to fully explain the antibacterial mechanism of these compounds. However, according to existing studies, one of the main mechanisms of action is the inhibition of cell wall synthesis and membrane function in Gram-positive bacteria, which are generally more sensitive to antibiotics and antimicrobial chemical compounds/herbal

drugs compared to Gram-negative bacteria [160]. The lipopolysaccharide layer and periplasmic space in Gram-negative bacteria are among the reasons of their relative resistance [161].

3.2.4.3. Pre-Clinical Safety Assessment

3.2.4.3.1. Evaluation of the Cytotoxicity Potential

The results of the *in vitro* cytotoxicity evaluations of the *A. bento-rainhae* (AbRb) 70% and 96% hydroethanolic extracts are presented in Figure 3.2.4.

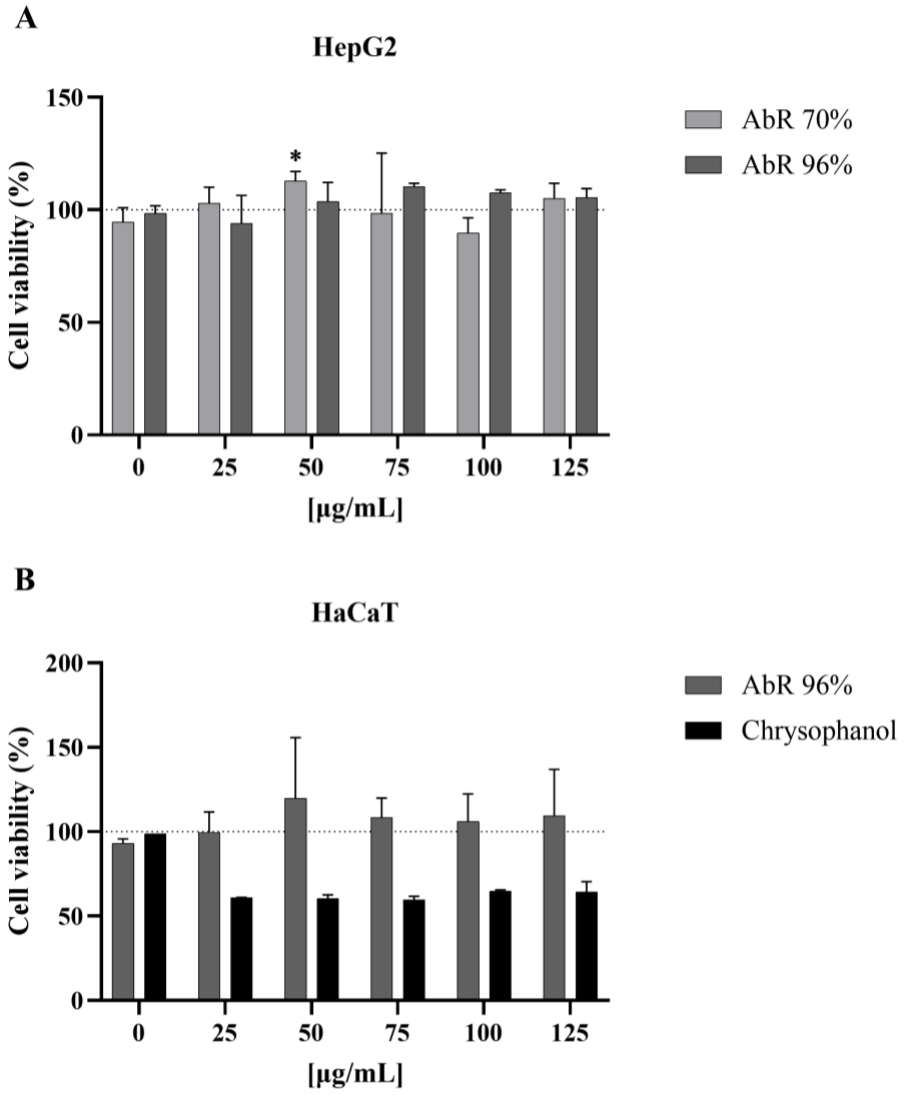

Figure 3.2.4. HepG2 (**A**) and HaCaT (**B**) cell viability after 48 h of incubation with *A. bento-rainhae* root tuber 70% and 96% hydroethanolic extracts and chrysophanol, evaluated by methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay. Data are shown as the percentage of solvent control (dashed line) and mean ± standard deviation; n = 2–5. * $p < 0.05$.

The analysis of these data obtained through the cell viability assay clearly showed that none of the tested extracts induced cytotoxicity in HepG2 cells. However, since this medicinal plant is commonly used for the treatment of skin disorders, we further assessed cytotoxicity using a skin cell type. For this, the AbRb 96% hydroethanolic extract, as the most active extract with the highest contents of marker secondary metabolites, and its major constituent, chrysophanol, were selected.

As previously observed with HepG2, the AbRb 96% extract did not reduce HaCaT viability, indicating its safe use through topical application at concentrations up to 125 µg/mL, but the same was not observed with chrysophanol, which reduced cell viability by up to 50% with concentrations higher than 25 µg/mL.

3.2.4.3.2. Evaluation of the Genotoxicity/Mutagenicity Potential

Although the negative results of the genotoxic/mutagenic potential of the root tuber 70% hydroethanolic extracts of both species were previously reported by the authors [155], as suggested by the guidelines [162], a genotoxicity assessment of different herbal preparations should be evaluated in order to reflect, as far as possible, the full spectrum of the extracted components. Additionally, since the AbRb 96% hydroethanolic extract exhibited the highest antimicrobial activity and quantity of the active secondary metabolites, it was selected for further genotoxicity/mutagenicity evaluations.

The obtained results of the Ames test for the AbRb 96% hydroethanolic extract are presented in Table 3.2.5. According to the genotoxicity guidelines [129,130], a mutagenic substance in the bacterial reverse mutation (Ames) test should exhibit a reproducible dose-related increase in the number of revertant colonies for at least one of the tester strains. Additionally, the number of revertant colonies must be more than twice the number of colonies produced on the negative (solvent) control plates. For cytotoxicity, a reduction in the number of revertants and/or clearing or diminution of the background lawn might be detected [146,163,164].

Table 3.2.5. Mutagenicity of *A. bento-rainhae* root tuber 96% hydroethanolic extract in the bacterial reverse mutation test (Ames test).

AbRb 96% µg/Plate	Number of Revertant Colonies Without Metabolic Activation, Mean (n = 3) ± Standard Deviation (SD)				
	TA98	TA100	TA102	TA1535	TA1537
500	39 ± 2	150 ± 3	319± 8	15 ± 5	17 ± 2
1000	37 ± 5	166 ± 8	306 ± 9	16 ± 3	18 ± 6
2000	38 ± 1	142 ± 6	305 ± 12	13 ± 3	22 ± 6
2500	42 ± 4	152 ± 9	301± 4	10 ± 1	24 ± 8
3750	45 ± 1	163 ± 8	327 ± 3	13 ± 3	24 ± 3
5000	49 ± 3	143 ± 11	320± 8	16 ± 1	20 ± 6
NC	38 ± 6	142 ± 2	320 ± 4	15 ± 2	20 ± 2
PC	2-NF	SA	tBHP	SA	9-AA
	488 ± 30	1048 ± 43	881 ± 26	827 ± 13	1354 ± 5
	Number of Revertant Colonies With Metabolic Activation (S9), Mean (n = 3) ± Standard Deviation (SD)				
1000	43 ± 1	145 ± 1	221 ± 6	12 ± 4	11 ± 4
2000	33 ± 3	147 ± 1	217 ± 5	12 ± 6	13 ± 2
4000	33 ± 4	162 ± 2	215 ± 5	11 ± 6	11 ± 3
5000	36 ± 1	159 ± 6	237 ± 2	13 ± 3	14 ± 1
NC	44 ± 8	157 ± 6	172 ± 2	11 ± 2	12 ± 1
PC	2-AA	BaP	2-AA	2-AA	2-AA
	832 ± 35	947 ± 148	732 ± 12	266 ± 1	306 ± 50

Abbreviations: AbRb 96%, *A. bento-rainhae* root tuber 96% hydroethanolic extract; Nd, not determined; NC, negative control/solvent control (DMSO 30%); PC, positive control reference; 2-NF, 2-nitrofluorene; SA, sodium azide; tBHP, *tert*-butyl hydroperoxide; 9-AA, 9-aminoacridine; 2-AA, 2-aminoathracene; BaP, benzo(*a*)pyrene.

The analysis of the results showed that none of the tested concentrations of this extract (up to 5000 µg/plate) enhanced the number of revertant colonies in any tested strains with or without metabolic activation compared to the negative control. Moreover, toxicity did not occur since none of the above-mentioned requirements

occurred at any tested concentration. Therefore, under the conditions of this study, the mutagenic potential essential to ensure the safety of these extracts was not observed.

Even though, there are studies indicating the genotoxic potential of chrysophanol in the Ames test with metabolic activation [165], the obtained negative results of tested chrysophanol (up to 200 μg/plate) without metabolic activation (S9) and the AbRb 96% hydroethanolic extract (with and without metabolic activation), showed that the presence of chrysophanol does not influence the genotoxicity of the crude extract. Insufficient amounts of the mutagenic constituents and their interactions in the extracts/complex mixtures are among the various theories that could explain this phenomenon. Additionally, human exposure to chrysophanol and its derivatives through AbRb 96% hydroethanolic extract is expected to be negligible, concerning the expected mode of administration (topical application), since they need to undergo bioactivation, mediated by different isoforms of cytochrome P 450, to become genotoxic [145].

Chapter 4

A. bento-rainhae and *A. macrocarpus* Leaves - Identification of Marker Compounds and *In vitro* Toxicity Evaluation

Main part of this chapter was published as follows with minor modifications:

Identification of Marker Compounds and *In vitro* Toxicity Evaluation of Two Portuguese *Asphodelus* Leaf Extracts

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4.1. Abstract

The leaves of *Asphodelus bento-rainhae* subsp. *bento-rainhae*, an endemic Portuguese species, and *Asphodelus macrocarpus* subsp. *macrocarpus* have been used as food, and traditionally as medicine, for treating ulcers, urinary tract and inflammatory disorders. The present study aims to establish the phytochemical profile of the main secondary metabolites, together with the antimicrobial, antioxidant and toxicity assessments of both *Asphodelus* leaf 70% ethanolic extracts. Phytochemical screenings were conducted by the TLC and LC-UV/DAD-ESI/MS chromatographic technique and quantification of the leading chemical classes was performed by spectrophotometric methods. Liquid-liquid partitions of crude extracts were obtained using ethyl ether, ethyl acetate and water. For *in vitro* evaluations of antimicrobial activity, the broth microdilution method, and for the antioxidant activity, the FRAP and DPPH methods were used. Genotoxicity and cytotoxicity were assessed by Ames and MTT tests, respectively. Twelve known compounds including neochlorogenic acid, chlorogenic acid, caffeic acid, isoorientin, *p*-coumaric acid, isovitexin, ferulic acid, luteolin, aloe-emodin, diosmetin, chrysophanol, and *β*-sitosterol were identified as the main marker compounds, and terpenoids and condensed tannins were found to be the major class of secondary metabolites of both medicinal plants. The ethyl ether fractions demonstrated the highest antibacterial activity against all the Gram-positive microorganisms, (MIC value of 62 to 1000 µg/mL), with aloe-emodin as one of the main marker compounds highly active against *Staphylococcus epidermidis* (MIC value of 0.8 to 1.6 µg/mL). Ethyl acetate fractions exhibited the highest antioxidant activity (IC_{50} of 800 to 1200 µg/mL, respectively). No cytotoxicity (up to 1000 µg/mL) or genotoxicity/mutagenicity (up to 5 mg/plate, with/without metabolic activation) were detected. The obtained results contribute to the knowledge of the value and safety of the studied species as herbal medicines.

Keywords:

Aloe-emodin; antimicrobial; antioxidant; *Asphodelus*; chemical profile; herbal medicines; preclinical safety; *Staphylococcus epidermidis*

4.2. Introduction

Medicinal plants have been used as potential functional foods or resources to prevent various diseases worldwide in different traditional medicine systems. Medicinal plants and their respective phytochemicals, mainly secondary metabolites, are used not only to combat specific nutrient deficiencies, but to sustain secure food and primary healthcare medicines [166].

The species *Asphodelus* L. (*Asphodelaceae*) is consumed in large quantities in the cuisines (e.g., soups, pastries, etc.) of several countries and cultures. The leaves of *Asphodelus aestivus* Brot., for instance, are commonly consumed as a cooked vegetable dish in Turkey, where they are known as "çiriş otu" [38]. In Puglia, on the southeast coast of Italy, burrata cheese is always wrapped in *Asphodelus ramosus* L. leaves to indicate the freshness of the cheese before it dries out [39]. In addition to their nutritional value, *Asphodelus* spp. leaves are widely used in traditional medicine to treat ulcers and urinary and inflammatory disorders [36]. In North African countries and the Iberian Peninsula, decoctions of leaves and stems have also been used to treat withering and paralysis [22,37]. Previously reported phytochemical studies of *Asphodelus* spp. extracts from leaves and aerial parts have revealed the presence of phenolic acids [61,72], flavonoids [37,49,51,61,66,72] and anthracene derivatives [43,59,61,63,67,71] as the main chemical classes of their marker secondary metabolites. Several *in vitro* and *in vivo* biological activities of *Asphodelus* spp. leaf and aerial parts extracts have been reported and documented for their antimicrobial [42,44,50,71,72,94] antioxidant [38,42,70,102,107] and antitumoral [70–72,93] activity.

Asphodelus bento-rainhae subsp. *bento-rainhae* P. Silva is an endemic species from Serra da Gardunha and is considered as "vulnerable" on the Red List of Threatened Species of the International Union for the Conservation of Nature (IUCN) and co-exists with *Asphodelus macrocarpus* subsp. *macrocarpus* Parl. in the same geographical area. They are known by the common Portuguese name "abrotea" (Ancient Greek: Ἀβρότονον), and their leaf is used as fertilizer and fodder in Portugal [155]. To date,

no data related to the phytochemical characterization, pre-clinical safety, and biological potential of *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* leaves have been found in the literature. Therefore, the present study was conducted to identify the main chemical constituents, antimicrobial and antioxidant activities of leaf extracts of these species along with their *in vitro* toxicity assessments, using samples collected at different times of the year to determine the most appropriate period for the collection of material and to contribute to the knowledge of safety and their value as herbal medicinal products.

4.3. Materials and Methods

4.3.1. Chemical and Biological Reagents

Acetone, aluminum chloride, 2-aminoanthracene, 9-aminoacridine hydrochloride monohydrate, ammonium sodium phosphate dibasic tetrahydrate, ascorbic acid, benzo(*a*)pyrene, chlorogenic acid, chrysophanol, *d*-(+)-biotin, dimethyl sulfoxide/ DMSO, 2,2-diphenyl-1-picrylhydrazyl/DPPH, gallic acid, glucose monohydrate, glucose-6-phosphate, diosmetin, neochlorogenic acid, nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2-nitrofluorene, *tert*-butyl hydroperoxide/T-BHP, 2,4,6-tris(2-pyridyl)-*s*-triazine/TPTZ and vanillin were obtained from Sigma-Aldrich (St. Louis, MO, USA). *p*-Anisaldehyde, ferric chloride hexahydrate, hydrochloric acid, l-histidine monohydrochloride monohydrate, magnesium acetate tetrahydrate, magnesium sulfate heptahydrate, methanol, perchloric acid, potassium iodate, sodium acetate trihydrate, sodium carbonate, sodium hydroxide, and sodium nitrite were purchased from Merck (Darmstadt, Germany). Aloe-emodin, caffeic acid, (+)-catechin, ferulic acid, isoorientin, isovitexin, luteolin, oleanolic acid, *p*-coumaric acid and rhein were acquired from Extrasynthese (Genay, France). Citric acid monohydrate, di-sodium hydrogen phosphate dihydrate, and sodium dihydrogen phosphate monohydrate were purchased from PanReac AppliChem (Barcelona, Spain). Sodium chloride and di-potassium hydrogen phosphate were from Honeywell

Fluka™ (Seelze, Germany). β-sitosterol and 2-aminoethyl diphenylborinate were obtained from Acros organics (Geel, Belgium). Bacto™ agar was acquired from Becton Dickinson & Co (Franklin Lakes, NJ, USA), *n*-butanol came from Thermo Fisher Scientific™ (Waltham, MA, USA), ethanol (CH_3CH_2OH) was sourced from Carlo Erba Reagents (Val-de-Reuil, França), ferrous sulfate heptahydrate came from M&B laboratory chemicals (Dagenham, UK), Folin-Ciocalteu was acquired from Biochem chemopharma (Cosne-Cours-sur-Loire, France), glacial acetic acid came from Chem-Lab NV (Zedelgem, Belgium), polyethylene glycol 400/PEG was sourced from VWR Chemicals (Rosny-sous-Bois, France), sulfuric acid (H_2SO_4) was acquired from PanReac AppliChem (Barcelona, Spain), sodium azide came from J.T. Baker Chemical Company (Phillipsburg, NJ, USA) and nutrient broth (NB) Nº 2 was sourced from Oxoid (Basingstoke, UK). Aroclor 1254-induced rat liver S9 was purchased from Trinova Biochem (GmbH, Giessen, Germany). In preparing all solutions, dilutions, and culture media, ultra-pure water from a Milli-Q water purification system, Millipore (Molsheim, France), was used.

4.3.2. Plant Materials

The leaves of *A. bento-rainhae* (AbL) and *A. macrocarpus* (AmL) were collected from Serra da Gardunha, Portugal, first at the early flowering stage (AbLa, AmLa) in Spring, and then for the second time (AbLb, AmLb), during the Summer of 2019. All samples were dried in a well-ventilated dark space at room temperature. Corresponding voucher specimens were deposited in the Laboratory of Pharmacognosy, Department of Pharmacy, Pharmacology and Health Technologies, Faculty of Pharmacy, Universidade de Lisboa (Voucher specimens' number: OSilva_201901- *A. bento rainhae* and OSilva_201902- *A. macrocarpus*).

4.3.3. Preparation of Extract

Powder of the dried samples was obtained by grinding, and extraction was performed using the maceration method (1:10 with a mixture of ethanol/water 70% and 96%) under agitation and filtration (3×, 24 h each). Hydroethanolic extracts were evaporated under reduced pressure at a temperature of less than 40 °C using a rotary evaporator and subsequently freeze-dried. The selected extracts (AbLa, AmLa 70%) were then submitted to liquid-liquid partitioning (L-L), generating the diethyl ether (AbLa-1, AmLa-1), ethyl acetate (AbLa-2, AmLa-2), and aqueous (AbLa-3, AmLa-3) fractions.

4.3.4. Chromatographic Conditions

Silica gel 60 F_{254} and 60 RP-18 F_{254} pre-coated plates (Merck®, Darmstadt, Germany) were used for TLC screenings. Different spray reagents [122], including anisaldehyde–sulfuric acid for the detection of terpenoids, natural product polyethylene glycol reagent (NP/PEG = NEU) for the detection of phenolics, and potassium hydroxide (KOH) 5% ethanolic solution for the detection of anthracene derivatives were used.

A LC-UV/DAD analysis was performed using a Waters Alliance 2690 Separations Module (Waters Corporation, Milford, MA, USA) coupled with a Waters 996 photodiode array detector (UV/DAD) (Waters Corporation, MA, USA). An Atlantis T3 column, RP-18 end-capped (5 µm, 150 × 4.6 mm), connected to a pre-column with the same stationary phase was used. The injection volume was 25 µL with a flow rate of 1 mL/min. A mixture of water + 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used as the mobile phase, and gradients (95% A and 5% B), 20 min (71% A and 29% B), 30 min (67% A and 33% B), 35 min (64% A and 36% B), 45 min (50% A and 50% B), 65 min (100% B) and 75 min (95% A and 5% B) were applied.

Crude extracts (20 mg/mL) were solubilized in water and standard solutions were prepared in acetonitrile (1 mg/mL) and filtered through a polytetrafluoroethylene syringe filter (0.2 µm). Data were collected and analyzed using a Waters Millennium®

32 Chromatography Manager (Waters Corporation, Milford, MA, USA). The chromatogram was monitored and registered on *Maxplot* wavelength (240–650 nm).

Quantification of major marker compounds was done by external standardization, using the respective standards, at the wavelengths of maximum absorption of the compounds. Standard curves were constructed with 10 different concentrations, covering the expected concentrations of the samples, each concentration being injected in triplicate. Linearity was evaluated by 3 injections of standard solutions at concentrations varying from 1 to 10 ppm (μg/mL). Limits of Detection (LOD) and Quantification (LOQ) were calculated as the concentrations that gave signal-to-noise ratios of 3 and 10, respectively. Peak retention time and area were evaluated by 3 consecutive injections of the standard solutions.

A LC-UV/DAD-MS/ESI analysis was carried out using an HPLC (Waters Alliance 2695), with an autosampler and photodiode array detector (Waters PDA 2996) in tandem with a triple quadrupole mass spectrometer (Micromass®Quatro Micro™ API, Waters®, Drinagh, Ireland) using an electrospray ionization source (ESI) operating in negative mode. A LiChrospher 100 RP-18 (5 μm) 250 × 4 mm column with respective pre-column (Merck, Darmstadt, Germany) was used. A mixture of water + 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used as the mobile phase (Table 3.2.1.). Data were acquired and analyzed using MassLynx™ V4.1 software (Waters®, Drinagh, Ireland). Peaks assignment and the identification of compounds were based on a co-chromatography technique with the comparison of retention times and spectral data with those of standards and published data.

4.3.5. Quantification Assays for Determination of the Main Classes of Secondary Metabolites

Total phenolic content (TPC) of the 70% hydroethanolic crude extracts was determined using the Folin-Ciocalteu method [123], and an increasing gallic acid calibration curve (10–70 μg/mL) was used to obtain the standard equation of Y =

0.0087X + 0.0264, R^2 = 0.994. Total flavonoid content (TFC) was obtained following the method by Olivera et al., 2008 [124] and catechin concentrations (50–200 µg/mL) were used to obtain a standard curve with an equation of Y = 0.0039X + 0.027, R^2 = 0.993. Total triterpenoid content (TTC) was assessed using the procedure developed by Chang & Lin, 2012 [127], and oleanolic acid concentrations (100–800 µg/mL in methanol) were used to obtain a standard curve with an equation of Y = 0.0012X + 0.0849, R^2 = 0.994. For the determination of the total condensed tannins (TCTC) [123], catechin concentrations (200–2000 µg/mL) were used to obtain a standard curve with an equation of Y = 0.0002X + 0.0324, R^2 = 0.981 and for quantification of total hydrolysable tannins (THTC) [125], gallic acid concentrations (100–600 µg/mL) was used to obtain a standard curve with an equation of Y = 0.001X + 0.054, R^2 = 0.977. Total anthraquinones content (TAC) was evaluated according to the method described by Sakulpanich & Gritsanapan, 2008 [126], and rhein concentrations (3–18 µg/mL) were used to obtain a standard curve with an equation of Y = 0.0215X−0.0016, R^2 = 0.998.

All of the above-mentioned colorimetric techniques were assessed in triplicate for method validation, and a UV-Vis spectrophotometer (Hitachi, U–2000, Tokyo, Japan) was used. Values were obtained using standard equations (where X was the concentration of standard equivalents expressed as milligrams per gram of dried extract and Y was the measured absorbance). All of the obtained data were treated statistically by a one-way analysis of variance (ANOVA) with the *Asphodelus* species as the source of variance. Once both of the *Asphodelus* species were collected in two different seasons, the obtained data were also analyzed by ANOVA, with the season as the source of variance. The significant value was set for a *p*-value < 0.05.

4.3.6. *In vitro* Antimicrobial Activity

The antibacterial assay was carried out by the broth microdilution method [156], in 96-well tissue culture plates (VWR®, Radnor, PA, USA) to determine the activities by testing minimum inhibitory concentrations (MIC) of extracts against twelve reference

(ATCC, LGC Standards S.L.U., Barcelona, Spain) and clinical strains (INSA clinical strains collection) of both Gram-positive (*Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*) (Table 4.1) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*) bacteria representing the skin-related antimicrobial resistance status.

Samples to be tested were initially prepared in water or DMSO 10% and were screened at the concentration of 2–2000 µg/mL for crude extracts or L-L partitions and 0.2–200 for pure compounds. Serial dilutions were performed in a Mueller-Hinton medium and were distributed (50 µL) in each of the microplate wells using a microplate liquid handler (Precision^{TM} BioTek, Winooski, VT, USA).

Table 4.1. Composition of the Gram-positive pathogen panel under study.

Bacteria (Gram +)	Demonstration of resistance to the antibiotics											
	CIP	**DAP**	**ERY**	**FA**	**FOX**	**GN**	**LNZ**	**OXA**	**PEN**	**TEI**	**TET**	**VAN**
S. aureus ATCC 29213								S	MS			
S. aureus CQINSA4923	R		R	S	R	R	S	R	R	S	S	S
S. aureus INSArefV					R					R		R
S. aureus INSA936		R										
S. aureus INSA896	R			R	R		R					
S. saprophyticus INSA842			R	R								
S. saprophyticus INSA867										R		
S. epidermidis INSA796	R				R		R			R		
S. epidermidis INSA958							R			R		
S. epidermidis INSA960										R		
S. haemolyticus INSA982	R				R					R		
S. haemolyticus INSA984	R	R			R							

Abbreviations: ATCC: American Type Culture Collection, INSA: Instituto Nacional de Saúde clinical strains collection, CIP: ciprofloxacin, DAP: daptomycin, ERY: erythromycin, FA: fusidic acid, FOX: cefoxitin, GEN: gentamicin, LNZ: linezolid, OXA: oxacillin, PEN: penicillin, TEI: teicoplanin, TET: tetracycline, VAN: vancomycin, MS: methicillin-susceptible, R: resistant, S: susceptible.

For the preparation of inoculum from a pure bacterial culture on agar, a suspension in Mueller-Hinton medium (10^8 CFU/mL) with a turbidity of 0.5 for Gram-negative and 0.25 for Gram-positive bacteria on the McFarland scale (Grant Bio™ DEN-1B, Cambridgeshire, UK) were prepared and stored at 4 °C until use.

For MIC determination, the prepared suspensions were diluted at a ratio of 1:10, and from this dilution, 50 µL was added to all the wells. Two controls were included for each extract, fraction, or compound, one plate in the absence of the extract solution and the other in the presence of the solvent (DMSO), to verify the absence of contamination and to check the validity of the inoculum. After incubation at 37 °C for 18 h, the plates were read in a lighted place, and the MIC was determined. All experiments were carried out in triplicate, as previously described, to obtain consistent values.

4.3.7. *In vitro* Antioxidant Activity

The antioxidant potential was determined by two methods (Figures 4.1 and 4.2), initially started by a modified free radical scavenging activity (DPPH method) [167]., followed by the ferric reducing antioxidant power test (FRAP assay). DPPH solution (3.9 mL, 6×10^{-5} M in methanol) was mixed with 100 µL of diluted extracts or standard (ascorbic acid). After 30 min of incubation at room temperature, the absorbance of samples and standard solutions was measured at 517 nm.

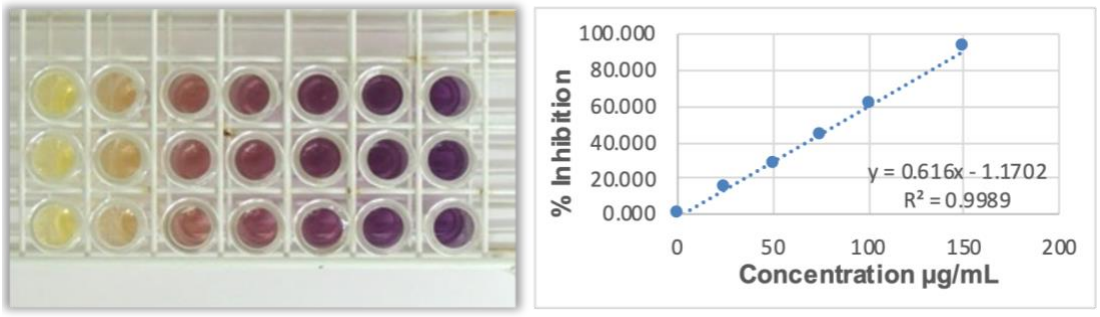

Figure 4.1. Colour changes of ascorbic acid at different concentration levels and its calibration curve as standard in determination of the free radical scavenging activity.

The percentage of DPPH free radical scavenging activity was calculated using the following formula: % scavenging = [absorbance of control−absorbance of test sample/absorbance of control] × 100. Results were expressed as mean ± standard deviation and presented in inhibitory concentration (IC_{50} value), representing the sample concentration required to scavenge 50% of the DPPH free radicals.

For the Frap assay [168], 100 µL of plant extracts (1000–5000 µg) were mixed with 3 mL of working FRAP reagent (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$. $6H_2O$ in the ratio of 10:1:1 at the time of use); thereafter, samples were placed in the water bath at 37 °C. The reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) can be monitored by measuring the change in absorption at 593 nm, measured after 4 min. Ascorbic acid concentrations (25–175 µg/mL) were used to obtain a standard curve with an equation of Y = 0.0031X−0.0398, R^2 = 0.994. The FRAP reagent was used as a blank, and results were expressed as mmol ascorbic acid/g dry extract. Values were obtained in three sets of experiments and assessed in triplicate for method validation.

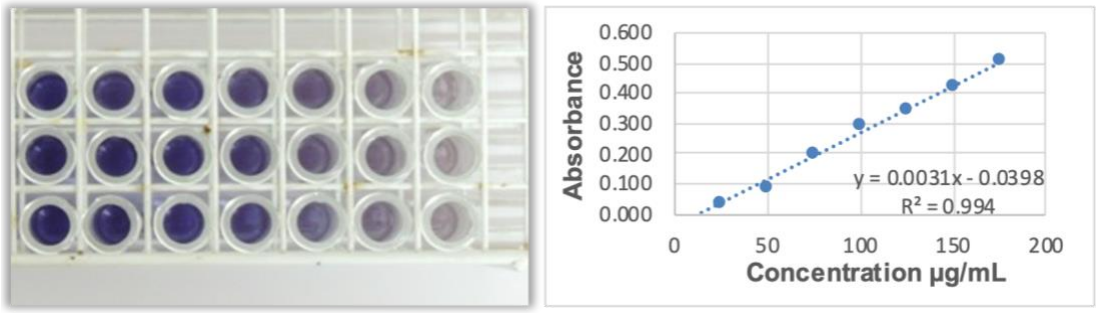

Figure 4.2. Colour changes of ascorbic acid at different concentration levels and its calibration curve as standard in determination of ferric reducing antioxidant power.

To ascertain if both methods were equally valid in measuring the antioxidant activity, they were correlated through the Pearson coefficient index (−1 < *r* < 1). A Pearson coefficient absolute value higher than 0.9 shows a strong correlation between the two methods. The Pearson index was also used to correlate the data of antioxidant activity

with the quantification of the several chemical classes of compounds to ascertain their relationship with antioxidant power. Once both *Asphodelus* species were collected in two different seasons, the obtained data were also analyzed by ANOVA, with the season as the source of variance. The significance value was set for a *p*-value < 0.05.

4.3.8. *In vitro* Genotoxicity/Mutagenicity Evaluation by Ames Test

A bacterial reverse mutation test (Ames test), commonly employed as an initial screening of the genotoxicity potential of herbal substances/preparations, was used to detect relevant genetic changes and genotoxic carcinogens [146]. The assessment of mutagenicity was performed according to the OECD No. 471 [129], the ICH S2 (R1) [130] guidelines, and following the published protocols [128], using five *Salmonella enterica* serovar Typhimurium tester strains (TA98, TA100, TA102, TA1535, and TA1537) in a direct plate incorporation method with and without metabolic activation. TA100, TA98, TA102 and TA1535 were kindly provided by the Genetic Department of the Nova Medical School of the Universidade Nova de Lisboa (Portugal), having received them from Professor B.N. Ames (Berkeley, CA, USA). TA1537 was from ATCC, NUMBER: 29630™, LOT: 7405375. The strains were inoculated in nutrient broth medium and incubated for 12–16 h, at 37 °C in the dark, shaking at 210 rpm in an orbital incubator, and kept at 4 °C until use. S9 mix (10%, *v/v* rat liver S9, 0.4 M $MgCl_2$, 1.65 M KCl, 1 M glucose-6-phosphate, 0.1 M nicotinamide adenine dinucleotide phosphate, and 0.2 M sodium phosphate buffer, pH 7.4) was freshly prepared and kept on ice during the experiment.

The extracts (25 mg/mL) were dissolved in DMSO (up to 30%), which also served as the negative control. An amount of 200 µL of extract dilutions were mixed with 500 µL sodium phosphate buffer (0.1 M, pH 7.4) (assay without metabolic activation) or S9 mix (assay with metabolic activation), 100 µL of the bacterial culture, and 2 mL of melted top-agar, supplemented with 0.05 mM biotin and histidine, at 45 °C. This mixture was then vortexed and plated on Petri dishes with Vogel-Bonner agar

medium and supplemented with 2% glucose. After a 48h incubation at 37 °C, manual counting of His+ revertant colonies for each concentration was performed. All assays were performed in triplicate.

The results were expressed as the mean number of revertant colonies with the standard deviation (mean ± SD). The positive controls were sodium azide (SA, 1.5 µg/plate for TA100 and TA1535), 2-nitrofluorene (2-NF, 5 µg/plate for TA98), 9-aminoacridine (9-AA, 100 µg/plate for TA1537), and tert-butyl hydroperoxide (tBHP, 50 µg/plate for TA102) in the assay without metabolic activation, and 2-aminoathracene (2-AA, 2 µg/plate for TA98 and 10 µg/plate for TA102, TA1535 and TA1537) and benzo(*α*)pyrene (BaP, 5 µg/plate for TA100) in the assay with metabolic activation.

4.3.9. *In vitro* Cytotoxicity Evaluation by MTT Assay

Cytotoxicity was evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay [157] on a human liver cell line HepG2 (ATCC Cat. No. HB-8065, Middlesex, UK). HepG2 were seeded in 96-well plates at a density of 8.5×10^4 cells/cm^2 in *α*-MEM (Sigma-Aldrich®, St. Louis, MO, USA) with 1 mM sodium pyruvate (PAN Biotech, Aidenbach, Germany) and 1% non-essential amino acids (NEAA, PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco®- Thermo Fisher Scientific™ (Waltham, MA, USA), in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. After 48h incubation, the cell culture medium was replaced by fresh medium with AbLa and AmLa extracts (9:1) at final concentrations of 50, 125, 250, 500, and 1000 µg/mL.

Cells were also incubated with a complete cell culture medium, DMSO 1% and DMSO 20% in *α*-MEM as a positive, solvent, and negative control, respectively. After 48 h, the cells were carefully washed with 100 µL PBS, and 200 µL 0.5 mg/mL MTT (Sigma-Aldrich®) in a cell culture medium was added. HepG2 were incubated for 3 h in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. The purple crystals were

solubilized with 200 µL DMSO and measured at 570 nm using a microplate spectrophotometer (SPECTROstar Omega; BMG LabTech, Ortengerg, Germany). The results were expressed as a percentage relative to the solvent control. Four wells were used for each sample, and at least two independent experiments were performed. Data analysis and graphs were plotted using GraphPad Prism® software (version 9.0.0.121, GraphPad Software, San Diego, CA, USA). Results are presented as mean ± standard deviation. $p < 0.05$ was considered significant.

4.4. Results and Discussion

4.4.1. Drug-Extract Ratio

The drug−70% hydroethanolic extract ratio for *Asphodelus bento-rainhae* leaf (AbL) were 4.5: 1 and 4.8: 1 for the first (AbLa) and second (AbLb) collection seasons, respectively. For *Asphodelus macrocarpus* leaf (AmL), these values were obtained as 2.9: 1 for the first (AmLa) and 6.3: 1 for the second (AmLb) collection season. The drug–extract ratio was calculated as 12.6: 1 and 12.3: 1 for the AbLa and AmLa 96% hydroethanolic extracts, respectively.

4.4.2. Phytochemical Analysis

Thin-layer chromatography (TLC), followed by high-performance liquid chromatography (HPLC) coupled to a photodiode detector (UV/DAD), and electrospray ionization spectrometry (ESI/MS) techniques were applied for the rapid and reliable detection of several samples. The obtained chromatographic profiles of *Asphodelus bento-rainhae* and *Asphodelus macrocarpus* leaf extracts (AbLa, and AmLa, respectively) and their subsequent liquid-liquid partition with increasing polarity solvents, namely ethyl ether (AbLa-1, AmLa-1), ethyl acetate (AbLa-2, AmLa-2) and water (AbLa-3, AmLa-3), showed qualitative similarity in their chemical composition (Figure 4.3), characterized by the presence of terpenoids, phenolic acids, flavonoids, and anthracene derivatives.

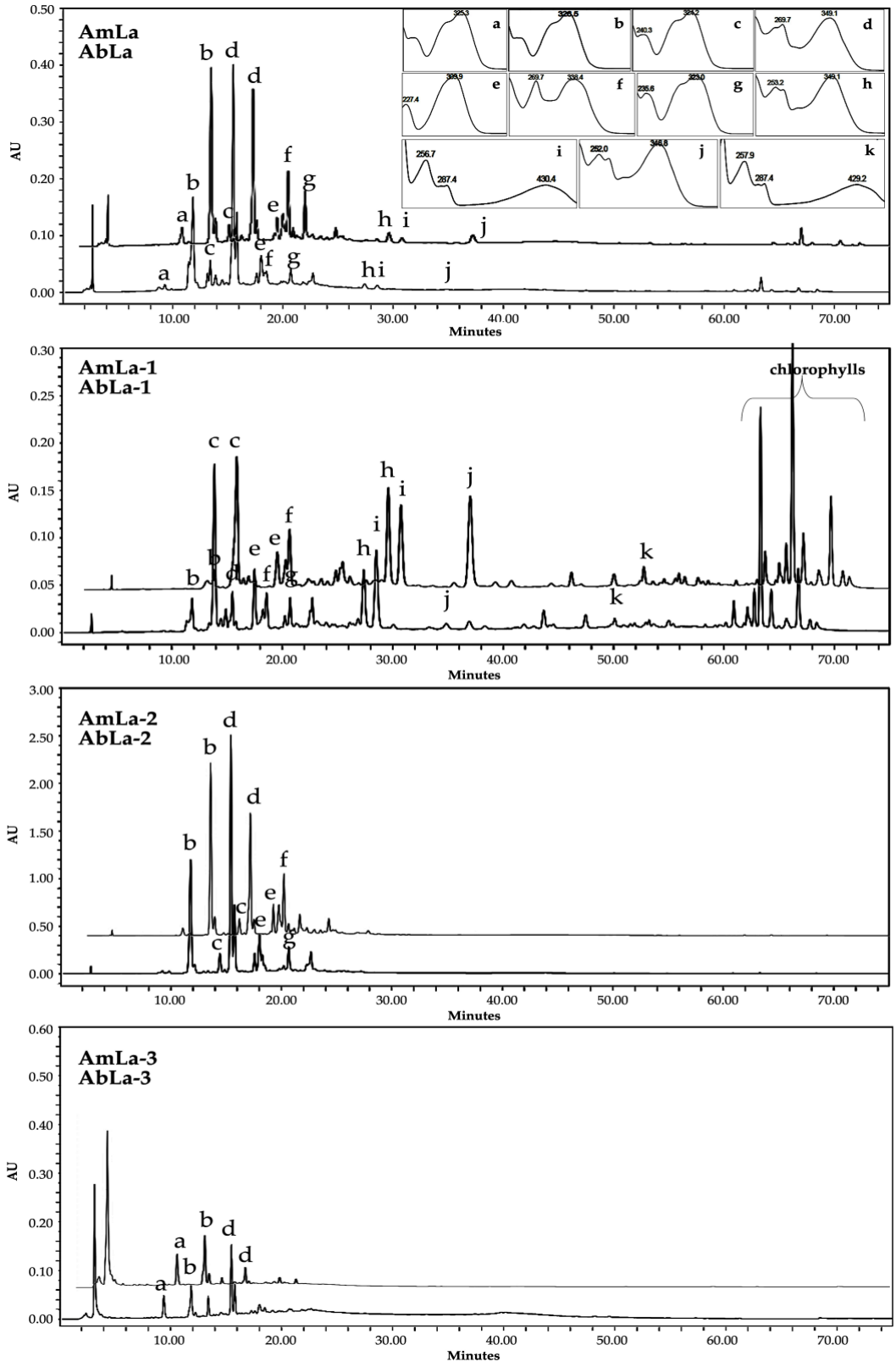

Figure 4.3. Comparative LC-UV/DAD chromatographic profiles of marker secondary metabolites of *A. bento-rainhae* and *A. macrocarpus* leaf 70% hydroethanolic extracts and their subsequent L-L partitions.

Abbreviations: AbLa: *A. bento-rainhae* leaf first collection, AmLa: *A. macrocarpus* leaf first collection, (−1): ethyl ether fractions, (−2): ethyl acetate fractions, and (−3): aqueous fractions.

Based on both TLC and HPLC spectral analysis, using the authentic standards (co-chromatography) and comparison with literature data, twelve known compounds, namely, neochlorogenic acid (a), chlorogenic acid (b), caffeic acid (c), isoorientin (d), *p*-coumaric acid (e), isovitexin (f), ferulic acid (g), luteolin (h), aloe-emodin (i), diosmetin (j), chrysophanol (k), and *β*-sitosterol (l) were identified as the main marker compounds of both species (Table 4.2, Figure 4.4).

Table 4.2. Characterization of the peaks of interest obtained from *A. bento-rainhae* and *A. macrocarpus* leaf 70% and 96% hydroethanolic extracts and their subsequent L-L partitions.

Peak	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Identified Compound
a	9.29	325.3	353	191 (100), 179 (3)	* neochlorogenic acid
b	11.79	326.5	353	191 (100), 179 (67)	*chlorogenic acid
c	13.82	240.3, 324.2	179	135 (100)	caffeic acid
d	15.43	269.7, 349.1	447	357 (43), 327 (100), 297 (76)	isoorientin
e	17.46	227.4, 309.9	163	119 (100)	*p*-coumaric acid
f	18.55	269.7, 338.4	431	341 (23), 311 (72), 283 (100)	isovitexin
g	20.53	235.6, 323.0	193	178 (62), 149 (68), 134 (100)	ferulic acid
h	27.38	253.2, 349.1	285	175 (13), 151 (100), 133 (22)	luteolin
i	28.73	256.7, 287.4, 430.4	269	239 (100)	aloe-emodin
j	34.78	252.0, 346.8	299	284 (100)	diosmetin
k	50.46	257.9, 287.4, 429.2	253	225 (100)	chrysophanol

Abbreviations: t_R: Retention time, λ_{max}: wavelength. *neochlorogenic acid is a synonym name of 5-*O*-caffeoylquinic acid and chlorogenic acid is a synonym name of 3-*O*- caffeoylquinic acid

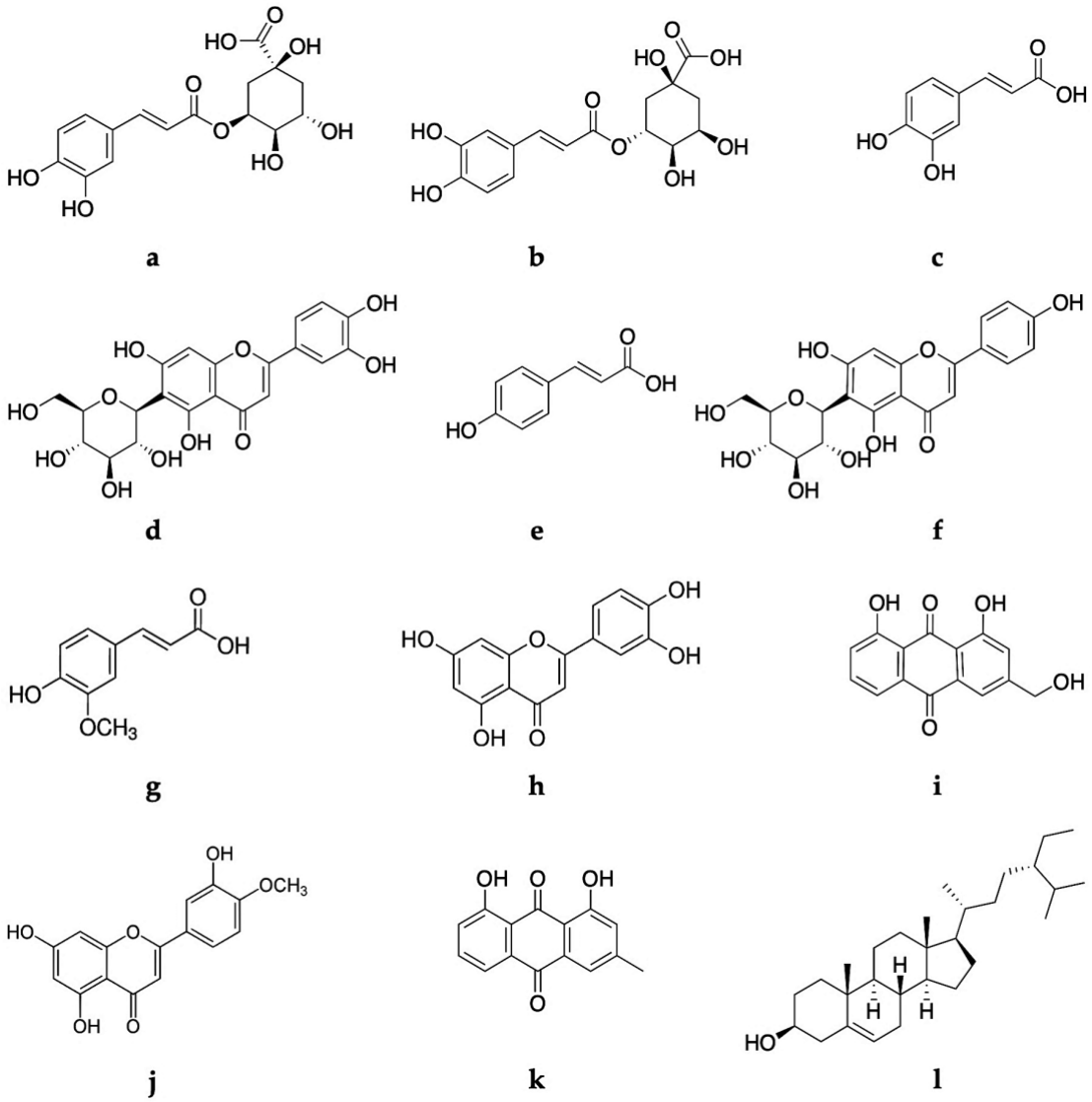

Figure 4.4. Structures of the marker secondary metabolites (a to l) from *A. bento-rainhae* and *A. macrocarpus* leaf 70% and 96% hydroethanolic extracts, mentioned in **Table 4.2.**

Isoorientin and chlorogenic acid were found as the major marker compound of both species crude, ethyl acetate and aqueous extracts and aloe-emodin was found among the major compounds of the diethyl ether fractions. Isoorientin was quantified using a calibration curve (equation of Y= 3673.4x + 1351.7, R^2= 0.9875, LOD= 2.57 µg/mL and LOQ= 7.80 µg/mL) as 184 and 107 µg/mL and chlorogenic acid (equation of Y= 5021.2x – 643.27, R^2= 0.999, LOD= 1.01 µg/mL and LOQ= 3.06 µg/mL), as 66 and 42

µg/mL for *A. bento-rainhae* leaf 70% and 96% hydroethanolic extracts, respectively. Isoorientin was also quantified as 112 and 30 µg/mL and chlorogenic acid, as 90 and 27 µg/mL for *A. macrocarpus* leaf 70% and 96% hydroethanolic extracts, respectively. Aloe-emodin was quantified as 13 and 15 µg/mL for *A. bento-rainhae* leaf 70% and 96% hydroethanolic extracts, respectively and 7 and 8 µg/mL for *A. macrocarpus* leaf 70% and 96% hydroethanolic extracts, respectively, using a calibration curve with equation of Y= 163422x – 2E+0.6, R^2= 0.9984, LOD= 0.08 µg/mL, and LOQ= 2.45 µg/mL.

Previously reported phytochemical studies of *Asphodelus* spp. revealed the presence of chlorogenic acid in the leaf and aerial parts extracts of *Asphodelus aestivus* Brot. [61] and *Asphodelus ramosus* L. [84], while caffeic acid was only reported from the flower extract of *A. ramosus* [80]. Isoorientin from *Asphodelus aestivus* [61], *Asphodelus albus* Mill. subsp. *delphinensis* [66], *Asphodelus cerasifer* Gay [59,66], *Asphodelus microcarpus* Salz. et Viv. [37] and *Asphodelus ramosus* [49], together with isovitexin from *Asphodelus aestivus* [61] and luteolin from *Asphodelus acaulis* Desf. [59], *Asphodelus albus* [59,66], *Asphodelus cerasifer* [59,66], *Asphodelus fistulosus* L. [43], *Asphodelus macrocarpus* Parl. subsp. *rubescens* [59], *Asphodelus microcarpus* [66,72], *Asphodelus ramosus* [66] and *Asphodelus tenuifolius* Cav. [51] have also been recorded as the most common flavonoids of these species.

Aloe-emodin from *A. aestivus* [61], *A. albus* [59,63], *A. cerasifer* [59], *A. fistulosus* [43,67], *A. macrocarpus* subsp. *rubescens* [59], and *A. microcarpus* [63,67] as well as chrysophanol from *A. albus* [59,63], *A. fistulosus* [43,67], *A. macrocarpus* subsp. *rubescens* [59] and *A. microcarpus* [67,71] have been frequently detected and therefore found to be the most common anthracene derivatives. β-sitosterol, a common phytosterol, was only found in the root extracts of *A. albus*, *A. microcarpus*, and *A. tenuifolius* [37,64,78,86] and seeds extract of *A. fistulosus* and *A. microcarpus* [68].

Quantification results of the main chemical classes of secondary metabolites, namely total phenolics (TPC), total flavonoids (TFC), total anthraquinones (TAC), total

condensed and hydrolysable tannins (TCTC and THTC, respectively), together with total terpenoids, (TTC) are presented in Table 4.3.

Table 4.3. Quantification of the principal classes of secondary metabolites of *A. bento-rainhae* and *A. macrocarpus* leaf 70% hydroethanolic extracts.

Assays	AbLa	AbLb	AmLa	AmLb
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
TPC				
(mg GAE/ g dried extract)	44.16±21.62	38.83±17.1	37.15±14.32	38.28±15.63
(mg GAE/ g dried leaf)	9.23±4.52	8.57±3.78	12.63±5.38	6.09±2.49
TFC				
(mg CAE/ g dried extract)	**40.79±4.45	29.56±1.43	33.46±0.89	*35.52±1.51
(mg CAE/ g dried leaf)	8.16±0.89	6.53±0.32	5.32±0.14	12.08±0.51
TAC				
(mg RhE/ g dried extract)	*1.16±0.13	1.07±0.11	0.55±0.07	0.81±0.09
(mg RhE/ g dried leaf)	0.24±0.05	0.24±0.04	0.19±0.02	0.13±0.01
TCTC				
(mg CAE/ g dried extract)	*180.96±10.98	149.71±12.98	132.60±2.73	142.98±6.71
(mg CAE/ g dried leaf)	37.82±2.30	33.06±2.87	45.09±0.93	22.73±1.07
THTC				
(mg GAE/ g dried extract)	67.61±9.22	*55.16±6.64	60.53±8.04	37.03±3.87
(mg GAE/ g dried leaf)	14.13±1.93	12.18±1.47	20.58±2.74	5.89±0.62
TTC				
(mg OAE/ g dried extract)	111.72±22.77	88.78±23.22	*165.47±26.54	125.74±20.72
(mg OAE/ g dried leaf)	23.35±4.76	19.60±5.13	56.26±9.03	19.99±3.29

Abbreviations: AbLa: *A. bento-rainhae* leaf first collection, AbLb: *A. bento-rainhae* leaf second collection, AmLa: *A. macrocarpus* leaf first collection, AmLb: *A. macrocarpus* leaf second collection, TPC: total phenolic content, TFC: total flavonoid content, TAC: total anthraquinones content, TCTC: total condensed tannin content, THTC: total hydrolysable tannin content, TTC: total triterpenoid content, GAE: gallic acid equivalents, CAE: catechin equivalents, RhE: rhein equivalents, OAE: oleanolic acid equivalents. * Significantly higher content (p-value < 0.05) when compared between different species of the same collection season analyzed by ANOVA test.** Significantly higher content (p-value < 0.05) when compared between different seasons of collection of the same species.

Concerning the analysis between the different collection seasons for the same species, the results showed that the total content of TCTC and TFC in *A. bento-rainhae*

(*p*-values: 0.034, 0.01, respectively) and THTC in *A. macrocarpus* leaf extracts were significantly higher in the first collection season (*p*-value: 0.01).

The analysis of the results between different species of the same collection season showed that TTC content in the first collection season and TFC content in the second collection season were significantly higher in *A. macrocarpus* when compared to those of *A. bento-rainhae* (*p*-values: 0.0021 and 0.01, respectively). However, TAC, TCTC, and TFC contents in the first collection season (*p*-value: 0.002, 0.007, and 0.006, respectively) and THTC content in the second collection season (*p*-value: 0.028) were significantly higher in *A. bento-rainhae* when compared to those of *A. macrocarpus*.

The obtained data showed the TCTC (180.96 ± 10.98 and 142.98 ± 6.71 mg CAE/g DW) and TTC (111.72 ± 22.77 and 165.47 ± 26.54 mg OAE/g DW) contents with the highest and TAC (1.07 ± 0.11 and 0.55 ± 0.07 mg RhE/g DW) with the lowest content in comparison to the other quantified chemical classes in both *A. bento-rainhae* and *A. macrocarpus* leaf extracts.

Previously reported *Asphodelus* spp. leaf extracts quantified values of TPC, TFC, and TCTC indicated the important role of solvent selection for the extraction procedure. In fact, *A. microcarpus* ethanol extract showed a higher amount of TPC and TFC (54.44 ± 13.6 mg GAE/g of DW and 31.13 ± 1.96 mg QUE/g of DW, respectively) in comparison to the aqueous and methanol extracts [70]. However, in *A. ramosus*, the aqueous extract exhibited a higher amount of TPC (33.51 ± 0.33 mg GAE/g of DW) when compared to the methanol, methanol/water (50%), and ethyl acetate extracts [105]. *A. aestivus* acetone extract also showed an elevated amount of TFC (17.74 ± 0.46 mg CAE/g of DW) in comparison to the aqueous, ethanol and methanol extracts [38,42]. Contrary to the data mentioned above and that obtained by us, significantly higher amounts of TPC (183.7 ± 3.5, 128.5 ± 2.1 and 109.7 ± 1.5 mg GAE/g of DW) and lower amounts of TCTC (59.8 ± 0.6, 49.2 ± 0.5 and 41.4 ± 0.3 mg CAE/g of DW) were reported from *A. tenuifolius* methanol, ethanol, and petroleum ether extracts[20]. It was also observed that both TPC and TFC contents have increased with the increase of the extraction temperature in the experiments done with *A. ramosus* [105].

4.4.3. Determination of *In vitro* Antioxidant Potential

In this study, the antioxidant activity was evaluated by two complementary methods, DPPH assay to determine the 50% inhibition of free radical scavenging activity, and FRAP, which evaluates the reducing potency of the antioxidants to react to the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex.

Concerning the results shown in Table 4.4, overall, *A. bento-rainhae* exhibited stronger antioxidant activity when compared to *A. macrocarpus* extracts. Among all the tested extracts, ethyl acetate fractions (AbLa-2, AmLa-2) showed the highest antioxidant activity when compared to all the other fractions (IC_{50}: 800 µg/mL and IC_{50}: 1200 µg/mL, respectively).

Table 4.4. *In vitro* determination of the antioxidant activity of *A. bento-rainhae* and *A. macrocarpus* leaf 70% hydroethanolic extracts and their subsequent L-L partitions.

Extracts code	Assays	
	DPPH (IC_{50} µg/mL)	FRAP (mmol AA/g dry extract)
AbLa 70%	2000	0.337±0.042
AbLb 70%	2540	0.306±0.023
AmLa 70%	2990	0.280±0.046
AmLb 70%	3070	0.271±0.072
AbLa-1	2950	Nd
AbLa-2	800	Nd
AbLa-3	2910	Nd
AmLa-1	3009	Nd
AmLa-2	1200	Nd
AmLa-3	4000	Nd
AA	83	Nd

Abbreviations: AbLa: *A. bento-rainhae* leaf first collection extract, AbLb: *A. bento-rainhae* leaf second collection extract, AmLa: *A. macrocarpus* leaf first collection extract, AmLb: *A. macrocarpus* leaf second collection extract, DPPH: 2,2-diphenyl-1-picrylhydrazyl, IC_{50}: The half maximal inhibitory concentration, FRAP: Ferric reducing antioxidant power, AA: ascorbic acid, Nd: not determined.

When comparing FRAP and DPPH, the obtained an r value of −0.975, showing a strong correlation between them, validating the results of both techniques, although the data of the FRAP test correlate better with the quantifications data. The classes of compounds that correlate better with the antioxidant power of the extracts are the flavonoids (TFC, r value of 0.943) and phenolic compounds (TPC, r value of 0.949), in which a higher content of these compounds is related to higher antioxidant power. In accordance with these results, phytochemical screenings of the crude extracts and their L-L partitions revealed the presence of homoorientin and chlorogenic acid as the main marker compounds of most active fractions (AbLa-2, AmLa-2).

There is no report on the antioxidant activity of our studied *Asphodelus* species; however, the previously reported results of DPPH analyses of the other *Asphodelus* spp. showed that *A. microcarpus* leaf ethanol and methanol extracts exhibited the highest antioxidant activity (IC_{50}: 55.9 µg/mL and IC_{50}: 98 µg/mL, respectively) [70,102]. On the contrary, *A. aestivus* leaf methanol extracts noticeably showed a higher antioxidant activity when compared to ethanol extract (IC_{50}: 160 µg/mL and IC_{50}: 9540 µg/mL, respectively) [38,42]. *A. tenuifolius* leaf methanol extract exhibited the lowest IC_{50} (18370 µg/mL) levels among the others, including our studied species [107].

4.4.4. Assessment of the Antibacterial Potential

The *in vitro* quantitative method of susceptibility testing (determination of MIC values) was used for the evaluation of the antimicrobial potential against both selected Gram-positive and Gram-negative resistant pathogens in this study.

Concerning the obtained results, the selected leaf 70% hydroethanolic extracts (AbLa, AmLa), and their subsequent ethyl acetate (AbLa-2, AmLa-2) and aqueous (AbLa-3, AmLa-3) L-L partition fractions did not exhibit antimicrobial activity against both Gram-positive and Gram-negative microorganism pathogens at any of the tested concentrations (MIC > 2000 µg/mL). However, as shown in Table 4.5, diethyl ether

fractions (AbLa-1, AmLa-1) demonstrated considerable antibacterial activity against all the Gram-positive microorganisms, with MIC values ranging from 62 to 1000 µg/mL.

Table 4.5. *In vitro* antimicrobial activity of *A. bento-rainhae* and *A. macrocarpus* leaf etheric L-L partition extracts against Gram-positive strains.

Bacteria (Gram +)	MIC (µg/mL)				
	AbLa 96%	AmLa 96%	AbLa-1	AmLa-1	Aloe-emodin
S. aureus ATCC 29213	500	> 2000	500	500	3.2
S. aureus CQINSA4923	250	2000	62	125	50
S. aureus INSArefV	500	> 2000	500	500	1.6
S. aureus INSA936	500	> 2000	250	250	12.5
S. aureus INSA896	500	2000	125	125	3.2
S. saprophyticus INSA842	1000	> 2000	125	250	100
S. saprophyticus INSA867	1000	> 2000	1000	1000	25
S. epidermidis INSA796	1000	2000	250	500	1.6
S. epidermidis INSA958	1000	2000	250	500	0.8
S. epidermidis INSA960	1000	2000	125	125	1.6
S. haemolyticus INSA982	125	> 2000	125	125	25
S. haemolyticus INSA984	125	> 2000	125	125	12.5

Abbreviations: AbLa: *A. bento-rainhae* leaf first collection extract; AmLa: *A. macrocarpus* leaf first collection extract; AbLa-1, AmLa-1: diethyl ether fractions; ATCC, American Type Culture Collection; INSA, Instituto Nacional de Saúde clinical strains collection; MIC, minimum inhibitory concentration.

The AbLa 96% hydroethanolic extract was found to be the most active crude extract with higher contents of anthracene derivatives among the main marker metabolites in comparison to AmRb 96% and both species' 70% hydroethanolic extracts. In general, *A. bento-rainhae* exhibited higher activity when compared to *A. macrocarpus,* and no activity in the tested range of concentrations (MIC > 2000

µg/mL) was found against Gram-negative microorganisms (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) for any of the tested extracts, fractions or pure compounds.

Aloe-emodin (compound i, Table 4.2) identified as one of the main marker compounds of the diethyl ether fractions and AbLa 96% hydroethanolic extract, and since the other main compounds (e.g., chlorogenic acid and isoorientin) were present in higher quantity in the other fractions and did not exhibit the antimicrobial activity, therefore was selected and tested against the pathogen panel under the study. This compound was found to be highly active against all the Gram-positive strains, particularly against all *Staphylococcus epidermidis* strains with a MIC between 0.8 to 1.6 µg/mL. These findings were also confirmed by the obtained results of aloe-emodin quantification and could explain the higher activity of AbLa 96% in comparison with the 70% hydroethanolic extracts.

In accordance with our results, aloe-emodin was previously reported as a potential antimicrobial that was active against several Gram-positive bacteria [160]; however, in a recent study, aloe-emodin with MIC values of 4 to 32 µg/mL exhibited deformities in the morphology of *S. epidermidis* cells and the destruction of the selective permeability of the cell membranes [169].

Results of studies involving the determination of the antimicrobial activity of other *Asphodelus* spp. against a similar pathogen panel revealed their lower antimicrobial potential. For instance, a leaf ethanol extract of *A. aestivus* exhibited a MIC of 42,000 µg/mL against *S. aureus*, and of 60,000 µg/mL against *Klebsiella pneumoniae* [91]. The *A. fistulosus* leaf ethanolic and aqueous extracts showed activity against *S. aureus* (MIC 2200 µg/mL and 7600 µg/mL, respectively) [170].

A methanol extract of *A. luteus* aerial parts showed an MIC between 1250 to 2500 µg/mL against methicillin-resistant *S. aureus* (MRSA) [94]. A methylene-chloride extract of the aerial parts of *A. tenuifolius* was found to be more active against *S. aureus* (MIC = 1600 µg/mL), *Enterococcus faecalis* (MIC = 1000 µg/mL), and *E. coli* (MIC = 1800 µg/mL) in comparison to the *n*-butanol and ethyl acetate extracts of the

same species[51]. Recently, an *A. tenuifolius* whole plant chloroform extract was shown to be active against *S. epidermidis* (MIC = 580 µg/mL) [171]. *A. microcarpus* leaf extracts also showed antimicrobial activity against several Gram-positive strains, with MIC values of 78 to 5000 µg/mL [46,71,72,94,104].

A. bento-rainhae and *A. macrocarpus* leaf extracts seem to be more active against the tested Gram-positive strains in comparison to the other tested *Asphodelus* spp. extracts. The antibacterial activity of *A. fistulosus* leaf aqueous extract against *E. coli* (MIC = 62 µg/mL) and of *A. tenuifolius* aerial parts methylene-chloride extract against the same microorganism (*E. coli*, MIC = 1800 µg/mL) and also against *P. aeruginosa* (MIC = 150 µg/mL) are examples of the few studies relating the antimicrobial activity of *Asphodelus* spp. to Gram-negative strains. Overall, the observed antimicrobial activity of both *A. bento-rainhae* and *A. macrocarpus* leaf crude extracts were similar to those obtained and reported form the other *Asphodelus* spp. tested against a similar panel of pathogens. However, the fractionation of crude extracts enabled the detection of significant antimicrobial activity in the diethyl ether L-L partition fractions, quantitatively the richest in 1,8-dihydroxyanthracene derivatives, a known chemical class of secondary metabolites with antimicrobial activity [160].

4.4.5. Pre-Clinical Safety Assessment

Following the guidelines of the genotoxicity by the Ames test, which is commonly used as an initial screen of genotoxicity, for a substance to be considered genotoxic in the test, the number of revertant colonies on the plates containing the test compounds/substance must be more than twice the number of colonies produced on the solvent control plates (i.e., a ratio above 2.0). In addition, a positive dose-response should be evident for the various concentrations of the tested mutagen [151,152]. Since the crude extracts obtained from the first collection season (AbLa, AmLa) exhibited higher contents of the main classes of secondary metabolites, they were subsequently selected for further safety examination.

The obtained results of the Ames test for both AbLa and AmLa extracts are presented in Table 4.6.

Table 4.6. Mutagenicity of *A. bento-rainhae* and *A. macrocarpus* leaf crude extracts in the bacterial reverse mutation test (Ames Test).

AbLa µg/plate	Number of revertant colonies without metabolic activation, mean (n=3) ± standard deviation (SD)				
	TA98	TA100	TA102	TA1535	TA1537
250	17±4	160±7	355±13	19±4	10±1
625	20±4	158±5	349±34	24±1	10±2
1250	17±2	182±16	429±25	20±1	7±1
2500	21±2	178±8	458±16	22±2	8±2
3750	24±3	175±19	472±29	21±4	9±3
5000	24±2	175±14	485±31	18±1	13±3
AmLa µg/plate					
250	17±2	186±10	357±14	22±3	9±1
625	20±2	155±15	365±3	20±3	9±2
1250	22±5	150±5	394±8	16±1	10±5
2500	21±3	170±15	441±2	17±3	12±5
3750	24±5	168±4	454±24	17±3	8±2
5000	23±3	165±20	407±28	24±2	15±1
NC	19±2	156±17	320±4	21±3	7±1
PC	2-NF	SA	tBHP	SA	9-AA
	488±30	1048±43	881±26	827±13	1354±5
AbLa µg/plate	Number of revertant colonies with metabolic activation, mean (n=3) ± standard deviation (SD)				
500	Nd	166±22	221±16	19±4	15±1
1250	63±6	164±9	248±11	15±7	16±1
2500	59±5	174±4	248±11	17±2	11±1
5000	52±6	178±15	254±12	15±1	16±1
NC	44±8	157±6	172±2	11±2	12±1
PC	2-AA	BaP	2-AA	2-AA	2-AA
	832±35	947±148	732±12	266±1	306±50

Abbreviations: AbLa: *A. bento-rainhae* leaf first collection extract, AmLa: *A. macrocarpus* leaf first collection extract, Nd: not determined, NC: negative control /solvent control (DMSO 30%), PC: positive control reference, 2-NF: 2-nitrofluorene, SA: sodium azide, tBHP: *tert*-butyl hydroperoxide, 9-AA: 9-aminoacridine, 2-AA: 2-aminoathracene, BaP: benzo(*a*)pyrene.

Neither extract induced an increase in the number of revertant colonies in any of the tested strains at any tested concentration, with (500, 1250, 2500, and 5000 µg/plate) and without (250, 625, 1250, 2500, 3750, and 5000 µg/plate) metabolic activation, when compared to the negative control. Moreover, cytotoxicity did not occur since there was neither a decrease in the number of spontaneous revertants nor a decrease on the background lawn of the plates at any of the concentrations tested. Therefore, under the conditions of this study, neither extract of the two species showed mutagenic activity.

Our cell viability assay (Figure 4.5.) concurrently indicated that none of the AbLa and AmLa extracts reduced HepG2 viability.

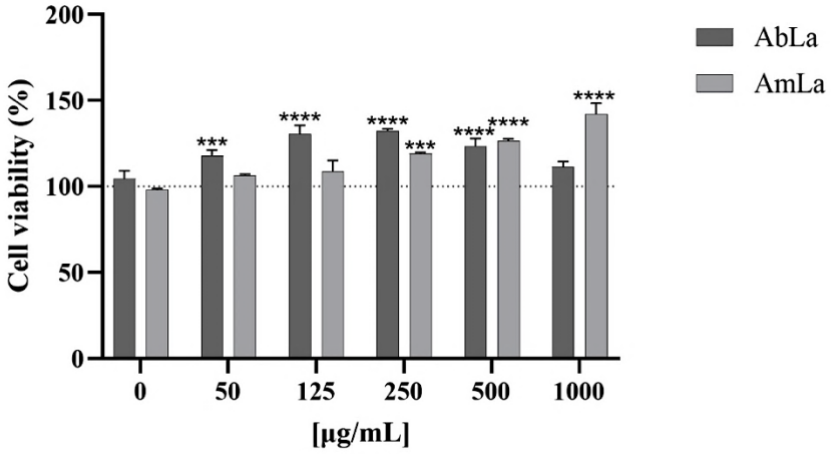

Figure 4.5. HepG2 viability after 48 h of incubation with AbLa and AmLa extracts evaluated by MTT reduction assay. Data are shown as the percentage of solvent control (dashed line) and as mean ± standard deviation; n= 2–5. *** $p < 0.001$; **** $p < 0.0001$.

The AbLa extract (50–500 µg/mL) enhanced HepG2 viability/proliferation up to ~30% when compared to the 0 µg/mL concentration, whereas the same was observed for AmLa, i.e., it promoted HepG2 viability/proliferation by up to 40%, especially at higher concentrations (250–1000 µg/mL; $p < 0.001$ and $p < 0.0001$). Therefore, under the conditions of this study, the extracts of both species did not show mutagenic activity and *in vitro* cytotoxicity of HepG2, which is crucial to ensure their safety [128,147,152].

Chapter 5

General Discussion and Conclusion

The original findings presented in this thesis were certainly directed towards the discovery of active antimicrobial drugs effective against resistant pathogenic agents from two Portuguese *Asphodelus* species, namely *Asphodelus bento-rainhae* and *Asphodelus macrocarpus*. These findings are reported here for the first time for both species' root tubers and leaves.

In **Chapter 2** of this thesis, we performed a comprehensive review of the existing data regarding the genus *Asphodelus,* and our findings showed that among the species of this genus*,* only 30% of the species, namely *A. aestivus*, *A. fistulosus*, *A. microcarpus*, *A. ramosus*, and *A. tenuifolius*, have been documented for their traditional uses. In phytochemical studies 50% of the species (*A. acaulis*, *A. aestivus*, *A. albus*, *A. cerasifer*, *A. fistulosus*, *A. macrocarpus*, *A. microcarpus*, *A. ramosus*, *A. tenuifolius*) have been evaluated for their constituents.

All the species with ethnomedical documented data were submitted to biological activity tests, showing a total or partial correlation with their traditional use as anti-microbial, anti-fungal, anti-parasitic, cytotoxic, anti-inflammatory, or antioxidant agents.

Root tubers plant part was mainly reported to have anthraquinone derivatives, triterpenoids, and naphthalene derivatives, while aerial parts mostly exhibited the presence of flavonoids, phenolic acids, and few anthraquinones. Considering the previous phytochemical studies, 1,8-dihydroxyanthracene derivatives (e.g., aloe-emodin and chrysophanol) were the most common reported anthraquinones of *A. aestivus*, *A. luteus* and *A. microcarpus* extracts, which could be responsible for the reported antimicrobial/fungal activities [108,110]. Aloe-emodin as a potent cytotoxic compound might be also related to the reported anti-tumoral activity of *A. aestivus* [92,108].

Flavonoids, namely luteolin and apigenin derivatives reported from the aerial parts and Phenolic acids, namely caffeic acid and chlorogenic acid reported from aerial parts and root tubers, were frequently reported from *Asphodelus* species, which according to their known antioxidant and anti-inflammatory properties [111,112],

could be correlated to their traditional use for treatment of inflammatory diseases in agreement with the reported biological studies performed on these species.

Phytosterols (e.g., fucosterol, *β*-sitosterol, and stigmasterol) and *β*-amyrin were the most common found triterpenoids from roots and seeds. According to the literature, *β*-amyrin possess antibacterial/antifungal properties [113], which complement the reported biological activities of *A. tenuifolius*.

The obtained findings emphasized the importance and potential of the *Asphodelus* genus as a source of new compounds with biological activities and also significance of progress in development of new herbal products based on the *Asphodelus* medicinal plants, which are used in traditional medicine. Furthermore, the research explored the quality, mode of action, and safety of these products. It should be pointed out that, to the best of our knowledge, the latter aspect (the safety of *Asphodelus* species) has not yet been the object of in-depth studies by others.

In **Chapter 3,** identification and preparation of the specimens were described, and the botanical characterization of the selected samples were performed as initial quality assessment tools for the identification of root tubers of *A. bento-rainhae* and *A. macrocarpus* as herbal raw materials.

The observed botanical characteristics, such as multiseriate epidermis (velamen), the large parenchyma water-storing cells, the cortex cells containing soluble sugars, the oil cells containing lipid material of possibly defense character, and the cell idioblasts, which contain raphide crystals of calcium oxalate, were found among numerous anatomical similarities, between our *Asphodelus* species and several other species from *Liliaceae* and *Orchidaceae*. Moreover, evident morphological differences in the development of tubercles and microscopically in the arrangements and characteristics of the vascular cylinder (metaxylem and protoxylems) were found.

These characteristics, which are in fact, the means of synchronization and adaptation of these plants with the seasonality of the Mediterranean climate [142], enabled the

establishment of the morphological parameters needed for the proper identification of both species root tubers as raw materials for pharmaceutical use.

The observed antimicrobial activity of both *A. bento-rainhae* and *A. macrocarpus* root tuber 70% hydroethanolic extracts (AbRb and AmRb) tested against multidrug-resistant skin-related pathogens were similar to those obtained and reported from the other *Asphodelus* spp. tested against a similar panel of pathogens [36]. However, the bioguided fractionation of these extracts using diethyl ether L-L partition fractions and an enriched 96% hydroethanolic extract certainly enhanced their significant antimicrobial activity, as they contain the highest content of 1,8-dihydroxy anthracene derivatives, a known chemical class of secondary metabolites with potential antimicrobial activity [160].

These data were confirmed by TLC and LC-UV-DAD co-chromatographic techniques and more importantly by the quantification of chrysophanol as the major marker compounds of root tubers extracts of both species. Moreover, chrysophanol and its derivatives could be regarded as significant chemotaxonomic markers for both of the studied *Asphodelus* species.

Considering the obtained results of the *in vitro* cytotoxicity and genotoxicity evaluated by Ames test, 70% hydroethanolic extracts of both species tested up to 5.0 mg/plate (maximum test concentration recommended by the OECD guidelines for testing chemicals) did not induce an increase in the number of revertants, per plate, in any of the tested bacterial strains; therefore, under the conditions of our study, the extract did not show toxicity and genotoxic potential. Same was also confirmed by the tested root tuber 96% hydroethanolic extract of *A. bento-rainhae*, the extract with highest antimicrobial activity and quantity of the active secondary metabolites.

In **Chapter 4.** the antimicrobial activity verified with leaf 70% hydroethanolic extracts of *A. bento-rainhae* and *A. macrocarpus* was also consistent with the results obtained when testing other *Asphodelus* spp. against a similar panel of pathogens [36]. However, similar to the root tuber extracts, an enriched 96% hydroethanolic extract and fractionation of crude extracts, certainly enabled the detection of significant

antimicrobial activity, specifically, in the diethyl ether L-L partition fractions, quantitatively the richest in 1,8-dihydroxyanthracene derivatives content. Aloe-emodin, a well-known antibacterial agent was identified as the main compound responsible for this activity. These findings were also confirmed by TLC and LC-UV-DAD co-chromatographic techniques and more importantly, the quantification of aloe-emodin. The quantification values and the calculated LOD and LOQ values also indicate the possibility of determining the quantified compounds at the low concentration.

On the other hand, the ethyl acetate L-L partition fractions, which are quantitatively the richest in phenolic acids and flavonoid derivatives contents, exhibited the highest antioxidant activity, confirming the major role of the different classes of the identified phenolic compounds in the activity of *A. bento-rainhae* and *A. macrocarpus* leaves as medicinal plants. Moreover, the negative results of the Ames and MTT tests indicate that the leaf hydroethanolic extracts of both of these medicinal plants are safe in terms of toxicity.

The comparison of the findings obtained from root tuber and leaf of both species collected in two different seasons, presented in **Chapter 3** and **Chapter 4**, indicated that different season of collection has a significant effect in the quantity of plant metabolites. Leaf samples of the first collection season (Spring) and root tuber from the second collection season (Autumn) exhibited the higher amounts of secondary metabolites. Leaf 70% hydroethanolic extracts in general, exhibited the higher quantity of phenolics, flavonoids, condensed and hydrolysable tannins and the root tuber 70% hydroethanolic extracts showed higher amounts of anthraquinones and terpenoids.

Although, based on the quantitative assays, terpenoids and condensed tannins were found to be the main classes of marker secondary metabolites of leaf and root tuber extracts of both specie, however, when all the crude extracts and their L-L subsequent fractions were tested for their antimicrobial potential, the anthraquinones (anthracene derivatives), one of the minor quantified classes of secondary

metabolites, were found to be the main constituents of the most active fractions and could be responsible for their antimicrobial potential. Therefore, these results contribute to the concrete validation of the use of these medicinal plants as potential sources of antimicrobial agents in the treatment of skin disorders and infections.

Even though, the *in vitro* toxicity or mutagenicity potential of anthracene derivatives has been reported by others, the obtained negative results of the tested chrysophanol without metabolic activation (S9) and the 96% hydroethanolic extract (with and without metabolic activation), showed that the presence of these compounds does not influence the toxicity of the crude extract. Additionally, human exposure to these compounds through the crude extracts is expected to be negligible, concerning the expected mode of administration (topical application).

Overall, these findings, together with the phytochemical profile determination and quantification of the main constituents present in each of these medicinal plants, provide relevant and essential information for inclusion in their future quality and safety monographs, and more importantly, draw attention to the need for conservation action and ultimately, for the prevention of the extinction of the studied Portuguese endemic *Asphodelus* species.

Chapter 6

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